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Biosynthesis and Chemical Diversity of β-Lactone Natural Products

Serina L. Robinson,[a] James K. Christenson,[a,b] and Lawrence P. Wackett[a]

Covering: up to 2018

β-Lactones are strained rings that are useful organic synthons and pharmaceutical warheads. Over 30 core scaffolds of β-lactone natural products have been described to date, many with potent bioactivity against bacteria, fungi, or human cancer cell lines. β-Lactone natural products are chemically diverse and have high clinical potential, but production of derivatized drug leads has been largely restricted to chemical synthesis partly due to gaps in biochemical knowledge about β-lactone biosynthesis. Here we review recent discoveries in enzymatic β-lactone ring closure via ATP-dependent synthetases, intramolecular cyclization from seven-membered rings, and thioesterase-mediated cyclization during release from nonribosomal peptide synthetase assembly lines. We also comprehensively cover the diversity and taxonomy of source organisms for β-lactone natural products including their isolation from bacteria, fungi, plants, insects, and marine sponges. This work identifies computational and experimental bottlenecks and highlights future directions for genome-based discovery of biosynthetic gene clusters that may produce novel compounds with β-lactone rings.

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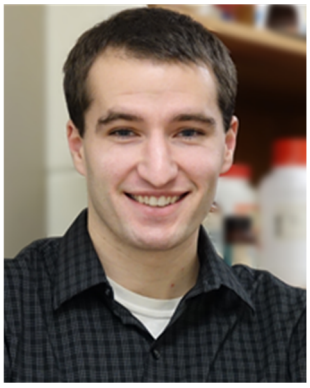


Lawrence P. Wackett received his Ph.D. at the University of Texas at Austin, working with David T. Gibson on enzymes that metabolize aromatic hydrocarbons. He went on to do postdoctoral studies at MIT with Christopher T. Walsh. He then travelled to the ETH-Zürich, collaborating with Thomas Leisinger, and started thereafter as an assistant professor at the University of Minnesota in the Department of Biochemistry, Molecular Biology and Biophysics and the BioTechnology Institute. In 2001, he became Distinguished McKnight University Professor at Minnesota. His research is in two main areas, novel microbial enzymes that biodegrade organic compounds and natural product biosynthesis.

1 Background and Reactivity

β-Lactones (2-oxetanones) are often referred to as 'privileged' structures as they have highly-reactive electrophilic scaffolds and serve as versatile synthetic intermediates. The first synthesis of a β-lactone was described by Einhorn in 1883,[1] but it was not until the mid-1950's that the first β-lactone natural product, the potent neurotoxin, anisatin, was isolated from the fruit of a Japanese spice tree.[2] The first complete asymmetric synthesis of a β-lactone was later reported in 1982,[3] laying the foundation for the use of chiral β-lactones in the total synthesis of natural products and their derivatives. A query of SciFinder revealed two spikes in the publication record of β-lactones (Fig. 1). The first spike in the 1960's – 1970's corresponded to commercial interest in β-lactones as building blocks to make biodegradable polyesters.[4,5] It was not until the second

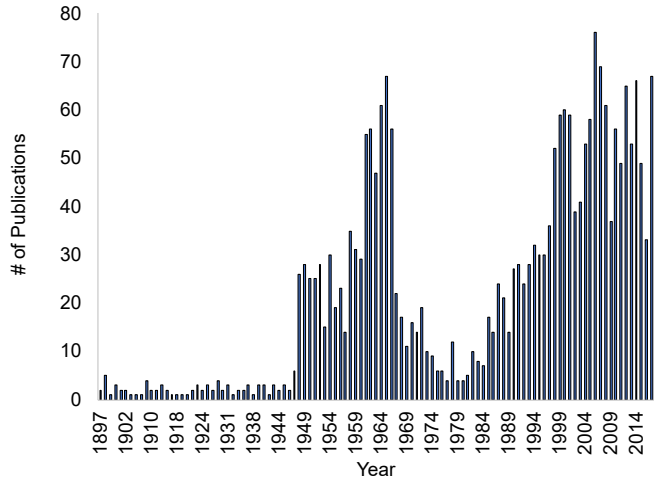


Figure 1. Number of publications in SciFinder Scholar with the word 'beta-lactone' in the title or keyword by year up to 2017.

wave, corresponding largely to a surge in β-lactone natural product discovery, that the potential clinical, synthetic, and chemoproteomic applications of β-lactones began to emerge. Interestingly, most β-lactone natural products were not reported until long after the post-World War II 'golden age' of antibiotic discovery. Only three distinct β-lactone natural product scaffolds (anisatin,[6] hymeglusin,[7] and oxazolomycin[8]) were discovered before 1975. From the 1980's through the 2000's, however, nearly 30 novel bioactive compounds with β-lactone functional groups were isolated from the culture broth of fungi and bacteria. As recently as 2015 and later, several distinct β-lactone analogs of known natural product scaffolds were discovered, including the cystargolides,[9] ostalactones,[10] bisoxazolomycins,[11] and vibralactoximes.[12] The wave of β-lactone natural product discovery may have been delayed partly due to the instability of β-lactone rings to chemical workup following biological extractions. β-Lactones readily undergo acid or base hydrolysis and are subject to thermal degradation. That may have caused β-lactone natural products to elude discovery by traditional extraction methods and they may represent an understudied class of compounds with regards to their biosynthesis. Recent developments in genome-based natural product discovery and synthetic biology techniques offer new potential to uncover the gene clusters encoding 'orphan' β-lactone compounds and promote discovery of novel β-lactone natural products.

Although overshadowed by the success of the β-lactams, β-lactones have received significant attention in their own right for their antimicrobial, anticancer, and antiobesity properties. Notably, in 1999, the β-lactone natural product derivative tetrahydrolipstatin (Xenical,

Alli) was approved by the U.S. Food and Drug Administration (FDA) as a weight loss drug.[13] Another β-lactone secondary metabolite, salinosporamide A, completed phase II clinical trials for multiple myeloma and glioblastoma and is currently in phase III clinical trials for combination treatment of glioblastoma.[14] Despite these few successes, the therapeutic potential for this potent class of molecules remains underexplored relative to the β-lactams, which comprise greater than 50% of antibiotics on the market today.[15]

The β-lactams and β-lactones belong to a class of strained four-membered heterocycles. The release of the inherent ring strain facilitates nucleophilic attack from catalytic serine, threonine, or cysteine residues in enzyme active sites (Fig. 2). Enzymes with these activated catalytic nucleophiles are susceptible to inhibition by forming a stable covalent adduct with an opened β-lactam or β-lactone ring. The major physiological targets of β-lactams are the penicillin-binding proteins (PBPs) involved in polymerization and cross-linking of peptidoglycan.[16-18] In contrast, chemical profiling studies have revealed that β-lactones target >20 different enzymes representing four different enzyme classes: hydrolases, transferases, ligases, and oxidoreductases.[19] Specific clinically-relevant targets of the β-lactones include lipases, proteases, esterases, and fatty acid synthetase thioesterase (TE) domains, which have applications in anticancer, antimicrobial, and antiobesity therapeutics.

There are a number of chemical factors that contribute to the broad target range of these reactive heterocycles. β-Lactone rings have dual modes of reactivity with two potential sites of nucleophilic attack (either the C_2 or C_4 positions) resulting in an ester or an ether, respectively (Fig. 2), whereas β-lactam rings typically undergo

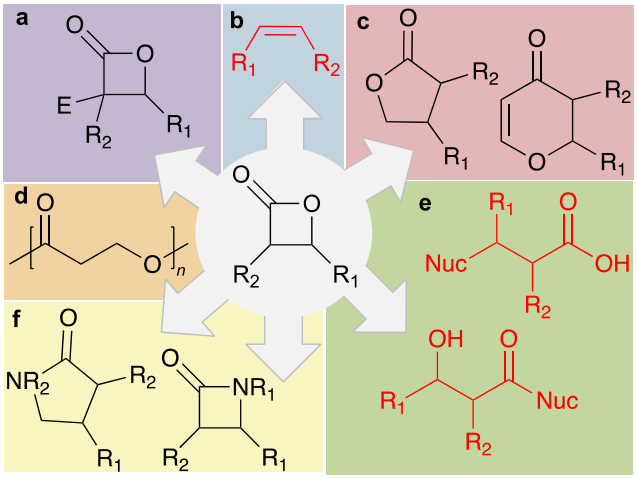


Figure 3. Known (bio)chemical transformations of β-lactones. Reactions for which enzymatic biocatalysis is also known (3b, 3e) are highlighted in red. E = electrophile. Nuc = nucleophile.

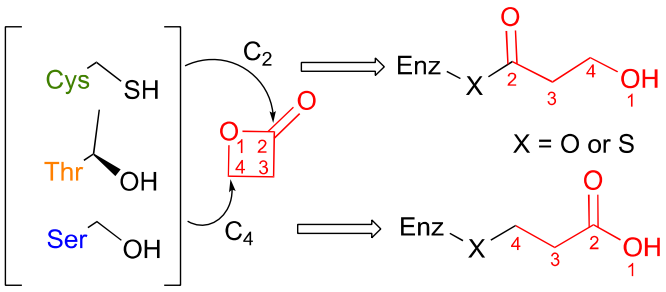


Figure 2. Dual sites of nucleophilic attack by cysteine, threonine, or serine residues results in two modes of ring opening.

nucleophilic attack exclusively at the acyl (C_2) position. The diversity of cellular targets is both a boon and a pitfall as the high reactivity can also cause off-target effects. Rational drug design and testing is paramount to realize the potential of this reactive but powerful class of molecules. For further insight into the mode of action of β-lactones we refer readers to in-depth reviews on targeted covalent inhibitors.[20,21]

Organic chemists have long used β-lactones as intermediates for the total synthesis of products with diverse functional groups that includes alkenes, heterocycles, and hydroxy acids (Fig. 3). While β-lactones are reported to have a similar ring strain and reactivity as epoxides (22.8 and 27.2 kcal/mol, respectively),[22] their dual sites of attack results in the capability to undergo different transformations than epoxides. The release of the strained β-propionolactone ring conformation is achieved through synthetic methods including electrophilic addition through an enolate intermediate (Fig. 3a), Lewis acid catalyzed rearrangements (Fig. 3c,) polymerization (Fig. 3d), nucleophilic attack (Fig. 3e), and decarboxylation (Fig. 3b).[31,32] The latter two of these are reactions also known to be catalyzed by enzymes in nature.[23,24] Romo and colleagues developed a direct one-pot conversion method to β-lactams,[25] while Namy and Machrouhi demonstrated conversion of β-lactones to γ-lactams (Fig. 3f).[26] The versatility of these synthons also led to their use in applications including the polymerization of β-propiolactone into polyesters found in plastics, clothing, and surgical materials.[27] The β-lactone ring has been used as an intermediate structure for total synthesis of >20 natural products, including enterobactin,[28] (+)-omphadiol,[29] and (+)-maculalactone A.[30]

Comprehensive reviews on organic synthesis of β-lactones and their utility as intermediates for the total synthesis of natural products have been published by Romo and others and will not be covered here.[25,31,32] However, a review focused specifically on the taxonomic diversity of β-lactone natural products, their biosynthetic

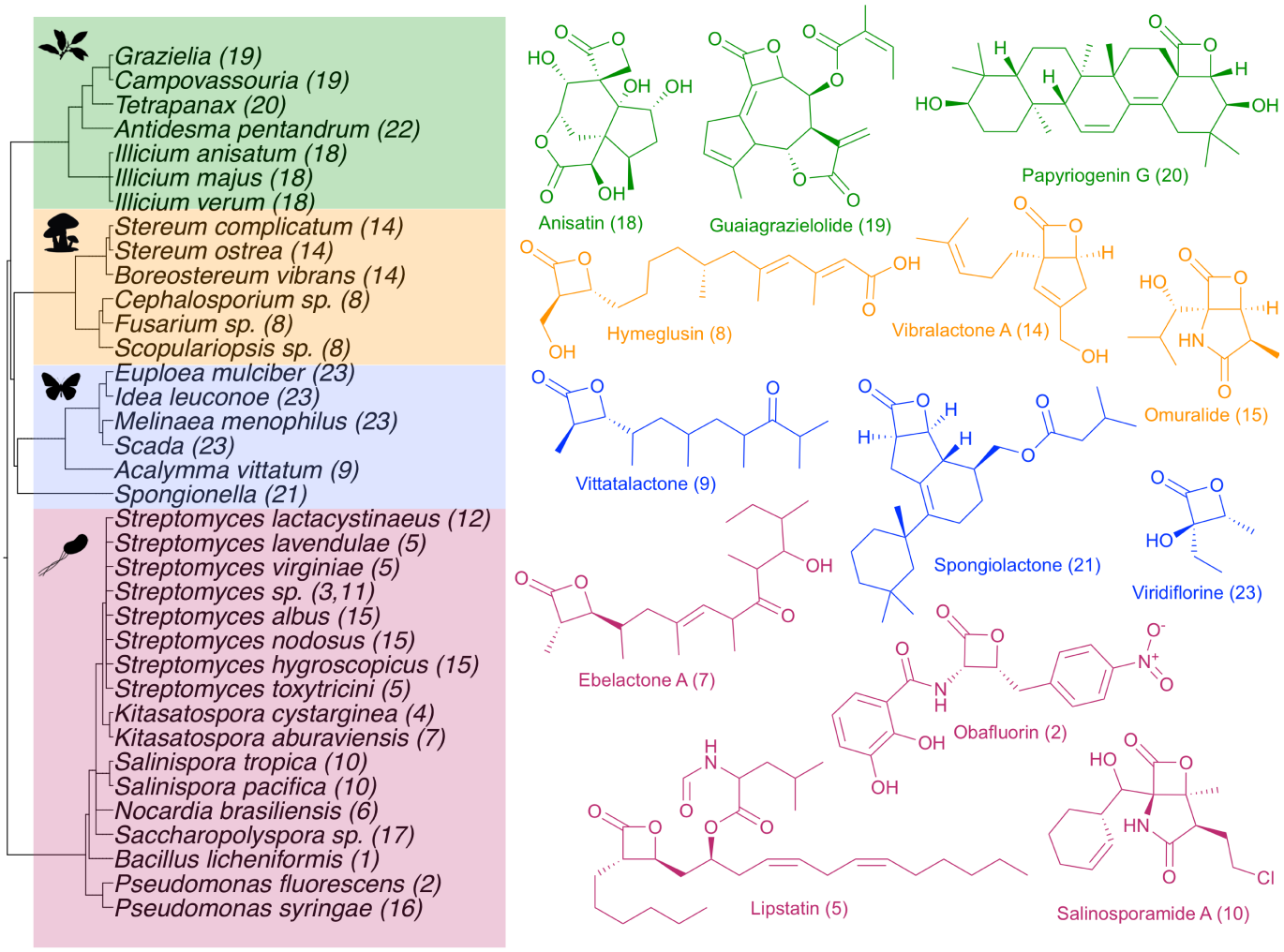


Figure 4. Phylogenetic diversity of source organisms from which β-lactone natural products have been isolated. Phylogenetic relationships correspond to the taxonomic ranking established by the National Center for Biotechnology Information (NCBI). Numbers on tree leaves correspond to the compound isolated from each source organism.

been published since 1995.[33,34] Recent breakthroughs uncovering mechanisms for enzyme-catalyzed β-lactone ring formation has opened new opportunities for genome-based prediction of the β-lactone functional group in nature.[23,35] Here we present an overview of the chemical family of β-lactone natural products, organized by their compound class and taxonomy of the source organisms (Fig. 4), with emphasis on the enzymatic mechanisms of β-lactone ring formation. This review aims to connect biosynthetic pathways with β-lactone natural products and highlight future directions in the field.

2 Mechanisms of β-lactone biosynthesis

2.1 Known Mechanisms

Enzymatic mechanisms for β-lactone formation primarily rely on carboxyl activation of a β-hydroxy acid (3-hydroxy acid) precursor to provide the energy for cyclization. The first report of an enzyme capable of catalyzing β-lactone ring formation was published in January 2017, discovered unexpectedly in a biosynthetic pathway for long-chain olefinic hydrocarbons from the plant pathogen *Xanthomonas campestris* (Fig. 5).[23] The first enzymes of the pathway, OleA and OleD, generate a β-hydroxy acid from the Claisen condensation of acyl-coenzyme A (CoA) precursors. The β-lactone synthetase, OleC, was found to convert *syn*- and *anti*-β-hydroxy acids to *cis*- and *trans*-β-lactones, respectively in an adenosine triphosphate (ATP)-dependent reaction (Fig. 6a).[23] The preservation of stereochemistry is consistent with the activation of the carboxylic acid followed by attack of the β-hydroxyl group to form the formation of a β-lactone ring. The disubstituted β-lactone then undergoes a physiological decarboxylation to an alkene catalyzed by a stereoselective β-lactone decarboxylase enzyme, OleB.[24]

The OleC-like β-lactone synthetases are members of the Acyl-CoA synthetase, Nonribosomal peptide synthase (NRPS) adenylation domains, and Luciferase (ANL) superfamily that includes CoA ligases, transfer RNA synthetases, and acyl- and amino acid adenylation domains.[36] Carboxylic acid activation by AMP is conserved within the ANL superfamily.[36] Four additional homologs of the OleC β-lactone synthetase from phylogenetically-diverse bacteria were purified and shown to be active in the cyclization of β-hydroxy acid precursors.[23] Additionally, the amino acid sequence identities of enzymes in the β-lactone natural product biosynthesis pathways for lipstatin (LstC) and ebelactone (Orf1) pathways are more similar to the sequence of *X. campestris* OleC than some experimentally confirmed OleC enzymes from the olefin pathway (Fig. 5).[23] These preliminary data suggest this mechanism of ring-closing may be widespread in β-lactone natural product biosynthesis, but further experimental work is required to explore this hypothesis.

Later in 2017, a second mechanism for β-lactone ring formation was reported as the final step of release from an NRPS assembly line. The *cis*-β-lactone ring in the compound obafluorin is formed by the type I TE domain of ObiF.[35] The ObiF TE has a rare catalytic cysteine instead of a serine in the GxSxG active site motif common in most TE domains. The direct β-hydroxy acid precursor to obafluorin is generated from upstream Obi enzymes and tethered via a thioester bond to the TE domain (Fig. 6b). Intramolecular attack of the β-hydroxyl group onto the thioester carbonyl simultaneously generates the β-lactone of obafluorin and releases it from the NRPS assembly line. Site-directed mutagenesis of the ObiF C1141 demonstrated that substitution of the cysteine with a serine or alanine abolishes β-lactone cyclization.[35] This method of lactonization is similar to the macrocyclization reactions that are known to occur in polyketide synthase (PKS) and NRPS systems that produce a macrolactone (erythromycin) and macrolactam (gramicidin S).[37] Homologous TE domains to ObiF with the rare catalytic cysteine were detected in other gene clusters that likely make obafluorin-like products in the pseudomonads *Burkholderia diffusa* and *Chitiniphilus shinanonensis*. The authors speculate that these TE domains with the GxCxG motif are rare (< 6.8% of type I TEs) and represent a specific signature for TE domains capable of catalyzing β-lactone ring formation.[35]

2.2 Proposed Mechanisms

Additional mechanisms of enzyme-mediated β-lactone formation have been proposed but further experimental evidence is required. Biochemical studies on salinosporamide A suggest the presence of a thioester-tethered intermediate with the peptidyl carrier protein (PCP) of the NRPS SalB as shown in Fig 6c.[38] A bicyclization reaction of this intermediate would simultaneously form the β-lactone/γ-lactam core and release the final salinosporamide product from the PCP domain. The stand-alone TE domain, SalF, may also participate, although it is also proposed to remove misprimed molecules from the PKS/NRPS assembly line.[39] SalF is a type II TE instead of a type I like the obafluorin TE domain and does not have a discernible GxCxG motif. In light of the recent discovery of β-lactam formation by a condensation (C) domain in nocardicin biosynthesis,[40] it is also possible the preceding C domain from SalA could contribute to ring formation.

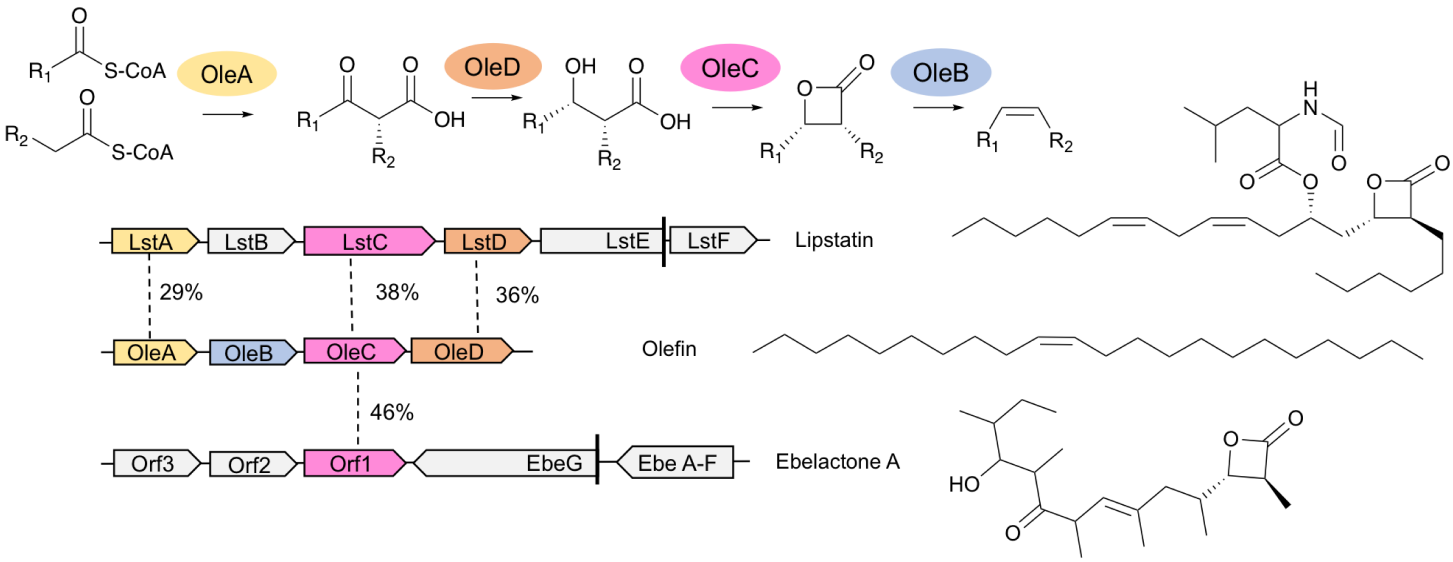


Figure 5. Olefin gene cluster and homologs in β-lactone biosynthesis identified by Christenson et al.[23]. Figure adapted with permission from *Biochemistry* **2017**, *56*, 348-351.[23] Copyright 2017 American Chemical Society.

Regardless of the exact mechanism, this route is distinct from that of obafluorin, although both appear to rely on the energy released from the thioester intermediate to drive β-lactone formation.

The formation of the β-lactone ring in vibralactone and ostalactone does not rely on activated β-hydroxy acids, but rather originates from a 2-(*3H*)-oxepinone structure, a seven membered lactone ring with double bonds at the four and six positions (Fig. 6d). Indeed, biochemical studies have validated this proposal as incubation of the 2-(*3H*)-oxepinone precursor with a cell-free crude enzyme preparation from the mycelia of its host organism, *Borostereum vibrans*, resulted in fused bicyclic β-lactone ring formation.[94] This reaction was not observed in a boiled enzyme control, suggesting ring closure of the oxepinone precursor is enzyme-mediated. We speculate similar mechanisms may be operative in the multi-cyclic plant β-lactones lupeolactone and guaiagrazieloide. However, an enzyme capable of catalyzing the intramolecular seven-membered ring cyclization has not yet been characterized.

Additional enzymatic mechanisms of β-lactone formation have been proposed for various natural products, but currently there are insufficient experimental data to confirm these different mechanisms. The proposed final NRPS module for the synthesis of the spiro-β-lactone oxazolomycin lacks a TE domain, but rather has an extra C domain.[41] Specialized C domains are known to perform cyclization reactions such as those of bacitracin and bacillamide, but none have been reported to form a four-membered lactone.[42] The C

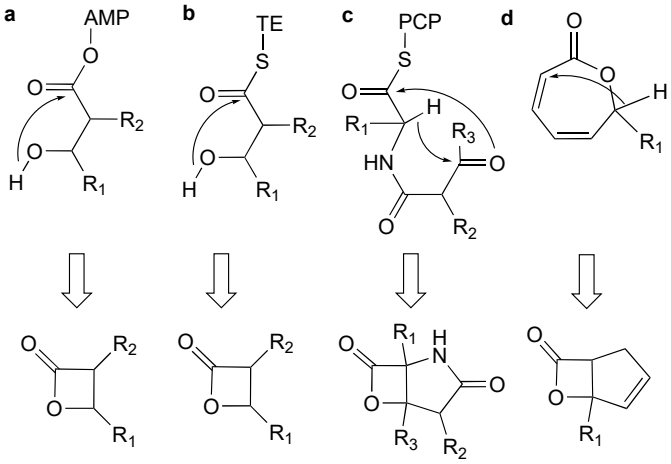


Figure 6. Enzymatic mechanisms of β-lactone ring biosynthesis. Reactions a) and b) have been confirmed by *in vitro* activity assays while c) and d) are inferred from indirect biochemical evidence.

domain in oxazolomycin lacks the conserved HHxxxDG motif of typical C domains as well as the DxxxxD motif of specialized cyclization C domains, but rather contains an unusual NYFCLDG motif. More experimental evidence is required to confirm the mechanism of spiro-β-lactone formation in oxazolomycin. A second proposal is from the polyketide-derived ebelactone. There is limited knowledge of the β-lactone ring biosynthesis in ebelactone, but the authors noted the presence of an extra KS domain at the end of the PKS and the absence of a TE domain.[41] The authors proposed a mechanism of non-enzymatic ring closure with concurrent release from the PKS assembly line but left open the possibility of a KS-facilitated cyclization.[41] In belactosin and cystargolide biosynthesis, the authors proposed lactonization from β-hydroxy methyl ester substrates, but it is unclear if these intermediates can energetically drive β-lactone ring formation non-enzymatically.[43]

Non-enzymatic β-lactone formation from activated precursors has been demonstrated in several systems. Lactacystin is the precursor to omuralide (*clasto*-lactacystin) and contains a hydroxyl group in the β-position to a thioester bond. The hydroxyl group spontaneously attacks the thioester linkage to form the lactone ring of omuralide.[44] Ebelactone is proposed to arise non-enzymatically from a β-hydroxythioester intermediate tethered to a PKS.[45] Wyatt and co-workers demonstrated a synthetic intermediate tethered to an *N*-acetylcysteamine moiety cyclizes to form the β-lactone.[45] Authors of lipstatin, cystargolide, and belactosin biosynthesis studies also proposed non-enzymatic β-lactone ring formation from β-hydroxy acids precursors activated by CoA, again utilizing thioester bond cleavage to drive ring formation.[13,43] However, recent bioinformatics analysis has also identified putative OleC-like β-lactone synthetases in many of these gene clusters, suggesting adenylation followed by intramolecular attack as an alternative of ring formation.[23] Concerning non-enzymatic β-lactone ring formation from β-hydroxythioesters, we speculate that future studies will find this is a rare occurrence compared with an enzyme-mediated process. Activated β-hydroxy thioesters are common intermediates in diverse NRPS, PKS, and fatty acid synthesis pathways, yet reports of spontaneous cyclization are not common. However, cyclization is clearly favourable enough under normal cellular conditions to allow for some non-enzymatic cyclization. An ongoing challenge in the field is differentiating between spontaneous and enzyme-catalyzed β-lactone cyclization from activated intermediates.

2.3 Comparisons with β-Lactam Biosynthesis

Table 1. General mechanisms of β-lactone and β-lactam ring closure showing unique and shared reaction types. TE = thioesterase; C = condensation; PCP = peptidyl carrier protein.

	β-lactones		β-lactams	
	Natural products	**Description**	**Natural products**	**Description**
ATP-dependent synthetase	Olefins	OleC-catalyzed adenylation of the COOH of β-hydroxyacid precursors[23]	Clauvlanic acid Carbapenems	β-lactam and carbapenem synthetase-catalyzed adenylation of the COOH of β-amino acid precursors[48]
NRPS TE-domain	Obafluorin	Catalytic cysteine residue (GxCxG motif) in TE domain forms a β-hydroxythioester for ring closure[35]	Sulfazecin	Catalytic cysteine residue (AxCxG motif) in TE domain forms a β-aminothioester for ring closure[50]
Oxidative cyclization	Unknown	Unknown	Isopenicillin N Cephalosporins Cephamycins	Isopenicillin N synthase (non-heme iron, non-α-ketoglutarate dependent)[51-53]
NRPS C-domain	Unknown	Unknown	Nocardicins	NRPS C domain condenses two amino acids and forms a β-lactam ring tethered to a PCP domain[40]
7-Membered ring closure	Vibralactones Ostalactones	Cyclization from a seven membered lactone to form a β/γ fused ring[10]	Unknown	Unknown

Mechanistic insights can be gained by comparing β-lactone and β-lactam biosynthesis. There are currently four known mechanisms to form β-lactam rings,[46] two of which are shared with β-lactones (Table 1). AMP-dependent activation of β-amino acids by β-lactam synthetase and carbapenem synthetase is a widespread mechanism for β-lactam formation.[46] Similar to the OleC-type β-lactone synthetases, the carboxylic group is activated with AMP followed by a ring closure step.[48] Interestingly, there is low sequence similarity between the β-lactone and β-lactam synthetases with the exception of the conserved ATP binding site. Both the β-lactam and carbapenem synthetases diverged from an ancestral asparagine synthetase which transfers ammonia to the AMP-activated carboxy side chain of aspartic acid.[48] OleC-like β-lactone synthetases, however, belong to the ANL enzyme superfamily and show the greatest sequence similarity to NRPS adenylation domains in the Protein Data Bank. Crystal structures of β-lactam synthetases have enabled detailed mechanistic understanding of these enzymes.[47,48] Currently no structures have been solved for OleC-like β-lactone synthetases, although synchrotron x-ray diffraction data for the *Xanthomonas campestris* enzyme has been collected to 3.4 Å resolution.[49]

The second shared mechanism between β-lactams and β-lactones is TE-mediated cyclization from NRPS domains. A recent publication demonstrated that the monobactam sulfazecin is formed during release from a specialized TE domain.[50] Similar to obafluorin, the TE active site contains a cysteine group rather than the conserved serine, suggesting a thioester bond is critical to drive cyclization. The reported sequence motif for the β-lactam TE, AxCxG, is structurally similar to that of β-lactone TE domains, GxCxG.[35,50] Both the β-lactam and β-lactone TE domains are part of the α/β hydrolase superfamily as is typical of TE domains. Both of these specialized TE domains were reported in 2017 and structures have yet to be published for these intriguing TE domains.

Currently, there appear to be two mechanisms of ring formation unique to β-lactam biosynthesis and one additional mechanism unique to β-lactone biosynthesis (Table 1). The iron and oxygen dependent isopenicillin N synthase is a 2-His-1-carboxylate facial triad enzyme[51] and no gene encoding a homolog has been identified in any β-lactone natural product gene cluster. Isopenicillin N synthase has a specialized mechanism to form a β-lactam ring from the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine in penicillin and cephalosporin antibiotics.[52,53] The second mechanism exclusive to β-lactams to date is that of C domain mediated cyclization in nocardicin biosynthesis.[40] This C domain catalyzes both the condensation and ring formation of two peptides to form the β-lactam ring with the peptide still attached to the PCP domain of the NRPS. This mechanism requires Michael addition of a secondary amine to a dehydroalanine intermediate generated from an unusual third histidine residue directly upstream of the canonical

Table 2. Biosynthetic gene clusters for β-lactone products. PKS = polyketide synthase; NRPS = nonribosomal peptide synthase. MiBIG = minimum information about a biosynthetic gene cluster database.

Natural product	Year	Medicinal properties	GenBank accession	MiBIG accession	Biosynthesis	Source organism(s)
Ebelactones[45]	1980	Esterase inhibitor	KC894072	BGC0000051	PKS	*Kitasatospora aburaviensis*
Obafluorin[35]	1984	Weak, broad-spectrum antibacterial and antifungal agent	KX134682-KX134695	BGC0001437	Amino acid	*Pseudomonas fluorescens*
Oxazolomycin[41]	1985	Cytotoxic to leukemia cell line (P-388) and gram-positive bacteria	EF552687	BGC0001106	Hybrid PKS/NRPS	*Streptomyces albus*
Lipstatin[13]	1986	Lipase inhibitor	KJ872771	BGC0000382	Fatty acid	*Streptomyces toxytricini; Streptomyces virginiae*
Belactosins[43]	2000	20S proteasome inhibitor	KY249118	BGC0001441	Amino acid	*Streptomyces sp.* UCK 14
Salinosporamides[38]	2003	20S proteasome inhibitor	EF397502	BGC0000145	Hybrid PKS/NRPS	*Salinispora tropica; Salinispora pacifica*
Cinnabaramides[89]	2007	20S proteasome inhibitor	FR687018	BGC0000971	Hybrid PKS/NRPS	*Streptomyces sp.* JS360
Cystargolides[43]	2015	20S proteasome inhibitor	KY249117	BGC0001440	Amino acid	*Kitasatospora cystarginea*

HHxxxDG motif of the C domain.[40] β-Lactone ring formation by the unexpected terminal C domain in oxazolomycin has been suggested by Zhao et al., but would likely follow a very different mechanism if proven.[41] Finally, there appears to be no equivalent β-lactam ring-formation mechanism to that of the fused bicyclic vibralactone-like compounds. From a chemical perspective, the intramolecular cyclization of a seven membered lactone ring should be energetically similar to that of a seven-membered lactam ring. However, to our knowledge, there are no β-lactams proposed to form by this mechanism.

3 Diversity of β-lactone natural products

While ~30 unique scaffolds of β-lactone natural products have been structurally characterized, only eight biosynthetic gene clusters (BGCs) have been reported to date (Table 2). The large number of these 'orphan' β-lactone compounds without known BGCs highlights the gap that remains between compound discovery and biosynthetic understanding. Recent breakthroughs in enzyme-catalyzed β-lactone ring biosynthesis may now facilitate genome-based discovery of β-lactone natural products and their respective BGCs. To provide a consolidated resource for β-lactone gene cluster and product exploration, we built an interactive web database, the β-Lactone Tool (z.umn.edu/bltool). This web application will be maintained and updated as more β-lactone natural products and their gene clusters are discovered.

The known β-lactone natural products can be grouped into five main classes based on their biosynthetic origins: 1) amino acids 2) fatty acids 3) polyketides, 4) hybrid PKS/NRPS pathways, and 5) terpenes. There are some products including alkaloid-derived compounds that do not fall into this classification. The classes can be further divided based on taxonomy of the source organisms. β-Lactones produced by microbes encompass the first four biosynthetic classes whereas β-lactones from plants are predominantly terpene-derived. β-Lactone products isolated from sea sponges and insect antennae closely

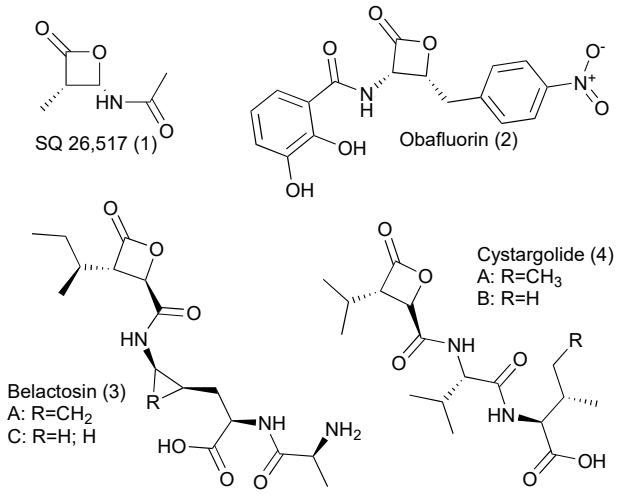


Figure 7. Amino acid-derived β-lactones.

resemble bacterial products, and it is unclear whether these compounds are produced by the eukaryotes themselves or their prokaryotic symbionts, as no gene clusters have yet been identified for their production.

3.1 Microbial β-lactones

3.1.1 Amino acid β-lactones

Monocyclic α-amino β-lactone antibiotics

The simplest of the amino acid-derived β-lactones (Fig. 7) is termed SQ 26,517 (1). This α-amino β-lactone compound was identified in 1982 by the Squibb Institute for Medical Research during a screen for β-lactam producing organisms.[54] The lactone ring of SQ 26,517 is likely derived from the cyclization of threonine. SQ 26,517 only displayed weak antimicrobial activity against *Streptococcus agalactiae* (50 μg/mL). The compound was produced by *Bacillus* sp. SC 11,480. The β-lactone compounds EM5395 and EM5357 were also identified during this screen as structural homologs of SQ 26,517 and the source organisms were tentatively assigned as a *Pseudomonas* and *Arthrobacter* sp. respectively. The poor antimicrobial properties of these compounds did not merit exact structural determination but the two compounds possessed threonine-derived β-lactone cores similar to SQ 26,517.

A more complex α-amino-acid based product, obafluorin (2), was isolated from the culture broth of *Pseudomonas fluorescens* ATCC 39502 in 1984.[55] It is a *cis*-monocyclic α-amino β-lactone disubstituted with a 2,3-dihydroxybenzoic acid and the non-proteinogenic amino acid β-hydroxy-*p*-nitro-L-homophenylalanine, identified as precursors by [^{13}C] stable-isotope feeding studies (Fig. 8).[56,57] The complete BGC, *obiA-F*, for obafluorin was published by Wencewicz and colleagues in 2017.[35] The NRPS-type biosynthesis is initiated by ObiB-mediated synthesis of chorismate via the endogenous shikimate pathway. ObiA, ObiC, and ObiE subsequently convert chorismate to 2,3-dihydroxybenzoic acid and ObiI, ObiJ, and ObiK transform that to *p*-aminophenylpyruvic acid (PAPPA). ObiL and ObiG reduce PAPPA to *p*-nitrophenylacetaldehyde (PNPAA). The authors demonstrated the L-threonine transaldoalolase ObiH converts L-threonine and PNPAA to β-hydroxy-*p*-nitro-L-homophenylalanine, which was confirmed in a concurrent publication on obafluorin biosynthesis by Wilkinson and colleagues.[58] The ATP-dependent NRPS A domain ObiF then activates this β-amino acid as an acyl adenylate to make the thioester which is released and cyclized to the β-lactone by an unusual TE domain as described in the mechanism section (Fig. 8).

Obafluorin is a broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria, but it has low potency (minimum inhibitory concentration (MIC) > 100 μg/ml)[55]. Like SQ 26,517, obafluorin has a (3*R*,4*S*) *cis*-β-lactone configuration[59], which is unusual since the majority of β-lactone natural products are *trans.* The β-lactone ring of obafluorin is unstable at neutral pH and rapidly hydrolyzes to the β-hydroxy carboxylic acid, which supports the necessity of

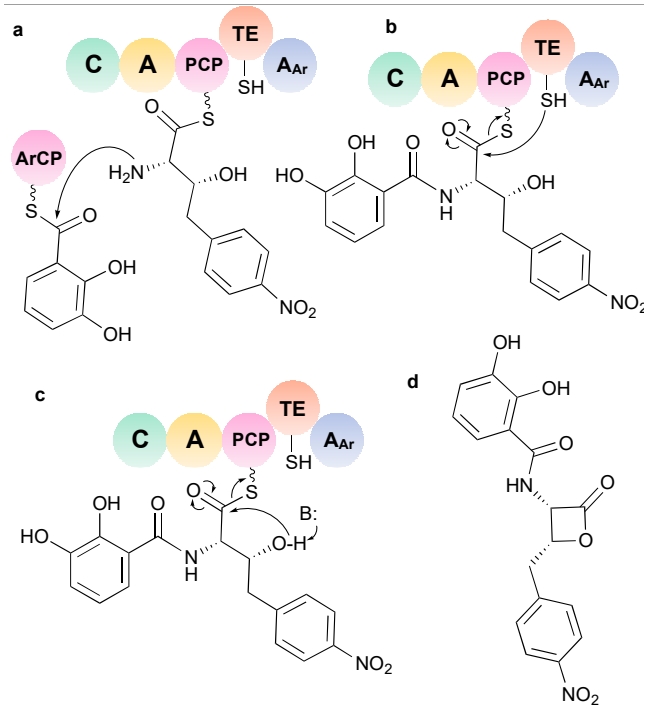


Figure 8. Obafluorin biosynthesis from 2,3 di-hydroxybenzoic acid and β-hydroxy-*p*-nitro-L-homophenylalanine (a), both derived from chorismate from the endogenous shikimate pathway.[35] Transesterification (b) is followed by intramolecular nucleophilic attack (c) to form the β-lactone ring prior to product release (d) from the ObiF NRPS.[35] ArCP = aryl carrier protein, C = condensation, PCP = peptidyl-carrier protein, TE = thioesterase domain, A_{Ar} = aryl adenylation.

enzyme-mediated cyclization.[35] Obafluorin is also chemically interesting in that it is the first β-lactone shown to be susceptible to hydrolysis by three common types of β-lactamases (P99, TEM-2, and K1).[55] We speculate the acylamino group in the α-carbon of the monocyclic β-lactone ring may contribute to its susceptibility to inactivation by β-lactamases.

Dipeptide β-lactones

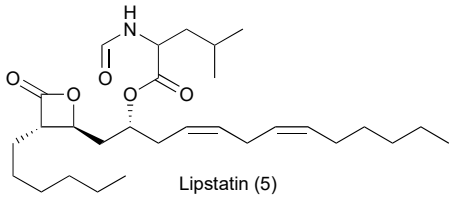
A study published in 2017 by Kaysser and colleagues reported the discovery of the BGCs for two dipeptide-derived β-lactones, the belactosins (3) and the cystargolides (4).[43] Belactosin A was first isolated in 2000 from the culture broth of a *Streptomyces* sp. UCK 14.[60] Cystargolides A and B were only recently isolated from *Kitasatospora cystarginea* NRRL B16505 in 2015.[9] Both the cystragolides and belactosins possess 3-carboxyl, 4-alkyl β-lactone moieties coupled to dipeptide backbones.[60] Isotope enrichment studies identified [$^{13}C_5$]-labeled valine and [1,2-$^{13}C_2$]-labeled acetate as peptide precursors that condense to yield the cystargolide β-lactone moiety. The belactosin β-lactone ring also incorporated one carbon atom from [1-^{13}C] isoleucine and two carbon atoms from [1,2-$^{13}C_2$]-acetate.[43]

Kaysser and colleagues sequenced the genome of *K. cystarginea* to search for the cystargolide BGC. Interestingly, the cystargolide gene cluster was not readily detectable by the BGC prediction software antiSMASH.[61] The authors then mined the *K. cystarginea* genome for isopropylmalate synthase (IPMS) homologs, which are precursors for biosynthesis of leucine. They identified an IPMS homolog encoded within an operon that had features of a putative cystargolide BGC. The putative BGC, *cysA-F*, was verified by genetic knockout studies. The belactosin BGC, comprised of 22 genes (*belA-V*), was identified via similar methods in the *Streptomyces* sp. UCK 14 genome and found to be homologous with the cystargolide BGC over several open reading frames. Cystargolide biosynthesis is initiated by IPMS-mediated Claisen condensation of acetyl-CoA and α-ketoisovalerate (a degradation product of valine) to yield 2-isopropylmalate. The authors purified the corresponding IPMS in the belactosin pathway (BelJ), to demonstrate its activity with 2-keto-3-methylvalerate (an isoleucine degradation product). The specificity of BelJ is novel for bacterial IPMS enzymes which previously only were known to exhibit narrow substrate specificity limited to α-ketoisovalerate. As mentioned above, the mechanism of β-lactone ring formation remains unknown; however, the authors speculated the ring may be formed by internal esterification catalyzed via the putative esterases CysE and BelR, or the acyl-CoA ligases CysC and BelV could promote enzymatic cyclization of a β-hydroxy acid intermediate.[43] These products are also interesting in that they are assembled by standalone amide ligase enzymes rather than classical NRPS or ribosomal systems.

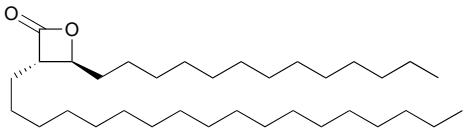
The belactosins and the cystargolides exhibit sub-micromolar IC_{50} levels of inhibition of the 20S subunit of the proteasome by covalent inhibition of its active site threonine.[9] The (3*S*,4*R*)-*trans*-β-lactone ring conformation is essential for proteasome inhibition and opening the β-lactone ring abolishes activity. Various groups have sought to improve the drug disposition of the belactosins and generated stabilized analogs with an IC_{50} in the low micromolar range for colorectal carcinoma cell lines[62,63] and phytopathogenic fungi.[64] The compounds and their derivatives have been patented,[65] but no compound has yet entered clinical trials to our knowledge. The potency of the *trans*-β-lactone belactosins and cystargolides contrast with the weak activity of *cis*-β-lactones obafluorin and SQ 26, 517. We speculate the *trans*-configuration of the β-lactone and β-positioning of the amino group are important structural features of the belactosins and cystargolides that confer proteasome inhibition properties.

3.1.2 Fatty acid β-lactones

Lipstatin (5) is a fatty acid-derived β-lactone produced by the terrestrial actinomycetes *Streptomyces toxytricini* and *Streptomyces virginiae* (Fig. 9).[66,67] In 2014, Bai and colleagues published the six-gene operon *lstA-F* for lipstatin biosynthesis and constructed single-gene knockouts to define gene cluster boundaries in the source organism, *Streptomyces toxytricini.*[13] Lipstatin precursors are reported to originate from activated octanoic acid and the incomplete β-oxidation of linoleic acid.[68] Lipstatin biosynthesis is initiated via 'head-to-head' Claisen condensation of these two fatty-acid substrates likely catalyzed by thiolase enzymes LstA and LstB.[13] The NRPS LstE attaches a leucine group, formylated by LstF, as a decoration on the lipstatin scaffold. LstD catalyzes reduction of the β-keto acid to a 3,5-dihydroxy fatty acid backbone. Bai and colleagues



Lipstatin (5)



Nocardiolactone (6)

speculated that the LstC functions as an acyl-CoA ligase to form acyl-CoA substrates from fatty acid precursors. However, it was recently shown that LstC it also shares significant amino acid sequence identity (46%) with the ATP-dependent β-lactone synthetase from *Xanthomonas campestris.*[23] It remains to be proven whether β-lactone ring formation from the β-hydroxy acid is mediated by the β-lactone synthetase homolog LstC or proceeds nonenzymatically as proposed by Bai et al. to complete total biosynthesis of the lipstatin natural product.

The hydrogenated derivative of lipstatin, tetrahydrolipstatin (THL), has garnered significant attention as an FDA-approved obesity treatment. THL is available over-the-counter (Alli) and by prescription (Xenical) to enhance weight loss through inhibition of gastric and pancreatic lipases to limit dietary fat absorption. THL also targets serine hydrolase enzymes in *M. tuberculosis*[70] and inhibits growth of the protozoan parasite *Giardia* with an IC_{50} in the low micromolar range.[69] However, THL is a highly lipophilic molecule with a very low solubility in water. Even with the use of pharmaceutical formulation methods or intravenous injection to improve absorption, THL's solubility is still far lower than desired, which limits its oral bioavailability and future applications.

Notably, there are many natural derivatives of lipstatin, specifically analogs with altered amino acid side chains. For example, *Streptomyces lavendulae* MD4-C1 produces esterastin, which has an *N*-acetyl asparagine, instead of *N*-formyl leucine, attached by an ester linkage to the fatty-acid backbone.[71] Similarly, valilactone has an *N*-formyl valine arm.[72] The panclicins A-E are also lipstatin analogs from *Streptomyces* sp. NR 0619 with *N*-formylglycyloxy or *N*-formylalanoxy side chains derived from the incorporation of glycine or alanine, respectively.[73,74] The BGCs for esterastin, the panclicins, and valilactone have not been published but are likely homologous to the lipstatin pathway with altered NRPS amino acid adenylation domains. A branched chain analog of lipstatin was also isolated that was shown to incorporate L-leucine into the lipstatin backbone instead of an activated octanoic acid.[75] In addition to natural analogs, numerous structure-activity relationship (SAR) studies have synthesized libraries of THL derivatives.[76-78] A 2018 publication inverted different stereocenters of the THL scaffold and demonstrated many stereoisomers still maintained potent inhibition of the *Mycobacterium tuberculosis* phospholipase Rv3802c and polyketide synthase Pks13 involved in mycolic acid biosynthesis.[78] Other SAR studies have substituted the *N*-formyl leucine with different side chains and found that *N*-formyl and *N*-acetyl prolyl esters resulted in an increase in Rv3802c inhibition in *M. tuberculosis.*[76] Taken together, the SAR studies and existence of numerous natural analogs suggest the THL scaffold is an attractive target for biosynthetic pathway engineering.

Another fatty acid-derived compound is the nocardiolactone (6), a 3,4 *trans*-disubstituted β-lactone natural product isolated from five pathogenic strains of *Nocardia brasiliensis.*[79] The highly lipophilic molecule has two alkyl chains of length C_{13} and C_{18} (Fig. 9). It displayed narrow spectrum antibiotic properties against the Gram-positive bacterium, *Bacillus subtilis* ATCC 6633. Apart from a sole report over two decades ago, no further work has been published on the biology of nocardiolactone production or its BGC. No other unique scaffolds for fatty acid-derived β-lactone natural products have been isolated to date. However, a query of sequence databases reveals a large number of lipstatin-like BGCs present in members of the Actinobacteria including the *Streptomyces, Nocardia, Mycobacterium,* and *Rhodococcoccus*.

3.1.3 Polyketide β-lactones

Polyketide-derived β-lactones have been isolated from the culture broth of bacteria and fungi as well as insect antennae that differ only subtly in backbone methylation and oxidation (Fig. 10). The most well-studied of these polyketide compounds, the ebelactones (7), were isolated in 1980 from the culture broth of *Streptomyces aburaviensis,* later reclassified to the genus *Kitasatospora.*[80] The genome of *K. aburaviensis* was sequenced and the ebelactone BGC was identified on a single contig.[45] The gene cluster encodes seven PKS proteins, EbeA-G, which assemble into a multimodular functional polyketide synthase. Biosynthesis is initiated by the KS domain of the loading module which decarboxylates malonyl-CoA to generate the starter acetate. Downstream acyltransferase domains are specific for methylmalonyl-CoA, consistent with the final ebelactone structure. [^{13}C]-Isotope studies further confirmed that six methylmalonate extender units are condensed in a 'head-to-tail' fashion to generate the final product. Ebelactones A and B have an impressive panel of cellular targets including esterases, lipases, cutinases, and homoserine transacetylases. They also modulate the mTOR pathway to enhance immune response.[45]

Wyatt and colleagues observed ^{18}O from [1-^{13}C, $^{18}O_2$] propionate was incorporated at all oxygen sites in ebelactone during biosynthesis suggesting conservation of C(β)-O bond in the lactone. These results support a model of intramolecular nucleophilic attack of the carbonyl carbon by a β-hydroxyl group. As mentioned earlier, the authors proposed the β-lactone ring formation step of ebelactone occurs spontaneously from a β-hydroxy thioester intermediate of the PKS. However, the

presence of a rare terminal KS domain as well as an adjacent gene (*orf1*) that resembles a β-lactone synthetase have also been proposed to facilitate ring formation.[23,35]

Another polyketide-derived β-lactone, hymeglusin (8), originally discovered as Antibiotic 1233A and F-244, was first isolated from fungal culture broth of *Cephalosporium sp*. by Aldridge et al. in 1971.[81] Hymeglusin was later re-discovered in other Ascomycota including *Scopulariopsis* and *Fusarium* sp.[7,82] Isotope labeling studies in *Scoulariopsis* sp. with [1,2-$^{13}C_2$]-acetate revealed the hymeglusin backbone is constructed similarly to ebelactone by 'head-to-tail' polyketide condensation of seven acetate units. Methyl side chains at C_2, C_8, C_{10}, and C_{12} were introduced by four units of [methyl-^{13}C] methionine.[7] This mechanism of biosynthesis was confirmed to be identical in *Fusarium* sp.[82] Insight into β-lactone ring closure in *Fusarium* sp. through [1-^{13}C, $^{18}O_2$] acetate labeling studies indicated lactonization occurred by C(β)-O attack on the terminal carbonyl group.[82] The BGC for hymeglusin has not yet been published although we speculate it proceeds via a polyketide biosynthetic pathway similar to the ebelactones.

Hymeglusin has a (3*R*,4*R*) *trans*-β-lactone ring in contrast to the (3*S*,4*S*) configuration of the ebelactones. The configuration of the *trans*-β-lactone as (3*S*,4*S*) or (3*R*,4*R*) is a subtle difference that appears to be critical for enzyme target specificity. Synthetic studies demonstrated that the (3*R*,4*R*) β-lactone weakly inhibits pancreatic lipases (IC_{50} > 100 μM) and instead has high specificity towards a 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase (IC_{50} = 98 nM), an essential metabolic enzyme involved in sterol biosynthesis.[83] A crystal structure of hymeglusin bound to the *Enterococcus faecalis* HMG-CoA synthase was recently published.[84] The co-crystal structure revealed hymeglusin forms a thioester adduct with the active site cysteine in a narrow tunnel in the bacterial enzyme that is sequestered from solvent. This results in tighter binding compared to plant and animal enzymes which have more solvent-exposed cavities. Despite this, significant cross reactivity between prokaryotic and eukaryotic HMG-CoA synthases remains a barrier to the development of hymeglusin.

Vittatalactone (9) is yet another compound that appears polyketide-like in origin. Vittatalactone is an insect sex pheromone isolated from airborne volatiles of male striped cucumber beetles (*Acalymma vittatum*) in 2005.[85] Due to its similarity to hymeglusin and the ebelactones, we speculate the compound may be of microbial origin. The absolute stereochemistry of the vittatalactone ring was determined to be (3*R*,4*R*) like hymeglusin, and the opposite of ebelactone.[83] Interestingly, vittatalactone was only produced by feeding male cucumber beetles and was not detected from female or non-feeding beetles. However, vittatalactone elicited electrophysiological responses from the antennae of both male and female beetles suggesting its role as an aggregation compound.[85]

The similarities between the ebelactone, hymeglusin, and vittatalactone structures are striking in light of their production by deeply-branching bacterial, fungal, and insect clades on the tree of life. The compounds all share a β-lactone ring with a $C_{8\text{-}12}$ chain length polyketide tail differing only in stereochemical configuration and backbone decoration patterns. The high level of structural conservation suggests that biosynthesis of polyketide-derived β-lactone natural products may have arisen independently in different domains of life, however, further investigation is warranted into the genetic conservation and evolution of polyketide-derived β-lactones.

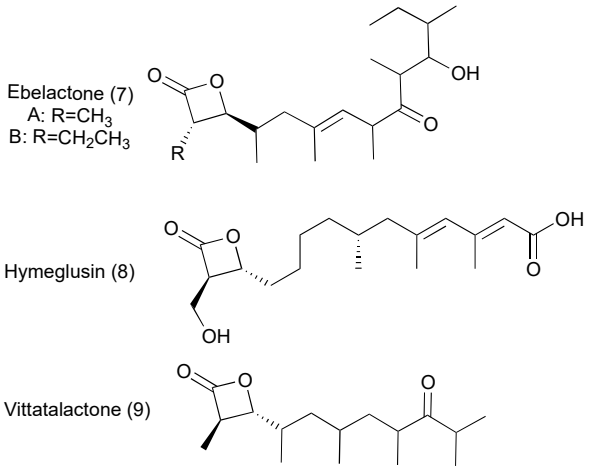


Figure 10. Polyketide-derived β-lactones.

3.1.4 Hybrid pathway β-lactones

Bicyclic fused β-lactone/γ-rings

Many β-lactone natural products that originate from hybrid biosynthetic pathways exhibit a bicyclic fused β-lactone/γ-lactam or cyclopentene configuration (Fig. 11). Salinosporamides (10) are the most clinically-relevant of these fused ring pathway compounds isolated to date. Eleven structural analogs (Salinosporamides A-K) have been isolated to date and they are all produced by obligate marine actinomycetes in the genus *Salinispora*.[38,86] Salinosporamide A, defined by its choroethyl side chain, entered clinical trials for treatment of multiple myeloma only three years after discovery. Like cystargolide and belactosin, salinosporamide inhibits the 20S subunit of the proteasome through nucleophilic attack on the β-

lactone ring by the threonine hydroxyl group.[39] Although the β-lactone is susceptible to hydrolysis, salinosporamide is unique in that a second attack from the C_3 hydroxyl group displaces the C_2 chloroethyl tail for cyclization to form a stable tetrahydrofuran adduct. This second cyclization step results in irreversible inhibition and a unique mode of action among the β-lactone natural products. For an in-depth review of the salinosporamides, we refer readers to a comprehensive review by Gulder et al.[39]

Elegant ^{13}C-isotope labeling experiments demonstrated that the salinosporamides are derived from acetate, β-hydroxy-2′-cyclohexenylalanine, and a chlorinated tetrose molecule.[87] The authors proposed the hybrid PKS/NRPS pathway was initiated by the condensation of acetyl-CoA and ethylmalonyl-CoA. The *sal* gene cluster was later identified by single gene disruption in *Salinispora tropica* CNB-392.[38] Eight biosynthetic enzymes were identified, two of which were unique to the salinosporamide pathway and six of which could be replaced by other primary metabolic counterparts. SalL, a S-adenosyl-L-methionine (SAM)-dependent chlorinase, in particular, appears to have evolved to form the precursor 5′-chloro-5′-deoxyadenosine which is converted to chloroethylmalonyl-CoA. Chloroethylmalonyl-CoA is of as interest as an unusual extender unit that expands the repertoire of synthetic biology tools that can be used to generate custom polyketide scaffolds with desirable halogenated side chains.[38] Notably, this biosynthetic logic was used to substitute the chlorinase SalL with a fluorinase from *Streptomyces cattleya*.[88] The engineered strain of *Salinispora* was then found to produce a non-natural fluorosalinosporamide analog when inorganic fluoride was supplemented in the media.[89]

The cinnabaramides (11) are structurally similar to the salinosporamides with a hexyl group instead of halogenated ethyl tails. The apparent lack of the chloroethyl tail results in weaker proteasome inhibition than salinosporamide A but enhanced antifungal properties.[89] The BGC was identified in a terrestrial *Streptomyces* sp. JS360 in 2011.[90] Unexpectedly, the relaxed substrate specificity in the cinnabaramide PKS extender module enabled incorporation of (*E*)-4-chloro-oct-2-enoic acid when it was supplemented in the growth media and resulted in the production of chlorinated cinnabaramide analogs.[90]

Another class of fused ring proteasome inhibitors are the omuralides (12) that form non-enzymatically from the β-hydroxy thioester lactacystin precursor.[91] The microbial natural product, lactacystin, was first isolated from terrestrial *Streptomyces* sp. OM-6519 in 1985, but only its lactonized form was found to block chymotryptic activity of the proteasome with an IC_{50} value of 47

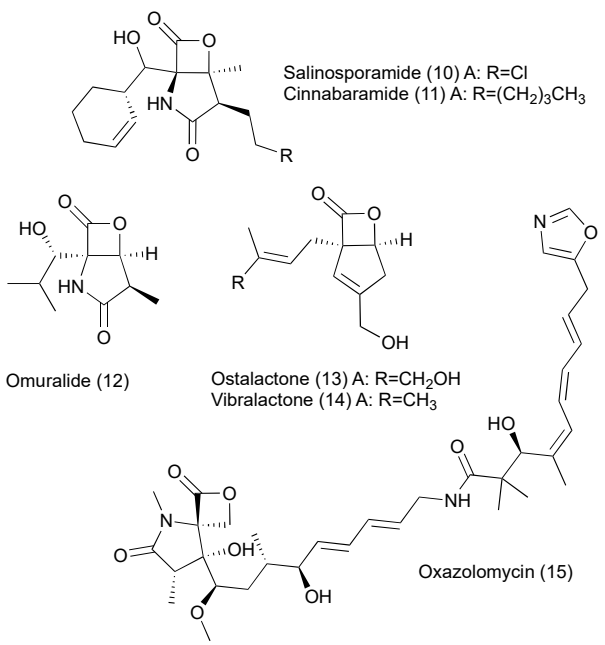


Figure 11. Examples of bicyclic fused ring and hybrid pathway β-lactones.

nM.[44,92] The BGC has not been published, but [^{13}C]-isotope labeling studies suggested the omuralides originate from condensation of valine-derived methylmalonic semialdehyde and leucine molecules.[93]

Structurally analogous bicyclic compounds with fused β-lactone/γ-cyclopentene rings include the fungal-derived ostalactones A-B and vibralactones. Vibralactones (13) were discovered in 2006 from the culture broth of the fungus *Boreostereum vibrans.*[93] Although the vibralactones appear to be polyketide or sesquiterpenoid-derived, [1-^{13}C] labeling studies unexpectedly revealed their biosynthetic origins in the endogenous shikimate pathway.[94] Zhao et al. demonstrated incorporation of [U-^{13}C]-phenylalanine into vibralactone and proposed the aryl intermediates arise from the shikimate and phenylalanine biosynthetic pathways.[94] The aryl intermediate is prenylated by a novel aromatic prenyltransferase gene, *vibPT.* The authors used RNA-Seq to demonstrate *vibPT* expression was correlated with increased vibralactone production in *Borostereum vibrans* over time. They also expressed VibPT recombinantly and demonstrated transfer of a dimethylallyl group to the aryl ring intermediate. Following prenylation, the aryl intermediate undergoes oxidative expansion to a seven-membered ring via epoxidation and rearrangement before final β-lactone formation via intramolecular attack.

Ostalactones A-C (14) were recently discovered from the basidiomycete *Stereum ostrea* in 2016.[10] Ostalactone A differs from vibralactone only by the presence of a

hydroxyl group on the dimethylallyl side chain (Fig. 11). Similar to the vibralactones, Kang et al. isolated a seven-membered ring indicative of an intramolecular cyclization mechanism.[10] The BGC for the ostalactones have not been reported, but we hypothesize the gene cluster might be identifiable by genome mining for prenyltransferase *vibPT* homologs in *Stereum ostrea*.

The fused β-/γ-rings are the largest cohesive class of β-lactone natural products isolated to date, which hints at the biological importance of the fused ring structure. An important taxonomic distinction is that β-lactones fused to cyclopentene rings have only been isolated from fungi to date while those fused to γ-lactams have exclusively been found in the Actinobacteria. While the bicyclic β-lactone/γ-lactam structures are derived from complex hybrid PKS/NRPS systems, the β-lactone/cyclopentene structures are non-PKS and originate from the shikimate pathway. The structural convergence of the fused β-/γ-ring is even more interesting when the ecology of source organisms is considered. The cinnabaramides are produced by terrestrial *Streptomyces* spp. while the salinosporamide-producing *Salinispora* spp. are obligate marine actinomycetes from diverse tropical and subtropical locations.[95] In contrast, the *Stereum* and *Borostereum* spp. are terrestrial wood decay fungi widespread in North America. The emergence of the fused β-/γ-ring scaffold from deeply branching fungal and bacterial domains invites speculation that the bicyclic scaffold may have arisen independently more than once and warrants further investigation into its evolution.

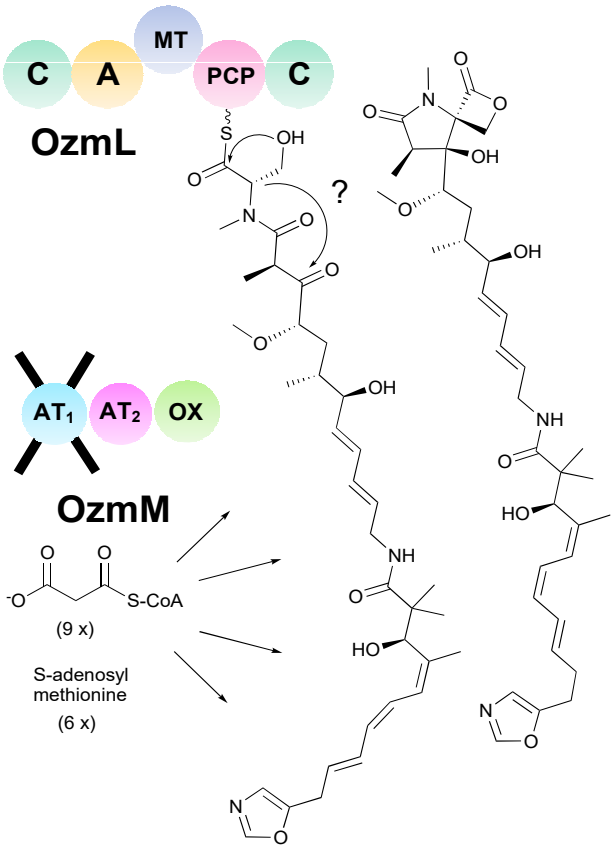


Figure 12. Proposed final step in oxazolomycin biosynthesis catalyzed by the nonribosomal peptide synthase OzmL. Domains: C = condensation, A = adenylation, MT = methyltransferase, PCP = peptide carrier protein, AT = acyltransferase, OX = oxidoreductase.

Spiro-β-lactone antibiotics

Oxazolomycin (15) was first identified as the antibiotic resistaphylin from *Streptomyces antibioticus* in 1971.[8] Numerous oxazolomycin analogs have been isolated from both marine and terrestrial *Streptomyces* spp. in subsequent decades, including one discovered in 2017 termed bisoxazolomycin.[11] Bisoxazolomycin was isolated as two oxazolomycin molecules that dimerized, likely by nucleophilic attack of an adjacent hydroxyl group on the spiro-β-lactone ring to form an ester.[11] A family of related oxazolomycin derivatives include the curromycins isolated from *Streptomyces hygroscopicus*[97] and the lajollamycins isolated from *Streptomyces nodosus*.[98]

The oxazolomycin gene cluster from *Streptomyces albus* JA3453 consists of 20 open reading frames, *ozmA–T*, including four PKSs, two NRPSs, and a hybrid NRPS-PKS megasynthase.[41] The oxazolomycin backbone is constructed from nine malonyl-CoAs, a glycine, a methoxymalonyl-ACP, and a serine (Fig. 12). Interestingly, all 10 of the type I PKS modules lack integrated acyltransferase (AT) domains. Instead, there are two standalone ATs, OzmC and OzmM, the latter of which is further comprised of two AT domains (OzmM-AT1 and OzmM-AT2).[41] Interestingly, gene knockout studies revealed that the OzmM-AT1 is a silent, non-functional AT domain and that OzmC and OzmM-AT2 are necessary and sufficient for acyltransferase activity in oxazolomycin biosynthesis (Fig. 12).[41] As no TE domain was found, the authors speculated that the addition of an extra terminal C domain in OxmL may catalyze β-lactone ring formation, but no experimental evidence has yet been published supporting this hypothesis.

The striking spiro-β-lactone/γ-lactam ring scaffold is unique to this class of molecules and warrants further study both for the potentially unique mechanism of spiro-β-lactonization and for their clinical utility as an antibiotic. Oxazolomycin has inhibitory activity against human immunodeficiency virus, Gram-positive bacteria, P-388 leukemia cells, and crown gall tumor formation by *Agrobacterium tumefaciens*,[8,99] suggesting versatile cross-species utility and reactivity. To date, no activity-based chemical profiling has yet been conducted to

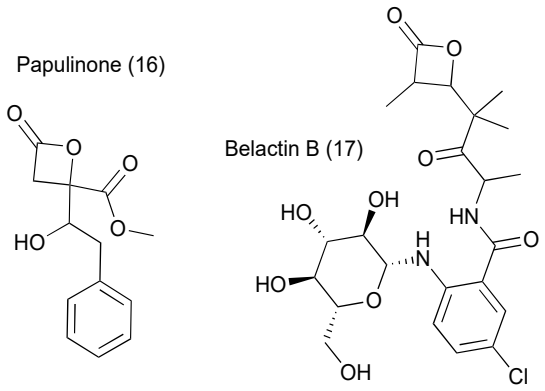


Figure 13. Examples of 'other' β-lactones: papulinone and a representative belactin compound.

identify cellular protein targets of the oxazolomycin-like compounds.

3.1.5 Other β-lactones

A few β-lactone natural products do not fit into major classes described above (Fig. 13). For example, papulinone (16) was isolated from liquid cultures of the plant pathogen *Pseudomonas syringae pv. papulans.*[100] The source organism is the causative agent of blister spot in apples and pears. Papulinone was, classified as a weak phytotoxin since application to bean and apple leaves at 2 mg/mL resulted in tissue necrosis. The biosynthetic origins of papulinone remain unknown, but it is likely derived from β-phenyl lactic acid, a plant growth hormone known to be produced by *Pseudomonas* spp. We speculate an aldol reaction between phenylacetaldehyde and oxaloacetate followed by a ring closure step of the resulting β-hydroxy acid represents a likely biosynthetic route to papulinone. Papulinone has a spiro-β-lactone ring but, unlike oxazolomycin, it is not fused to a γ-lactam. Identification of the biosynthetic gene cluster for papulinone remains an outstanding question in the field to further our understanding of spiro-β-lactone ring biosynthesis.

The belactins (17) are another interesting class of β-lactone products for which little is known about the biosynthesis. The belactins were first characterized as serine carboxypeptidase inhibitors isolated from fermentation broth of *Saccharopolyspora* sp. MK19-42F6 in 1995.[101] We hypothesize the 5-chlorobenzyl-amino backbone is formed via an NRPS-type pathway, but this must be experimentally verified. Belactin B has a β-glucopyranosylamino side chain likely attached enzymatically by a glycosyltransferase. Although many natural products are decorated with sugar residues, this is the first example of a sugar attached to a β-lactone scaffold.

3.2 Plant- and animal- β-lactones

3.2.1 Terpenoid-derived β-lactones

Plants and marine sponges are the only known source of terpenoid β-lactone natural products (Fig. 14). Sesquiterpene (C_{15}), diterpene (C_{20}), and triterpene (C_{30}) products have been isolated to date, all derived from isoprene units. These compounds display anticancer and neurotoxic activity. Although anisatin was the first β-lactone natural product to be discovered in 1952,[2] the biosynthesis of terpenoid β-lactones are understudied compared to their microbial counterparts.

Sesquiterpenes

The β-lactone anisatin (18), discovered in the *Illicium* plant species in east Asia, was isolated for its neurotoxic activity in 1952[2] but its structure was not determined until 1965.[6] Anisatin, and its derivatives that include neoanisatin,[102] majucin,[103] and the veranisatins,[104] act as potent convulsants and were found to act by binding γ-aminobutyric acid (GABA) neurotransmitters.[105,106] There is nothing reported about their biosynthesis.

Another class of unrelated sesquiterpene β-lactones includes the grazielolides and guaiagrazielolides (19) found in the South American flowering plants of the Asteroideae subfamily. The first grazielolide was isolated from *Grazielia intermedia* and contains a 10-membered ring structure.[107] The core structure of a guaiagrazielolide likely originates from the 1-5 linkage of grazielolide to generate 5- and 7- membered rings. To our knowledge, the biosynthesis or biological activity of these compounds has never been reported.

Diterpenes

Spongiolactone (21) and its analog 3′-norspongiolactone were isolated from the extract of the Mediterranean Sea sponge, *Spongionella gracilis.*[108,109] It shares a similar fused β-/γ-ring structure with the vibralactones and ostalactones but is diterpene-derived. No information is reported as to whether the biosynthetic source organism is the sponge itself or a microbial symbiont. Notably, the spongiolactones also share structural similarities with the sponge-derived gracilins, which are promising compounds for treatment of Alzheimer's disease.[110] The close analog, 3′-norspongiolactone, was also found to have cytotoxic activity against human chronic myelogenous leukemia cells and normal human peripheral blood mononuclear cells with IC_{50} values of 12 and 30 μM, respectively.[108]

The cellular targets of spongiolactone are poorly understood. A recent quantitative chemoproteomic study in leukemia cell lines with an alkynylated spongiolactone probe revealed a broad array of enzyme targets.[111]

Interestingly, in comparison to a lipstatin-like β-lactone probe which mainly targeted serine hydrolases, the targets of the spongiolactone probe were in multiple unrelated enzyme classes. This preliminary study suggests the rigid and complex scaffold of spongiolactone has a nonspecific polypharmacological profile that is distinct from other β-lactone compounds.[111] The majority of β-lactones to date have been isolated from terrestrial organisms. However, it is worth noting that the spongiolactones, oxazolomycins, and salinosporamides were all isolated from marine systems and each have unique target profiles and clinical potential. We emphasize that further research efforts should be focused on BGC mining of undersampled systems, including marine sediments and sponges.

Triterpenes

The β-lactone papyriogenin G (20) was isolated from the leaves of *Tetrapanax papyriferum*, an evergreen shrub, and its crystal structure determined in 1978.[112] It is an oleanane-type triterpene with the β-lactone moiety formed by carbons 17, 22, and 28. No data is available on its biosynthesis or its biological function. However, it is likely that the common oleanane plant triterpene scaffold is synthesized first and a β-lactone is installed as a later modification.

A related triterpene, lupeolactone (22), was isolated in 1983 from the aerial parts of the plant species *Antidesma pentandrum Merr*. native to Taiwan, the Philippines, and other regional Pacific islands.[113] The structure of lupeolactone contains a pentacyclic scaffold that is likely derived from triterpene precursors. It is unknown whether a macrolactonization reaction occurs prior to the formation of the final structure or if generation of the β-lactone occurs after the scaffold has been formed. Oral administration of lupeolactone was found to lower cholesterol levels in normal and hypercholesterolemic rats.[113] The β-lactone moretenolactone,[114] a hopanoid from *Ficus insipidia* (tropical fig tree), closely resembles lupeolactone. The latex, leaves, and unripe fruits of this tree are used by natives to treat worm diseases, but the bioactivity of purified moretenolactone is not reported. Total organic synthesis of nearly every natural product in this review has been achieved,[33,34] but a method for synthesis of triterpene β-lactones has not been reported to date.

3.2.2. Alkaloid β-lactones

Viridiflorine (23) and norviridiflorine are the only β-lactones isolated to date from butterflies.[115] These compounds were extracted from the antennae of the male giant danaine butterflies (*Idea leuconoe*) and function as sex pheromones to attract females. The viridiflorines

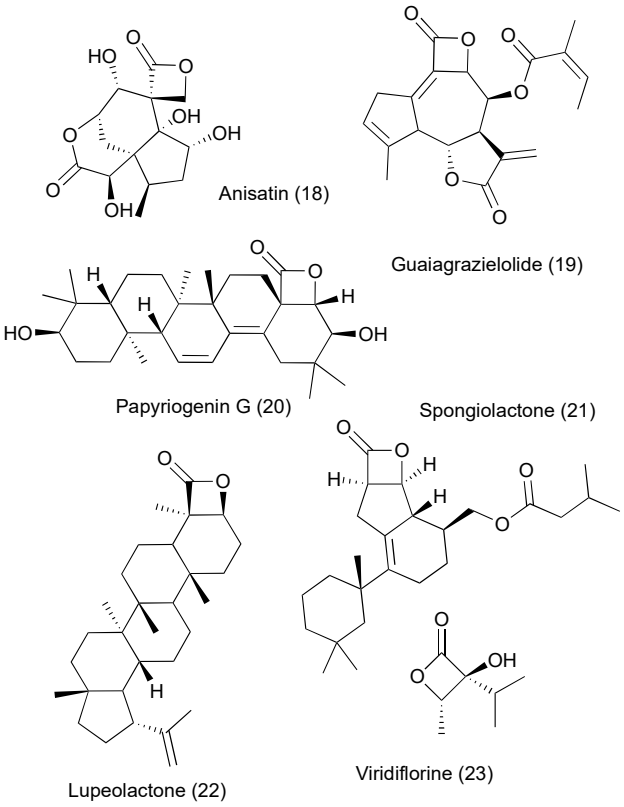


Figure 14. Terpenoid- and alkaloid- derived β-lactones from plants and animals.

were later re-isolated from the male abdominal scent glands of *Euploea mulciber* (danaine) and wing scales of *Melinaea menophilus* and *Scada kusa* (ithomiine), all phylogenetically related to *Idea leuconoe.*[116] The authors proposed these β-lactones are cyclized from β-hydroxy acids liberated from the cleavage of pyrrolizidine alkaloids lycopsamine and ideamine that are consumed by the butterflies. Danaine butterflies are known to consume pyrrolizidine alkaloids as a form of pharmacophagy primarily during the larval stage.[117,118] The β-hydroxy acid to β-lactone cyclization suggests a mechanism similar to that of lipstatin or obafluorin. These few examples of the β-lactone compounds vittatalactone and the viridiflorines playing a role in diverse insects as male sex pheromones highlights the potential biological importance of these compounds in insect mating. Further research could explore on whether these potent sex pheromones are produced by the insects themselves or their microbial symbionts.

3.3 β-Lactones as pathway intermediates

β-lactones have been used as synthons in organic synthesis schemes for decades, but only a few examples are known to date where β-lactones are intermediates in biosynthetic pathways. The biosynthesis of long-chain olefinic hydrocarbons by the four enzymes OleA, OleB,

OleC, and OleD relies on a β-lactone intermediate (Fig. 5). These olefinic membrane hydrocarbons are produced by hundreds of bacterial species possessing the *oleABCD* gene cluster from deeply-branching phyla including the *Verrucomicrobia, Chlorflexi, Proteobacteria* and *Planctomycetes*.[119] The taxonomic distribution of organisms containing *oleABCD*-like gene clusters span at least eight different bacterial phyla and over 600 unique species. This phylogenetic diversity is especially remarkable in contrast to the Actinobacteria, mainly *Streptomyces*, which are usual 'major players' in natural product biosynthesis. The structures of the final olefinic products are largely determined by the OleA enzymes which are pathway 'gatekeepers' with different substrate specificities that generally correspond to the available fatty acid pools of the organism.[119] Consequently, the downstream enzymes OleB, OleC, and OleD accept different β-ketoacid precursors produced by OleA to generate a diversity of olefin natural products with varied chain lengths, saturation, and branching patterns.[119] Interestingly, protein-protein interaction experiments in *X. campestris* revealed formation of large complex comprised of OleBCD enzymes assembled in a 4:1:4 stoichiometry.[120] Additionally, all Actinobacteria with the *ole* gene cluster contain an *oleBC* gene fusion in place of separate genes, thereby physically linking the β-lactone synthase and β-lactone decarboxylase activities in a single polypeptide chain.[119] We hypothesize these protein complexes may be required to prevent free diffusion of the reactive β-lactone throughout the cell. These findings also suggest that export mechanisms may be a typical feature for pathways in which β-lactones are the final product of a biosynthesis pathway.

Another example of β-lactones being used as synthons comes from the basidiomycete *Boreostereum vibrans*, a natural producer of vibralactone.[95] Researchers isolated derivatives of vibralactone, termed vibralactoxime, bearing an oxime group in place of the primary alcohol.[12] Short polymers of vibralactoximes were discovered in culture broth with an unusual oxime ester linkage which presumably arises from the oxime attack on the β-lactone of the adjacent monomer. Some monomer and polymer derivatives of the vibralactoximes were also found to lack the β-lactone entirely, and rather have a β-hydroxyl methyl ester in place of the β-lactone. Many of these monomer and polymer derivatives had IC_{50} values against pancreatic lipase that were lower than vibralactone. The prevalence of β-lactones as intermediates in biosynthetic pathways remains an important area for further research.

4 Future directions

Improvements in computational algorithms and synthetic biology methods have greatly expanded capabilities for natural product prediction and validation. While significant progress has been made using these techniques to predict β-lactone BGCs computationally, there are still countless unanswered questions. Major bottlenecks facing the field of natural products in general include linking orphan gene clusters with natural products, identifying cellular targets, and understanding the biological function of secondary metabolites in the host organism and microbial communities. In this section, we will address the key areas and knowledge gaps related to β-lactone biosynthesis.

Bioinformatics and 'orphan' BGC elicitation

Improved biochemical knowledge about enzyme-catalyzed β-lactone ring formation has enabled genome-mining techniques to identify sequence-specific motifs for ATP-dependent β-lactone synthetases and the rare GxCxG motif in the TE domain catalyzing lactonization in obafluorin. However, existing secondary metabolite gene cluster detection tools including antiSMASH[61] and PRISM[121] have not yet incorporated this information into their prediction algorithms. Machine learning using datasets of known β-lactone natural products could now be used to train accurate classifiers for improved bioinformatic predictions of novel BGCs, although these techniques may be limited by the small number of known β-lactone compounds.

Computational prediction is not the only hurdle in the discovery of new β-lactone compounds. BGC elicitation platforms have revealed that the majority of natural products are not produced under normal culture conditions. Thus, the ~30 β-lactone natural products isolated to date may only be the 'tip of the iceberg'. High-throughput platforms developed for natural product elicitation[122] and advanced genetic engineering tools, including developments in the use of site-specific recombinases,[123] could aid in production of β-lactone compounds from their predicted gene clusters. A genome-mining based approach is also particularly useful in mining BGCs from the 'uncultivated majority' of microbes for which BGCs can be cloned and expressed in heterologous hosts. Another hurdle to β-lactone natural product discovery is their propensity for thermal decarboxylation of the β-lactone ring when analysed by gas chromatography. Additionally, β-lactones can be broken down under the ionization conditions typically used for liquid chromatography mass spectrometry. One technique to overcome the challenges of rapid screening and detection of β-lactones rings is the use of reactive thiol-based probes to 'prospect' for these electrophilic rings in culture medium.[124]

Activity-based protein profiling

The broad cellular target range of β-lactones has largely been revealed through the use of activity-based protein profiling (ABPP). ABPP is a modern proteomic technique in which reactive chemical probes bind covalently to enzyme active sites residues to give insight into the activity and function of both the probes and their targets.[125,126] ABPP probes typically consist of a reactive 'warhead' and a tag, which can be an affinity label such as biotin, a fluorophore, or an alkyne or azide for 'click chemistry' via Huisgen 1,3-dipolar cycloaddition. β-Lactones are ideal warheads for ABPP due to their small size, electrophilicity, and covalent inhibition of multiple enzyme classes.[19,127] The first systematic profiling of β-lactone cellular targets using ABPP was conducted by Sieber and colleagues in 2008.[19] The authors constructed a large library of natural-product inspired monocyclic β-lactone probes and tested their targets using ABPP in a mouse model and six different pathogenic bacterial strains.[19] Sieber and colleagues reported a remarkable range of targets, including enzymes involved in primary metabolism, antibiotic resistance, virulence, and nucleic acid synthesis. In subsequent ABPP studies, the Sieber lab developed β-lactone probes targeting ClpP protease to attenuate virulence in *Staphylococcus aureus*[128] and serine hydrolase enzymes Ag85 and Pks13 to inhibit mycolic acid biosynthesis in *Mycobacterium tuberculosis.*[129] For a comprehensive overview of the uses of β-lactones and β-lactams in ABPP, we refer readers to a review by Böttcher and Sieber.[127]

While significant progress has been made to map the profile of certain classes of β-lactones by ABPP, there is still much to be uncovered using this powerful technique. Structure-activity relationships and target profiles have not yet been elucidated for the full palette of β-lactone natural product scaffolds. For example, an ABPP study with a fused bicyclic vibralactone-like probe in *Listeria monocytogenes* revealed unexpected insights into proteasome function.[130] The vibralactone probe was able to label two isoforms (ClpP1 and ClpP2) of the caseinolytic-like proteases while monocyclic β-lactones were only able to label one isoform (ClpP2). The fused ring probe enabled investigation into the assembly of a stacked homo-heptameric assembly of the ClpP1 and ClpP2 isoforms which was not discoverable using monocyclic probes. Another ABPP study using a synthetic class of α-amino β-lactone class of compounds resembling SQ 26,517 revealed a distinct pattern of penicillin-binding protein labeling (PBP2x and PBP2B) that differed from the PBP labeling of β-lactams.[131] Future ABPP studies with spiro-β-lactones, glucopyranosylamino compounds like belactin B, or β-lactones that are polyketide- or terpenoid-derived could yield further insight into the enzyme targets of these core scaffolds.

Enzyme engineering and synthetic biology

Major breakthroughs in enzyme-catalyzed β-lactone ring formation greatly facilitates a biosynthetic approach to yield custom β-lactones and generate non-natural product derivatives. The β-lactone synthetase, OleC, is a standalone (non-PKS/NRPS) enzyme that could be harnessed to catalyze β-lactone ring formation from a variety of disubstituted β-hydroxy acid precursors.[23] However, the natural preference of OleC for substrates with long alkyl tails causes product solubility to be a major hurdle. Directed evolution techniques could be applied to engineer the β-lactone synthetase enzyme to accept a range of shorter chain, bulkier, or more 'drug-like' side chains. OleC-like enzymes could also potentially be delivered as therapeutics to cyclize compounds with naturally-occurring β-hydroxy acid motifs in the cell such as intermediates in mycolic acid and fatty acid biosynthesis, thereby inhibiting these pathways.

Modification of obafluorin-like TE domains by mutagenesis or domain-swapping may also be a viable route to engineer non-natural β-lactone formation during product release from NRPS-assembly lines. Substituting the catalytic serine in type I TE domains with a catalytic cysteine coupled with directed evolution approaches to increase activity could potentially generate engineered TE domains capable of forming β-lactones. Past efforts to alter substrate selectivity in PKS/NRPS systems have revealed the complexity of swapping or substituting non-native assembly line domains. However, great progress has been made in recent years to generate non-natural cyclized natural product analogs by moving or mutating TE domains.[132,133] Obtaining a high-resolution crystal structures of the obafluorin TE domain would significantly enhance the potential for rational design or directed evolution of these enzymes. These promising studies hint at the possibility of a 'biobrick' future in which β-lactone-cyclizing TE domains or AMP-dependent synthases could be installed as standard building blocks for post-PKS/NRPS modifications.

5 Conflicts of interest

The authors declare no conflict of interest.

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