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Biosynthesis of tetronate antibiotics: A growing family of natural products with broad biological activities

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Tetronate antibiotics, a growing family of natural products featuring a characteristic tetronic acid moiety, are of importance and of particular interest for their typical structures, especially the spirotetronate structure, and corresponding versatile biological activities. Considerable efforts have persistently performed since the first tetronate was isolated, to elucidate the biosynthesis of natural tetronate products, by isotope-labeled feeding experiments, genetical characterization of biosynthetic gene clusters, and biochemical reconstitution of key enzymatic catalyzed reactions. Accordingly, the biosynthesis of spirotetronates has been gradually determined, including biosynthesis of a polyketide-derived backbone for spirotetronate aglycone, incorporation of a glycerol-derived three-carbon unit into tetronic acid moiety, formation of mature aglycone via Diels-Alder-like reaction, and decorations of aglycone with various deoxysugar moieties. In this paper, the biosynthetic investigations of natural tetronates are well documented and a common biosynthetic route for this group of natural products is summarized accordingly.

tetronate, natural products, spirotetronate, polyketide, biosynthesis

1 Introduction

Natural occurring metabolites of various organisms from bacteria to plants, are of importance for their great diversity of chemical structures and the high pharmaceutical potential of biological activities, which have been proven to be productive source for new drug discovery [1]. In the 20th century, numerous natural products were structurally elucidated and medicinally assigned on account of their characteristic activities, including antibacterial agents (i.e., penicillin, vancomycin), antimalarials (i.e., quinine, artesmisinin) and anticancer agents (i.e., doxorubin, bleomycin). Among which, tetronates featuring a characteristic tetronic acid moiety (i.e., dihydrofuran-2,4-dione), were isolated for their intriguing structures and biological acitivities (Figure 1). Most tetronates exhibit moderate antibacterial activity, beside, many of them also exhibit characteristic activities, i.e., abyssomicin C inhibits aromatic amino acid and purine nucleotides production [2]; tetrocarcin A induces a cell-type-dependent apoptosis [3, 4]; and kijanimicin shows antitumor activity besides its antimicrobial activity [5, 6].

Chlorothricin, the first characterized tetronate, was isolated from *Streptomyces antibioticus* DSM 40725 in 1969. Its chemical structure was elucidated based on spectroscopic and single-crystal X-ray analysis, that comprising of a chlorothricolide aglycone (the tetronic acid moiety spiro-linked to a cyclohexene ring), a characteristic 2-methoxy-5-chloro-6-methylsalicyclic acid and two deoxysugars (Figure 1) [7, 8]. Biological assay revealed a unique functional mechanism of chlorothricin to inhibit pyruvate carboxylase [9]. Of interest of the spirotetronate structure and biological activity, considerable efforts for synthetic study and screening of novel analogs has stimulated the discovery of numerous naturally occurring products (more than 60) sharing a chlorothricin-like spirotetronate architecture, including kijanimicin [6], saccharocarcins [10], versipe-

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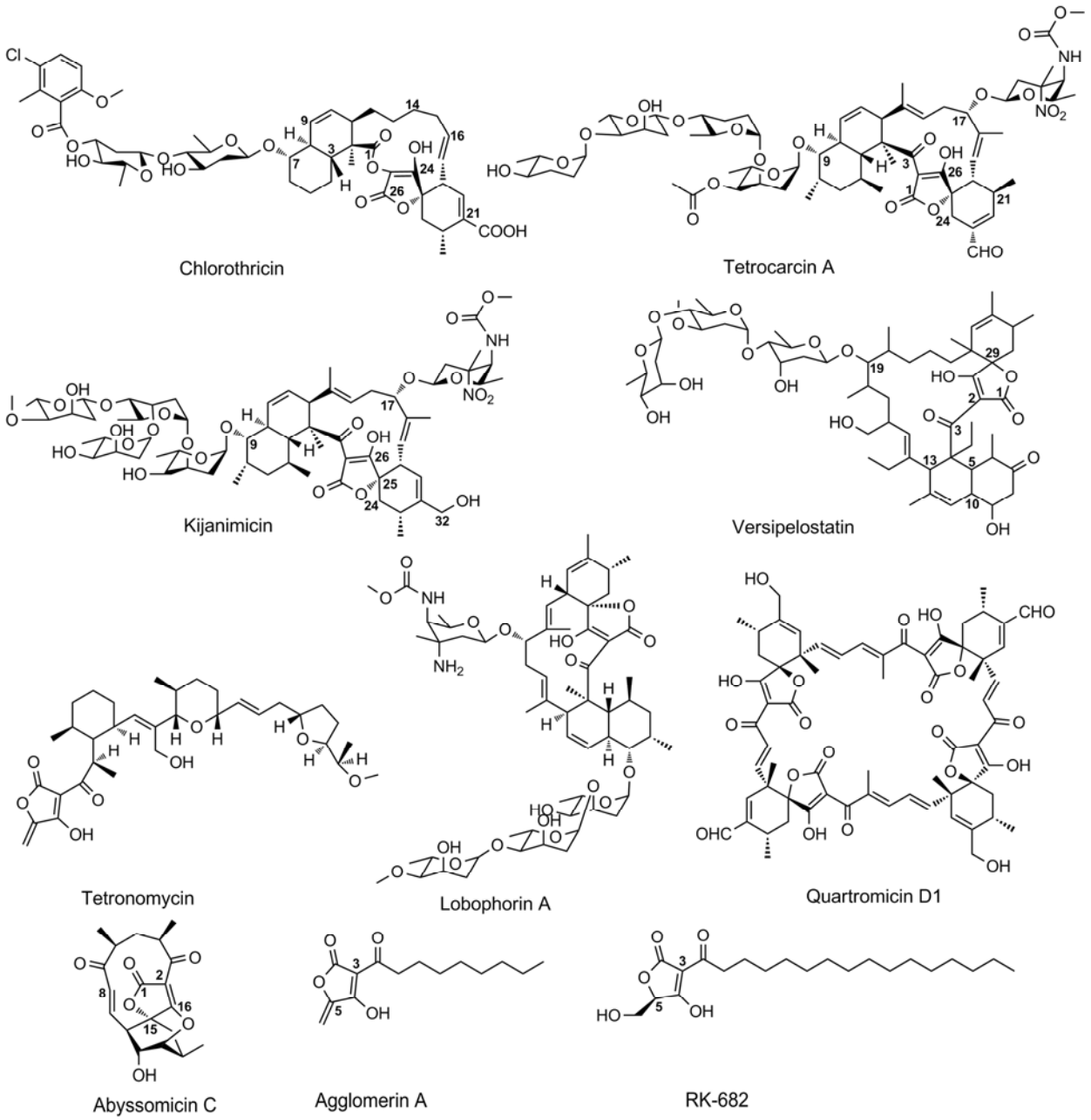

Figure 1 Structures of representative tetronate antibiotics.

lostatin [11], arisostatins [12], abyssomicin C [2] and lobophorin [13]. Screening simultaneously identified many tetronates with an acyl side chain or polyketide derived polyether substituted at C-3 (i.e., RK-682, agglomerins; tetronomycin, tetronasin) [14–17], C-5 (i.e., variabilin) [18] or both C-3 and C-5 of tetronare ring (i.e., pinastric acid) [19], indicating a great structural diversity of natural tetronates.

Both chemical and biosynthetic approaches were used to investigate the relationship between chemical structure and biological activity, and to generate tetronate analogs for medicinal potential. Synthetic study has achieved a great success initially. Yoshii E *et al.* [20] accomplished chemical synthesis of (+)-tetronolide in 1991; later, enantioselective synthesis of (−)-chlorothricolide was reported by Roush WR and Scitti RJ [21] in 1994. However, biosynthetic study was greatly retarded because little genetic information was provided, resulting in all early achievements confined to isotope-labeled feeding experiments. Recently, several biosynthetic gene clusters of tetronates were contiguously elucidated based on durative and intensive biosynthetic investigations. Liu W *et al.* [22] firstly reported cloning of chlorothricin gene cluster from *S. anibioticus* DSM 40725 in 2006. Cloning was performed using degenerate PCR for

highly conserved motifs of type I polyketide synthase (PKS), and the gene cluster was determined to span about 102 kb consisting of 35 open reading frames (orfs) [22]. An assembly model from chlorothricolide, two D-olivose, and 2-methoxy-5-chloro-6-methylsalicyclic acid building blocks for biosynthesis of chlorothricin was proposed in the literature. Kijanimicin gene cluster, consisting of 35 orfs, was following elucidated by probing with TDP-glucose-4,6-dehydratase gene from *S. fradiae* [23]. Analogously, a biosynthetic route for spirotetronates exemplified with kijanimicin was proposed, involving the assembly of backbone for kijanolide, incorporation of glycerol-derived three-carbon unit, two intramolecular cyclizations for kijanolide formation, and the final glycosyl transfer [23]. Following, gene clusters of tetronomycin [24], tetrocarcin A [25], RK-682 [26], abyssomicin C [27], quartromicin [28], lobophorin [29] and agglomerins [30] were subsequently identified. Comparison of these gene clusters has highlighted a set of three genes responsible for incorporation of glycerol-derived three-carbon unit to form the tetronate ring, which was well documented in biosynthetic studies of RK-682, quartromicin and agglomerins (Figure 2) [26, 28, 30].

In 2008, an overview by Schobert R and Schlenk A outlined an update on the new derivates of tetramic and tetronic acids, mainly concerning isotope-labeled feeding experiments, laboratory synthetic studies, and pharmacological aspects [31]. Whereafter, numerous biosynthetic investigations on tetronates have been reported, involving cloning and characterization of gene clusters, and *in vitro* biochemical reconstitutions [22–30]. Herein, we provide a general view focusing on *in vivo* genetic investigations and *in vitro* reconstruction of key enzymatic catalyzed reactions, to summarize an insight into the biosynthesis of tetronate antibiotics.

2 Biosynthesis of polyketide chain of tetronates

Investigations of tetronates biosynthesis were performed on a non-genetic level previously, by feeding tetronate pro-

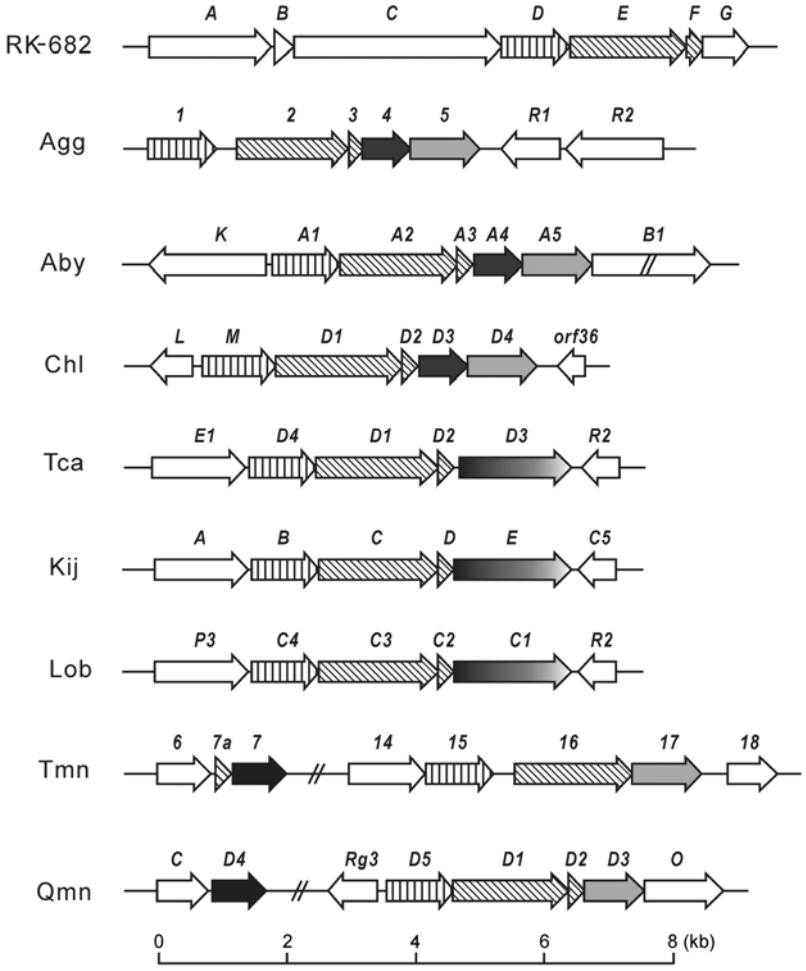

Figure 2 Comparison of gene cassettes for tetronate ring formation and dienophile biosynthesis. Agg, agglomerin; Aby, abyssomicin; Chl, chlorothricin; Tca, tetrocarcin A; Kij, kijanimicin; Lob, lobophorin; Tmn, tetronomycin; Qmn, quartromicin. Conserved genes in tetronate biosynthetic gene clusters are highlighted that responsible for tetronate ring condensation (FabH-like protein, vertical line), glycerate activation (FkbH-like and ACP proteins, oblique line), and formation of exocyclic dienophile double bond (acetyltransferase and eliminating enzyme, deep and light black).

ducing strains with isotope-labeled substrates/precursors, to determine the origin of tetronates based on incorporation of labeled atoms. Accordingly, the backbone of aglycone of tetronates and α-acyl chain are likely of polyketide origins. For chlorothricin, Floss HG *et al.* [32, 33] elucidated the origin of most carbon framework by feeding experiment: i) chlorothricolide was biosynthesized via a PKS assembly line from ten acetate and two propionate units, excluding a three-carbon unit (C22-C24, Figure 3); ii) two deoxysugars were generated from glucose; iii) 2-methoxy-5-chloro-6-methylsalisylic acid was derived from four acetate units via PKS pathway. Similarly, spirotetronates like tetrocarcin A [34], abyssomicin C [27] and versipelostatin [35], showed a PKS manner for biosynthesis of aglycone backbone that elucidated by feeding experiments (Figure 3). Straight acyl chains of acaterin [36] and agglomerin A [37], were originated from acetate units directly, however, branched three-carbon unit was failed to be isotope-labeled by feeding with three-carbon substrates excluding glycerol (Figure 3).

Biosynthesis of spirotetronate aglycone was presumed to assemble in a PKS pathway, thus it was reasonable to identify biosynthetic gene clusters by tracing PKS genes. Accordingly, chlorothricin and tetrocarcin A clusters were successfully cloned by probing PKS gene [22, 25]. In chlorothricin gene cluster, series modular PKSs have been identified that *chlA1-chlA6* consisting of a loading module and 11 elongation modules were predicted to be involved in chain elongation and modification, and inactivations of PKS genes completely blocked chlorothricin production, suggesting a collinear PKS assembly process as shown in Figure 4 [22]. Based on sequence analysis, multifunctional PKSs were found existing in all gene cluster characterized spirotetronates (i.e. chlorothricin, tetroncarcin A, kijanimicin, abyssomicin C and lobophorin), suggesting a common biosynthetic route of spirotetronate aglycone. Although modular PKSs also validated in quartromicin gene cluster, biosynthesis of quartromicin differed from other spirotetronates owing to its unique architecture, that four spirotetronic acid moieties connected by enone linkers in a head-to-tail manner [28]. Tang GL *et al.* [28] identified a module skipping strategy for quartromicin biosynthesis that two alternative PKS chains synthesized via one PKS assembly line.

Besides, some tetronates append a simple straight acyl chain on α-position of tetronate ring. It seems the acyl chain to be synthesized via fatty acid synthase pathway. RK-682, a potent inhibitor of protein phosphatases and of HIV-1proteinase, obtained its linear precursor directly from palmitic acid, which is following activated and elongated to give 3-oxo-stearoyl-*S*-ACP on a modular PKS RkC [26]. Recently, biosynthesis of agglomerins in *Pantoea agglomerans* PB-6042 was well documented, which proposed a hijacking of linear fatty acid precursor from primary metabolism, because no *rkC*-like PKS gene was found from *P. agglomerans* chromosome and successful production of hydroxyl-agglomerins in *Escherichia coli* via *agg1-agg3* (coding for glycerate activation and tetronate ring formation) alone [30]. Moreover, biosynthesis of acaterin was determined to utilize octanoate directly followed by coupling with a lactone unit [38]. All of these mentioned above strongly suggest that tetronates like acaterin, agglomerins and RK-682 with a simple straight α-acyl chain obtain their acyl groups directly from primary metabolism other than synthesize via PKS pathway involving in biosynthesis of spirotetronates aglycone.

3 Incorporation of glycerol-derived three-carbon unit into tetronic acid moiety

Chlorothrolide was primarily synthesized as polyketide chain from ten acetates and two propionates accounting for all but a three-carbon unit (C22–C24), which was following specifically labeled with an intact isotope-labeled glycerol (Figure 3). Feeding experiments of other spirotetronates (i.e., tetrocarcin A, versipelostatin and abyssomicin C), and two simple α-acyltetronates (i.e., acaterin and agglomerin A), revealed an intact incorporation of glycerol-derived three-carbon unit, suggesting a common biosynthetic mechanism for tetronate ring formation. It is of interest but still not clear that how glycerol incorporates into tetronic acid moiety while have no genetic and biochemical information. Establishment of biosynthesis of chlorothricin was expected to address this question and a biosynthetic route was pre-

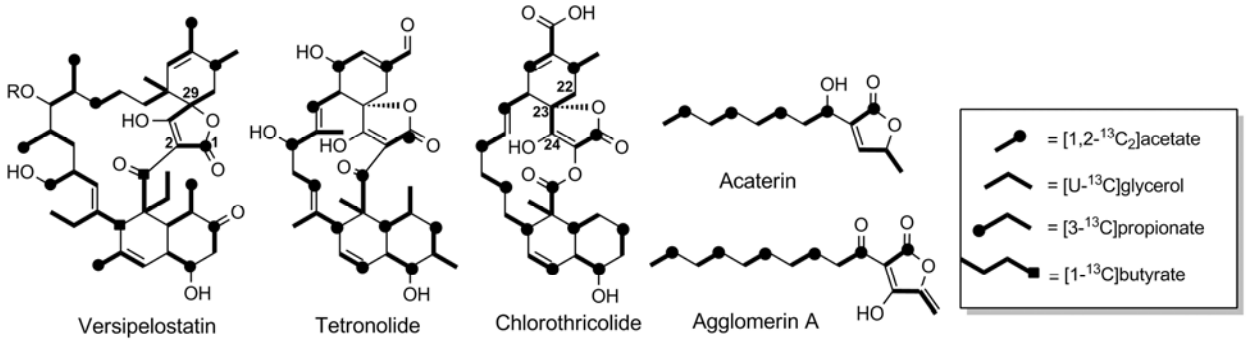

Figure 3 Incorporation of ^{13}C-labeled precursors into tetronate antibiotics.

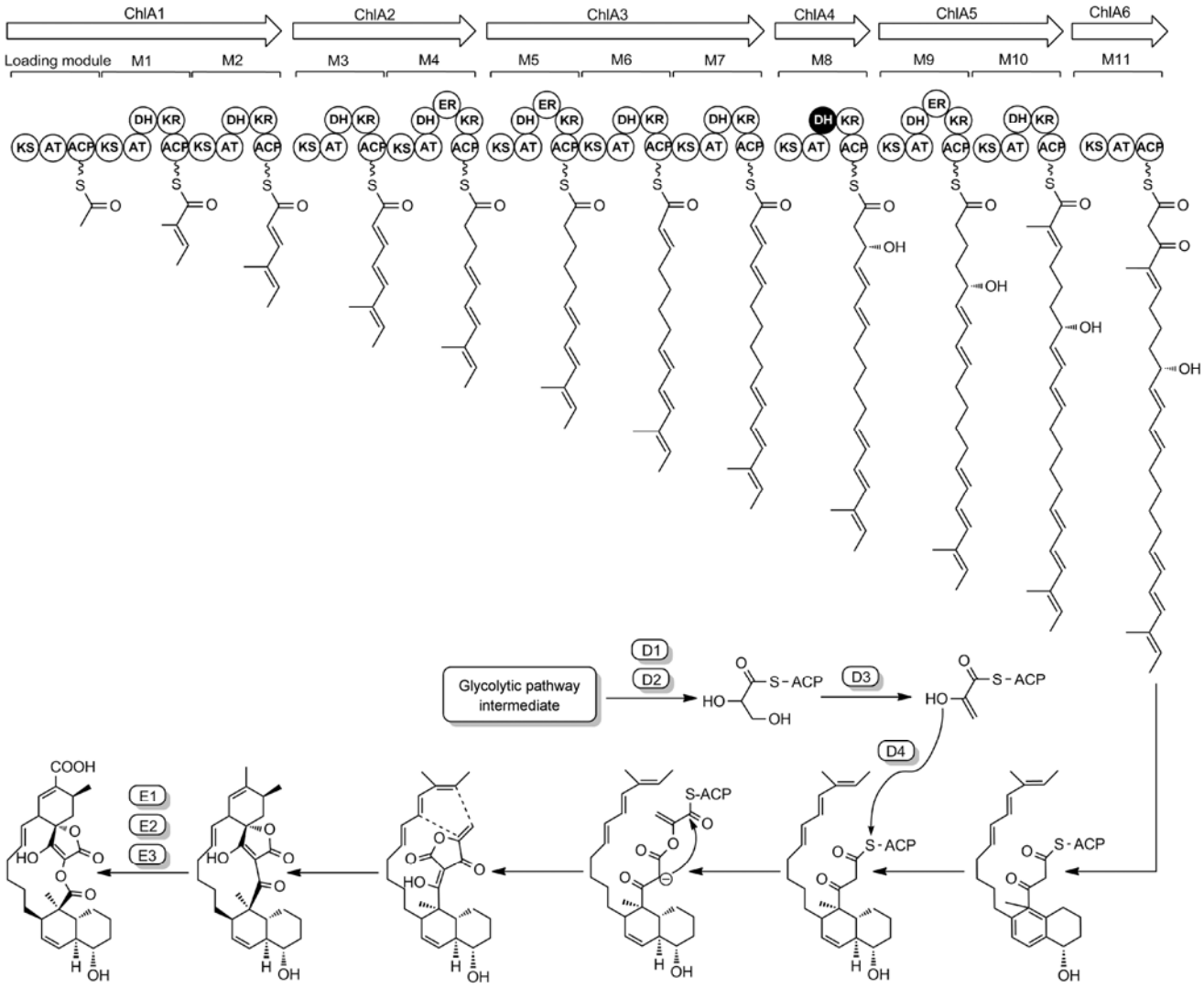

Figure 4 Proposed biosynthetic pathway of chlorothrolide [22].

dicted. ChlD1, a FkbH-like protein activates glycerate and transfers it to ACP protein ChlD2; ChlD3, a putative dehydratase, catalyzes 2,3-dehydration to generate enylpyruvoyl-*S*-ACP; ChlD4 may catalyze incorporation of enylpyruvoyl-*S*-ACP into chlorothricolide [22]. However, ChlM, a ketoacyl-ACP synthase III (FabH in *Escherichia coli*) that exits in most tetronate antibiotics, was excluded for chlorothricin biosynthesis because functional inactivation showed no influence on chlorothricin production [22].

Biosynthesis of kijanimicin elucidated by Liu HW *et al.* [23] in 2007, revealed that counterparts of ChlD1-ChlD4 and ChlM in kijanimicin cluster, KijB-KijE involved activation of glycerol-derived three-carbon unit, and its incorporation into tetronic acid moiety. KijC (a bifunctional phosphatase/glyceryltransferase, FkbH-like), activates glycerate from D-1,3-biophsphoglycerate which be following transferred to ACP (KijD); N-terminal of KijE (dihydrolipoyl acyltransferase (E2) subunit), transfers glyceryl group from glyceryl-*S*-KijD to coenzyme A (CoA) to generate glyceryl-*S*-CoA; KijB (FabH-like protein), is proposed to catalyze condensation of a C-2 carbanion of kijanolide polyketide-*S*-ACP and glyceryl-*S*-CoA; Following, C-terminal of KijE (α/β-hydrolase superfamily proteins), acts as thioesterase to catalyze formation of tetronate ring and release of polyketide from PKS assembly line [23].

Series of biosynthetic investigations on tetronates (i.e., chlorothricin, kijanimicin, tetroncarcin A and tetronomycin), have outlined a common biosynthetic mechanism for tetronate ring formation. However, evidence remains obscure in absence of biochemical evidence, until Sun YH and Leadlay PF [26] have reconstructed RK-682 biosynthesis *in vitro* (Figure 5). As previously reported in tetronomycin biosynthesis, RkE (Tmn16 in tetronomycin cluster, FkbH-like) catalyzes D-1,3-biphosphoglycerate to form glyceryl-*S*-RkE, which is then transferred to RkF (Tmn7a, ACP) and generates glyceryl-*S*-RkF. Following, glyceryl-*S*-RkF is cyclized

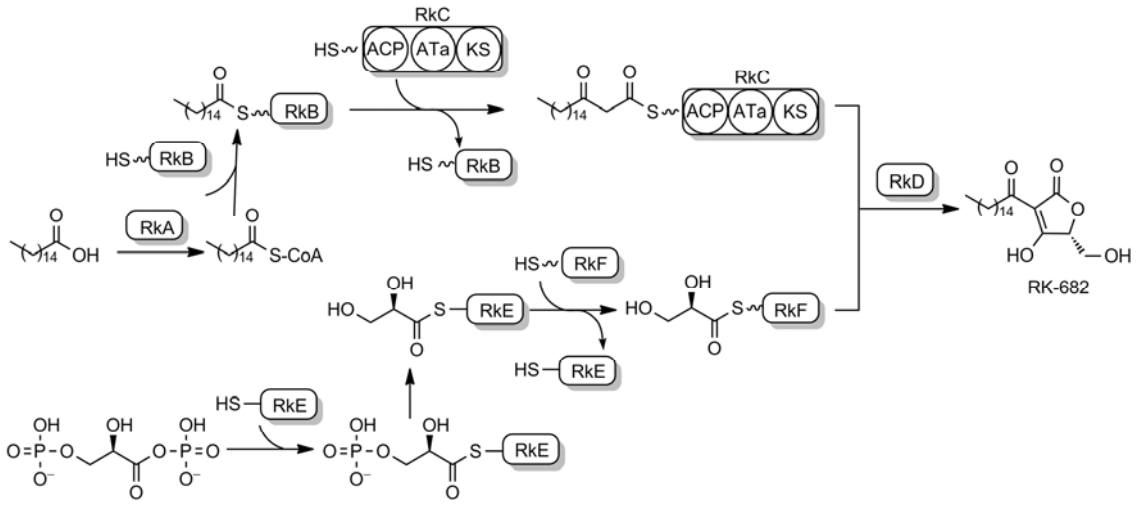

Figure 5 Biosynthetic mechanism of tetronate ring formation exemplified for RK-682 [26].

with 3-oxo-stearoyl-*S*-ACP in the presence of RkD (FabH-like) to produce RK-682. Analogous biosynthetic pathway also involved in biosynthesis of quartromicin and agglomerins, it seems highly likely to be involved in the biosynthesis of most tetronates except for acaterin. Endo A *et al.* [38] proposed a biosynthetic mode for acaterin that coupling of a five-carbon lactone with octanoate, rather than assembling via attachment of glyceryl-*S*-ACP to α-position of a decanoate derivative that commonly happens in other structure related tetronates.

4 Formation of tetronlides

Spirotetronates are of particular interest due to their intriguing structures, that tetronic acid moiety after formation is spiro-linked to a cyclohexene ring to form a mature aglycone. It involves an intramolecular [4+2] cycloaddition that falls into Diels-Alder reaction. In most spirotetronates, after polyketide backbone was produced, two intramolecular Diels-Alder-like reactions were tandemly happened, excluding abyssomicin C (one [4+2] cycloaddition) [27] and quartromicin (four intermolecular [4+2] cycloadditions) [28], to form the octahydronaphthalene ring and spirotetronate ring successively. Although Diels-Alder reaction has attracted substantial attention for a long time in tetronates biosynthesis, it remains inconclusive because there are no sequence similarity between proteins encoded in tetronate clusters and reported Diels-Alderases [22, 28]. Nevertheless, natural spirotetronates do appear to arise via Diels-Alder-like reactions after a specific dehydratin of initially-formed tetronate precursors to provide a dienophile. Formation of a dienophile on tetronate ring was crucial for [4+2] cycloaddition, of which the mechanism was documented recently in agglomerins biosynthesis [30]. Intensive comparison of tetronate clusters has highlighted a set of three-gene for glycerate activation and tetronate ring formation that existed in all characterized tetronate clusters, whereas another two-gene unit (in some cases fused as one, like *tcaD3* and *kijE*) involving dehydration was validated in all but RK-682 cluster (Figure 2). The dehydration actually involved two steps after formation of tetronic acid moiety in agglomerins biosynthesis. Firstly, Agg4 catalyzed O-acetylation and following Agg5 catalyzed the elimination of acetic acid to generate a dienophile (Figure 6) [30]. Analogously, the unusual two steps of acetylation-elimination should occur in all spirotetronates, catalyzing by enzymes homologous to Agg4 and Agg5 to generate the dienophile for [4+2] cycloaddition.

5 Glycosyl transfer on mature aglycone

Natural spirotetronates share a similar macrocyclic aglycone that a tetronic acid moiety conjugates with a trans-decalin system by a carboxylic ester in chlrothricin or a carbonyl group in kijanimicin-like spirotetronates (i.e., kijanimicin, tetrocarcin A, and versipelostatin). In general, the macrocyclic aglycones consist of 13-14 members, excluding abyssomicin C has 12-members, versipelostatin contains 17-members, and quartromicin has a characteristic 32-member carbocyclic architecture. Besides, deoxysugar decorations on aglycone greatly expand the structural diversity of spirotetronates, and render a versatile biological activity.

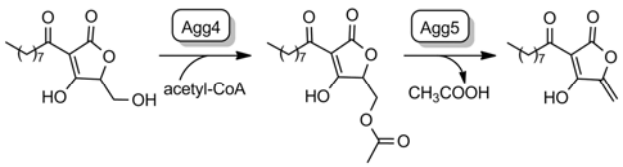

Figure 6 Biosynthetic mechanism of dienophile (double bond) formation exemplified for agglomerins [30].

Natural products possessing deoxysugar moieties that serve as recognition elements are crucial for biological activity, i.e., erythromycins and vancomycin dramatically decreased biological activity while removed sugar elements [40]. Spirotetronates have a similar glycosylation location, and show distance patterns in sugar moieties and chain length. Antitumor agent tetrocarcin A, contains the richest and most diverse unusual sugars. It has two sugar side chains appended, one comprising of alternate L-digitoxoses and L-amicetoses and the other with a rare 2,3,4,6-tetradeoxy-4-(methylcarbamyl)-3-C-methyl-3-nitro-D-xylo-hexopyranose named D-kijanose that also exists in kijanomicin and lobophorin [25]. Alterations of tetrasaccharide chain of tetroncarin A remarkably affect its Bcl-2 inhibition activity, indicating a glycodiversification of C-9 and a high potential to create novel tetrocarcin analogs with improved therapeutic value [41].

Glycosyltransferases with relaxed substrate specificity have been well characterized in natural products biosynthesis, which could be a powerful tool for combinatorial biosynthesis of analogs with altered glycolsylation patterns. In most cases, number of glycosyltransferases is generally identical to that of deoxysugars in spirotetronates. Interestingly, few glycosyltransferases are found in tetrocarcin A and lobophorin clusters, indicating that at least one involve twice in formation of saccharide chain [25, 29]. Recently, Zhang C *et al.* [29] dissected the glycosylation order in lobophorin biosynthesis based on systematic inactivation of individual glycosyltransferase genes and identification of the intermediates resulted. LobG3 was determined to act iteratively to append two L-digitoxoses at C-9 after lobG1 appending the unique kijanose at C-17; Finally, LobG2 appended terminal digitoxose to complete glycosylation for lobophorin biosynthesis (Figure 7) [29]. Inactivation of glycosyltransferases resulted expanding of structural diversity of lobophorin with altered glycolsylation patterns [29]. It provides a good example of combinatorial biosynthesis to generate novel products of interest by genetic engineering of the biosynthetic pathway.

6 Conclusions

Biosynthesis of natural tetronates elucidated to date shows a common biosynthetic route, comprising of a PKS manner/fatty acid pathway derived backbone, a typical condensation between 3-oxo-acyl-*S*-ACP and glyceryl-*S*-ACP catalyzed by FabH-like protein, formation of spirotetronate ring via a Diels-Alder-like reaction, and final decorations and tailoring of the cyclic aglycone unit. Most of which have been well characterized, except for the Diels-Alder reaction for spirotetronate ring formation, which still remains inconclusive. However, our recent report on biosynthesis of agglomerins has elucidated the formation of a dienophile that is essential for the subsequent Diels-Alder rearrangement in spirotetronate biosynthesis, and it facilitates the biosynthetic investigation of spirotetronate ring formation via chemoenzymatic approaches [30]. Meaningly, two newly identified compounds with tetronic acid moiety, malleilactone [42] and burkholderic acid [43] from human pathogenic *Burkholderia* species seems to be synthesized via a noncanonical nonribosomal peptide synthetase (NRPS)-PKS pathway and formation of tetronate ring appears to be performed via a FabH-independent manner. However, enzymes involved head-to-head fusion of two PKS derived precursors are not yet known. Recently, many novel natural products have been identified by newly developed screening strategies, i.e., mining genomes for cryptic biosynthetic pathways. Biosynthesis of tetronate ring via a highly conserved FabH-dependent pathway reviewed in this paper, which may be helpful for identifying more tetronate analogs by tracing the conserved FabH-FkbH-ACP encoding cassette from microbial genome database. Moreover, the combinatorial biosynthesis strategy also allows facile generation of novel tetronate analogs via genetic engineering of the biosynthetic pathway of interest.

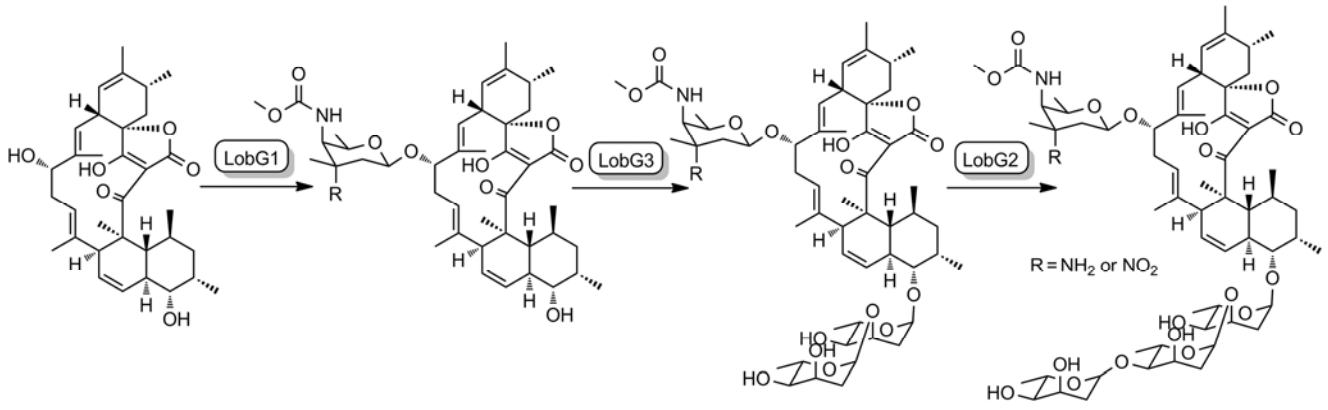

Figure 7 Glycosylations in lobophorin biosynthesis [29].

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