Published in final edited form as:

Methods Enzymol. 2018 ; 604: 207–236. doi:10.1016/bs.mie.2018.01.034.

Chemoenzymatic Dissection of Polyketide β -Branching in the Bryostatin Pathway

Samuel T. Slocum^{*,†}, Andrew N. Lowell^{*}, Ashootosh Tripathi^{*}, Vikram V. Shende^{*}, Janet L. Smith^{*,†}, and David H. Sherman^{1,*,‡,§,¶}

^{*} Life Sciences Institute, University of Michigan, Ann Arbor, MI, United States

[†] Life Sciences Institute, Department of Biological Chemistry, University of Michigan, Ann Arbor, MI, United States

[‡] Life Sciences Institute, Department of Medicinal Chemistry, University of Michigan, Ann Arbor, MI, United States

[§] Life Sciences Institute, Department of Chemistry, University of Michigan, Ann Arbor, MI, United States

[¶] Life Sciences Institute, Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI, United States

Abstract

β -Branching is an expansion upon canonical polyketide synthase extension that allows for the installation of diverse chemical moieties in several natural products. Several of these moieties are unique among natural products, including the two vinyl methylesters found in the core structure of bryostatins. This family of molecules is derived from an obligate bacterial symbiont of a sessile marine bryozoan, *Bugula neritina*. Within this family, bryostatin 1 has been investigated as an anticancer, neuroprotective, and immunomodulatory compound. We have turned to the biosynthetic gene cluster within the bacterial symbiont to investigate the biosynthesis of bryostatins. Recent sequencing efforts resulted in the annotation of two missing genes: *bryT* and *bryU*. Using novel chemoenzymatic techniques, we have validated these as the missing enoyl-CoA hydratase and donor acyl carrier protein, essential components of the β -branching cassette of the bryostatin pathway. Together, this cassette installs the vinyl methylester moieties essential to the activity of bryostatins.

1. INTRODUCTION

Bryostatins are a family of molecules that mediate chemical defense during the larval stage in the marine invertebrate *Bugula neritina* life cycle (Lopanik, Lindquist, & Targett, 2004). Bryostatin-1 is a high-affinity modulator of protein kinase C and has clinical efficacy for many pathologies (Blackhall et al., 2001; Guti'erez et al., 2016; Lucke-Wold et al., 2014; Mehla et al., 2010; Pagliaro et al., 2000; Schrott et al., 2015; Sun, Hongpaisan, & Alkon, 2009). Through a combination of bacterial enrichment and in situ hybridization experiments

¹ Corresponding author: davidhs@umich.edu.

(Davidson, Allen, Lim, Anderson, & Haygood, 2001; Davidson & Haygood, 1999; Sharp, Davidson, & Haygood, 2007; Woollacott, 1981), and cloning and sequencing of the biosynthetic gene cluster (Sudek et al., 2007), bryostatin biosynthesis has been attributed to a γ -proteobacterial obligate symbiont, *Endobugula sertula* (Davidson & Haygood, 1999). This bacterium has remained refractory to laboratory culture, adding difficulty to sourcing through direct fermentation (Schaufelberger et al., 1991), and has prevented developing a genetic system for investigating the *bry* system. Attempts to resolve these issues have proven to be either ineffective or prohibitively costly (Mendola, 2003). Syntheses of several bryostatins have been reported (Keck, Poudel, Cummins, Rudra, & Covel, 2010; Lu, Woo, & Krische, 2011; Manaviazar & Hale, 2011; Wender & Schrier, 2011), and a recent “scalable” total synthesis of bryostatin-1 has recently been accomplished (Wender et al., 2017). Bryostatin analogs (termed “bryologs”) show promise (DeChristopher, Fan, Felsher, & Wender, 2012; Staveness et al., 2016; Wender et al., 2011), however, a simplified and effective replacement for bryostatin-1 has yet to be developed.

To facilitate the study of bryostatin biosynthesis, we have turned to the natural biosynthetic pathway. Previously, we prepared a biosynthetic hypothesis from a cosmid library that enabled sequencing and bioinformatics analysis of the *bry* gene cluster (Sudek et al., 2007). Like many clinically important natural products, bryostatins are produced by an enzymatic assembly line comprised of polyketide synthases (PKS), multidomain complexes used by many branches of the tree of life to synthesize structurally diverse natural products. PKS have yielded dividends as a rich source of drug leads (Newman & Cragg, 2012) and more recently as effective biocatalysts for chemoenzymatic assembly of macrolide antibiotics (Hansen et al., 2013). The activities of PKS and associated tailoring enzymes hold solutions to difficult synthetic problems, and it is critical to understand the ability of PKSs to generate complex metabolites through regio- and stereoselective catalytic processes.

The basic catalytic cycle of type-I PKS can be related to that of mammalian fatty acid synthase (mFAS) (Maier, Leibundgut, & Ban, 2008). While mFAS is a single multidomain module that acts iteratively, type-I PKS pathways are instead made up by a series of synthetic modules that act as a linear assembly line, with each module containing domain deletions, substitutions, and additions that produce diverse chemical functionality. Another strategy for chemical diversification that a subset of PKS pathways has borrowed from primary metabolism involves the use of a β -branching cassette. Reminiscent of isoprene biosynthesis, β -branching employs a 3-hydroxy- 3-methylglutaryl-CoA (HMG-CoA) synthase homologue (HMGS) that catalyzes a two-carbon addition to the β carbonyl of the nascent polyketide chain (Campobasso et al., 2004). This HMG-like intermediate is in most cases modified by sequential dehydration and decarboxylation by enoyl-CoA hydratases (termed ECH₁ and ECH₂, respectively). A variety of activities including oxidation (Simunovic et al., 2006), epoxidation (Zhang et al., 2011), halogenation (Chang et al., 2004), and methylation (Simunovic & Müller, 2007) may also be included at different points in this process, introducing functionality that canonical PKS extensions cannot introduce.

The *bry* cluster is made up of five large *trans*-acyltransferase PKS genes with an adjacent group of smaller genes. *trans*-Acyltransferase PKS lack integrated acyltransferase (AT)

domains (Helfrich & Piel, 2016), and extender unit selectivity is provided by the activity of a separate gene product, in this case BryP. The biosynthetic hypothesis is described in Fig. 1, highlighting the role of the β -branching cassette. The present work describes the identification and confirmation of the roles of BryT and BryU in β -branching. The corresponding *bryT* and *bryU* genes were predicted to exist, but remained unidentified until recently when Kwan and coworkers completed a draft genome of *E. sertula* (Miller, Vanee, Fong, Lim-Fong, & Kwan, 2016).

β -Branching is relatively rare among PKS pathways, and the vinyl methylester product of the bryostatin β -branching cassette is especially unusual in this group (Fig. 2). Only a handful of natural products have similar moieties (Higa, Tanaka, Komesu, & Jefford, 1992; Kedei et al., 2015; Ohta et al., 2006), but they currently lack genetically characterized biosynthetic pathways. Additionally, the completed vinyl methylester moieties are situated at two key sites of interaction with its biological target, protein kinase C (Kedei et al., 2015; Kelsey et al., 2016). Understanding the biosynthesis of these molecules and perhaps engineering this ability to other pathways may allow the portability of bryostatin-like biological activity to nonnatural products.

In vitro studies confirmed the role of BryR as the HMGS (Buchholz et al., 2010), however, several key questions remained regarding the conversion of β -keto intermediate to vinyl methylester. The β -branching reaction catalyzed by BryR requires a phylogenetically distinct standalone ACP donor (ACP_D) carrying an acetyl group as a source of branching carbons, but no ACP_D was identified within the cosmid library and a surrogate ACP facilitated previous studies (Buchholz et al., 2010). The source of the final carboxyl *O*-methylation was unclear, but several candidate enzymes or catalytic domains were identified in the Bry pathway. Additionally, the double bond configuration and lack of decarboxylation of the final product suggest either an isomerization or an unique ECH₁; however, no ECH₁ was identified in the original sequenced and annotated *bry* cluster. A recent draft genome of the *E. sertula* bacterial symbiont revealed candidate genes and corresponding gene products for the missing β -branching components: BryT (ECH₁) and BryU (ACP_D) (Miller, Vanee, et al., 2016). Interestingly, these open reading frames (ORFs) were 15 bp away from one another. However, due to gaps in the genomic assembly and a lack of overlap with any of the earlier draft quality cosmid sequencing data, the distance from the bryostatin PKS genes may only be estimated to be >48 kb.

Both ECH₁ and ECH₂, like most members of the crotonase superfamily, proceed through a vinylogous enolate intermediate of the thioester carbonyl, allowing the selection of ² or ³ products. ECH₁/ECH₂ pairs can, therefore, generate either a vinyl methyl (² isomer) or exomethylene (³ isomer) moiety. The mechanism of ECH₂ selection can be rationalized structurally by comparing the curacin ECH₂ (Geders et al., 2007) to the primary sequence of the ECH₂ in the closely related jamaicamide pathway (Gu et al., 2009). In principle, ECH₁ may also be able to generate ² and ³ products, however, this is obfuscated by decarboxylation in most pathways and remains untested biochemically. The discovery of the bryostatin ECH₁, therefore, provides an opportunity to determine if the ³ structure in bryostatins is installed by ECH₁ dehydration or a different biochemical event.

In this chapter, we set out to investigate the unique bryostatin β -branching cassette in order to understand how these unusual vinyl methylester moieties are installed. Specifically, we probed newly identified genes as potential members of a second bryostatin gene locus in *E. sertula*. Using established techniques as well as specific biosynthetic probes, we scrutinized enzymatic products to determine the regiochemistry of the biochemical reaction. The methods described here can be generalized to other biosynthetic pathways to chemoenzymatically determine regiochemistries of complex secondary metabolites.

2. PREVIOUS STUDIES AND IDENTIFICATION OF CRYPTIC COMPONENTS OF BRYOSTATIN BIOSYNTHESIS

The previously described cosmid sequencing assemblies allowed for the preparation of a functional hypothesis of bryostatin biosynthesis (Sudek et al., 2007). The first in vitro biochemical studies focused on confirming the functions of BryP and BryR (Buchholz et al., 2010; Lopanik et al., 2008). Essential to the activity of BryR as HMGS is a standalone donor ACP (ACP_D) and an acceptor ACP (ACP_A), which is part of a PKS module. A malonyl unit is loaded onto the phosphopantetheine arm of ACP_D, which is then decarboxylated by a specific KS. The resulting acetyl unit is then transferred to a cysteine sidechain of HMGS, where it resides until hydrolysis or interaction with ACP_A. Upon binding of ACP_A, HMGS catalyzes an aldol addition of the α carbon of the acetyl unit to the β ketone carbon, resulting in an HMG-like product. Unlike HMG-CoA synthase that acts on CoA-linked substrates, the activity of HMGS has been found to be very specific to ACP_A and ACP_D, with little tolerance for substitution (Buchholz et al., 2010; Maloney, Gerwick, Gerwick, Sherman, & Smith, 2016). This ACP selectivity presents a challenge when the correct ACP is unknown, but may be overcome by use of surrogate carrier proteins that are closely related phylogenetically (Buchholz et al., 2010).

The functionality of BryR was demonstrated using a set of simplified and commercially available acyl-CoAs that had been used in the study of HMGS from the curacin and jamaicamide pathways: namely, acetyl-, acetoacetyl-, and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The activity and role of BryR in the pathway was confirmed, but several questions about the bryostatin β -branching cassette persisted, as no donor ACP or ECH₁ component were identified in the cosmid library (Buchholz et al., 2010). Somehow, components of the pathway are able to convert the HMG-like product of BryR into a ³ vinyl methylester found in the final product, and several hypotheses were considered. However, in order to more fully understand bryostatin biosynthesis, new insights into the *Ca. E. sertula* genome were required.

Thanks in large part to the development of next-generation sequencing and accompanying analysis software, great advances have been made in metagenomic sequencing (Miller, Weyna, Fong, Lim-Fong, & Kwan, 2016; Schofield & Sherman, 2013). These technologies enable samples containing DNA from the genomes of multiple species to be analyzed directly without the need of cosmid assembly and time-consuming, and significantly costlier Sanger sequencing. The ability to specifically isolate and analyze symbiont sequencing data in silico has led to several interesting results, including the complete genome (rv0.63 kb) of

the tunicate endosymbiont that produces ET-743, *Candidatus Endoecteinascidia frumentensis* (Schofield, Jain, Porat, Dick, & Sherman, 2015). These techniques are helping to characterize the genetic diversity hidden in symbiont genomes.

Using metagenomic DNA isolated from *B. neritina* larvae and targeted microdissections of adult ovicells, Miller and coworkers were able to achieve sufficient abundance of *Ca. E. sertula* DNA to conduct next-generation sequencing (Miller, Vanee, et al., 2016). Subsequent analysis and careful pooling of reads from the symbiont genome resulted in a draft genome of *Ca. E. sertula* (Miller, Weyna, et al., 2016). Made up of 112 contigs with a total of 3.3 Mbp, this genome is much larger than would be expected for an obligate symbiont (Moran, 2003), and only bears some of the hallmarks of genome reduction, indicating a relatively recent symbiosis. The GC content and pseudogene abundance also suggest that *Ca. E. sertula* may have been able to live independently in the recent past. This is a key question for the isolation of large quantities of bryostatins, as free-living *Ca. E. sertula* may be fermented to facilitate large-scale production of bryostatins. While this is a desirable target, *Ca. E. sertula* is likely dependent upon its host for nutrition, as several deficiencies in amino acid biosynthesis genes were identified. This genome is a very interesting resource for the study of an obligate symbiont that provides an advantage to its host by providing a chemical defense agent for protection (Lopanik et al., 2004).

The Kwan laboratory sequencing effort also delivered two interesting genes: an FAS-like acyl carrier protein adjacent to a PKS enoyl-CoA hydratase. Because of their annotation being unique among ORFs identified in the assembly, these two genes (termed *bryT* and *bryU*) were strong candidates for the missing components from the bryostatin gene cluster. Additionally, the accompanying RNA sequencing provided strong evidence that these genes are actively transcribed while *Ca. E. sertula* is proliferating, as is the entire bryostatin gene cluster.

BryU was identified by automated annotation within the draft genome of *Ca. E. sertula*, and no other hits were found that resemble BryU or any other ACP_D within the draft genome. BryU strongly resembles other FAS-like β -branch donor ACPs (Fig. 3), including mAcPC from the mupirocin pathway (El-Sayed et al., 2003) (40.5% identity) and CurB from the curacin pathway (43.0% identity), both of which were used in previous studies of BryR function. Comparison of BryU and BryR to a cocrystal structure of CurB interacting with the curacin HMGS (CurD, 67.5% identity to BryR) shows that the key residues for HMGS–ACP_D interaction are conserved in both pathways (Maloney et al., 2016). This is perhaps to be expected given the relatively high sequence identity and binding affinity of CurB for BryR as a surrogate ACP_D (Buchholz et al., 2010).

BryT was found immediately adjacent to *bryU* in the draft genome of *Ca. E. sertula*. Automatically annotated as a polyketide synthesis ECH, phylogenetic analysis and sequence comparisons provided strong evidence that this was the missing bryostatin ECH₁ (Fig. 4). Because several ECH₁ and ECH₂ proteins from PKS clusters have been biochemically characterized (Calderone, Iwig, Dorrestein, Kelleher, & Walsh, 2007; Gu et al., 2006), primary sequence alone is typically sufficient to classify a protein as ECH₁, ECH₂, or a primary metabolic homologue. In sequence alignment with ECH₁, BryT displays a 38.3%

identity with CurE (Chang et al., 2004) and 37.9% identity with JamI (Edwards et al., 2004). Compared to ECH₂, BryT has 15.7% identity with CurF (ECH₂) and 14.5% identity with JamJ (ECH₂), clearly indicating identity with ECH₁ (Gu et al., 2006).

3. INITIAL BIOCHEMICAL ANALYSIS OF NOVEL BRY GENE PRODUCTS

3.1 BryU, β -Branch Donor ACP

BryR, BryU, and BryA module-3 ACP were expressed and purified using methods described previously (Buchholz et al., 2010), and activity was assessed by LC/MS using the phosphopantetheine ejection assay (Meluzzi, Zheng, Hensler, Nizet, & Dorrestein, 2008). Purity of the protein was assessed by SDS-PAGE (Fig. 5). When acetoacetyl-ACP_A (BryA module-3 ACP) was added to BryR and acetyl-BryU, conversion to HMG-ACP_A was observed (Fig. 6C), thus confirming the role of BryU as the donor ACP for the bryostatin β -branching cassette.

Every β -branching cassette found in type I PKS pathways has been identified with an associated donor ACP that brings an acetyl (or propionyl Simunovic et al., 2006) unit to HMGS for incorporation into the polyketide, and bryostatin is no exception. HMGS has been shown in several pathways, including bryostatin, to have a high degree of selectivity for both donor and acceptor ACPs. The basis of this selectivity has been described structurally in the curacin pathway, and the residues that are key to this binding mode appear to be conserved in both BryR (D214, D222, E225, and R266, CurD numbering) and BryU (R42, I46, and M47, CurB numbering) (Maloney et al., 2016). While the affinity of the curacin donor ACP is far tighter for its native HMGS than for BryR, the measured affinity still suggests that many of these donor ACPs have high degrees of intersystem operability (Buchholz et al., 2010). If future attempts are made to transfer HMGS functionality to other pathways, the matching of donor ACP for native HMGS will likely be relatively straightforward.

3.2 BryT, Enoyl-CoA Hydratase

Rather than attempting to clone from metagenomic DNA, synthetic codonoptimized DNA coding for BryT was designed, and cloned into expression vectors (Sørensen & Mortensen, 2005; Stols et al., 2002). Coexpression with GroEL and GroES at relatively low growth temperatures was determined to be necessary, with low and inconsistent protein yields from other conditions (Fig. 7). Protein purified under optimized conditions was >90% pure, but was sensitive to cooling below $\sim 5^{\circ}\text{C}$, and would semireversibly precipitate at low temperatures. Other ECH₁ enzymes from bacterial PKS pathways were also obtained for comparison: CurE and JamI from the curacin and jamaicamide pathways, respectively.

HMG-CoA was used as a substrate as previously described (Gu et al., 2006): both as a reagent used to charge ACPs and as a direct substrate without further modification. Using the phosphopantetheine ejection assay, a mass shift equivalent to a dehydration was observed after incubation with CurE, JamI, or BryT, indicating the generation of 3-methylglutaconyl-CoA (3MG-CoA) (Fig. 6D). Upon detailed review of the data, a small

amount of dehydrated mass is still detectable in the absence of ECH₁. This was suspected to be ionization-mediated dehydration occurring during the mass spectrometry analysis.

3.3 BryA, Carboxyl O-Methyltransferase

Previous attempts to identify the carboxyl O-MT that generates the bryostatin vinyl methylesters were unsuccessful. Several candidate S-adenosyl methionine (SAM)-dependent methyltransferases were identified within the cluster: BryA module-3 and BryB module-4 both contain MTs, and a standalone MT was identified near the other β -branching cassette genes. None of these domains were able to catalyze methylation of the substrate mimics in initial tests. However, revisiting this search after the discovery of BryT proved fruitful.

After publication of the draft *Ca. E. sertula* genome and accompanying transcriptomic data, BryS could be confidently eliminated as a candidate for O-methylation. In a BLAST search, many close homologues (>50% identity) returned as tRNA C-methyltransferases. It is notoriously difficult to predict the substrate of methylation for class I MTs by sequence comparison alone, but the large number of BLAST hits for C-MTs raised suspicion. In addition, while transcripts were detected for the rest of the pathway, no mRNA transcripts of BryS were identified in the transcriptomic investigation performed by the Kwan group (Miller, Vanee, et al., 2016). Taken together, these results provide strong evidence that BryS was not the desired carboxyl O-MT.

Computational analysis and BLAST searches of other candidate methyltransferases against the MiBIG database (Medema et al., 2015) provided some guidance toward identification of the desired Bry methyltransferase. BryA module-3 MT returned hits for a few N- and O-standalone methyltransferases with modest sequence identity (rv26%). BryB module-7 MT, at 65% identity to BryA module-3 MT, returned similar hits. The other pair of bryostatin MTs provided different results. At 95% identity, BryB module-4 MT and BryC module-9 MT are almost identical and return a large number of module-integrated PKS C-methyltransferases with a typical sequence identity of rv45%. Considering that BryA module-3 MT and BryB module-7 MT are located within the PKSs that are predicted to mediate β -branching modifications, these BLAST results strongly suggested their role as the carboxyl O-MTs of the Bry β -branching cassette.

With the function of BryT confirmed, we hypothesized that the carboxyl O-MT may require dehydration before being able to methylate its substrate. Thus, BryA module-3 was cloned from the bryostatin cosmid library (Sudek et al., 2007), expressed and purified. Additionally, standalone BryA module-3 ACP was purified and charged using Svp (Sánchez, Du, Edwards, Toney, & Shen, 2001) and HMG-CoA. After incubating this HMG-ACP with BryT, BryA module-3, and SAM, a mass shift corresponding to dehydration and methylation was observed in the phosphopantetheine ejection assay (Fig. 6E). Several constructs of the excised O-MT domain were subsequently cloned and purified, greatly improving the specific activity and total turnover of the system. Both in the context of the module and as a standalone domain, the O-MT acted with a strict dependence upon BryT dehydration, ACP tethering, and supplemental SAM cofactor.

4. CHEMOENZYMATIC REGIOCHEMISTRY DETERMINATION

4.1 Analysis of the BryT Dehydration Product

While the detection of a loss of water is compelling evidence for ECH₁ activity, it does not allow for determination of the regiochemistry of the product. Several approaches were developed that take advantage of the ability of ECH₁ to use CoA-tethered substrates to unambiguously determine whether BryT was catalyzing dehydration to generate the α/β (d_2 product) or β/γ (d_3 product) regiochemistry. First, the similarity of PKS HMGS and HMG-CoA synthase in primary metabolism was leveraged to chemoenzymatically synthesize a specifically deuterated HMG-CoA (Fig. 8). Fully deuterated acetic anhydride was used to generate d_3 -acetyl-CoA, which was then employed as a carbon donor for MvaS, the HMG-CoA synthase from *Staphylococcus aureus* (Campobasso et al., 2004). This reaction series generated γ - d_2 -HMG-CoA, which would result in either a +1 Da or +2 Da difference from the natural isotopic abundance after ECH₁-mediated dehydration. Detection of the product masses was achieved through HPLC separation of the reaction components and MS confirmation of the individual masses.

The addition of BryT to this reaction system resulted in the generation of primarily d_2 -3MG-CoA, strongly indicating α/β dehydration and a d_2 product (Fig. 9). This is somewhat surprising, as no other enzymatic components are evident within the cluster that may isomerize this double bond to generate the β/γ configuration found in the final product. Because this is a simplified substrate mimic, we cannot rule out that dehydration of the native tetra- and octaketide substrates would result in different regiochemistry. However, this seems unlikely. The shared mechanism and high-sequence identity among BryT and other ECH₁ suggest that nonnative substrate structures would not alter product regiochemistry. Additionally, isolation and NMR analysis discussed in Section 4.1.3 indicate a single product configuration rather than a mixture as might be expected for a nonnative substrate.

Because of proton exchange with solvent, the timing of the reaction has a large impact on the isotope distribution detected in the products. However, proton exchange must be balanced with the generation of sufficient product to be reliably separated and detected. In the attempt to balance these demands, we found that shorter reaction times led to an even higher ratio of $d_2:d_1$ 3MG-CoA (Fig. 10), further indicating that the dideuterated molecule is the preferred product of BryT.

While analyzing these data, it is important to consider the possibility of a kinetic isotope effect altering the outcome. Because ECH₁ (and most of the crotonase family) proceed through an enolate intermediate stabilized by an oxyanion hole in the enzyme active site, the rate-limiting step is likely either the formation of the enolate or the first deprotonation of the substrate at the α -carbon. Because the abstraction of a γ -deuteron is highly unlikely to be the rate-limiting step of the reaction, a kinetic isotope effect is unlikely to alter the product distribution sufficiently to explain the abundance of γ - d_2 -3MG-CoA detected in these reactions.

4.1.1 Materials and Equipment

1. BryT, CurE, and JamI (ECH₁) were expressed and purified using methods described for CurD (Maloney et al., 2016)
2. *S. aureus* mvaS DNA, codon optimized for *E. coli* (DE3) (IDT, gBlocks)
3. (D/L)-3-Hydroxy-3-methylglutaryl coenzyme A sodium salt hydrate (Sigma #H6132)
4. Acetoacetyl coenzyme A sodium salt hydrate (Sigma #A1625)
5. Acetyl coenzyme A sodium salt hydrate (Sigma #A2056)
6. Coenzyme A sodium salt hydrate (Sigma #C3144)
7. Acetic anhydride-*d*₆ (Sigma #175641)
8. Magnesium chloride (Fisher #M35–500)
9. Dithiothreitol (DTT) (Sigma #43819)
10. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (Fisher #BP310–500)
11. Ammonium formate (Sigma #70221)
12. LC/MS water (Sigma #WX0001)
13. LC/MS methanol (EMD Millipore #MX0475)
14. LC/MS acetonitrile (EMD Millipore #AX0142)
15. LC/MS formic acid (Fisher #A117–50)
16. Reversed-phase C18 HPLC column (Waters, xBridge C18 column 3.6 μ , 2.1 $\times$ 250 mm)
17. Q-TOF LC/MS (Agilent G6545A)

4.1.2 In Situ γ -Deuterated HMG-CoA Synthesis and LC/MS Analysis

1. Coenzyme A was dissolved to a concentration of 10 mM in 0.5 M HEPES pH 7.0.
2. Acetic anhydride-*d*₆ was added to 1.5 mM and incubated for 20 min at 23°C to generate *d*₃-acetyl-CoA.
3. During the incubation, mvaS reactions were prepared by combining 25 μ M purified mvaS with 10 mM MgCl₂, 2 mM DTT, 50 mM HEPES pH 7.0, 10 μ M ECH₁ (BryT, CurE, or JamI), 2 mM acetoacetyl-CoA, and 10% glycerol.
4. Without further purification, crude *d*₃-acetyl-CoA reaction mixture was added to mvaS reactions to 4% (v/v) and incubated for 20 min at 23°C.
5. Reactions were quenched with three volumes LC/MS methanol with 5% formic acid.

6. Because of proton exchange with the deuterated material, expedience of the protocol is key. Quenched reactions were clarified by high-speed centrifugation and analyzed by LC/MS as quickly as possible.
7. Baseline separation of different CoA thioesters was achieved using a narrow gradient (4%–10% B) where buffer A was water with 50 mM ammonium formate pH 5.0 and buffer B was neat acetonitrile.
8. Extracted ion chromatograms were generated using masses calculated for both intact CoA thioesters and phosphopantetheine ejection adducts (Meluzzi et al., 2008). Phosphopantetheine ejection masses were found to be more reliably detected for all species.

4.1.3 Large-Scale Product Isolation and NMR Analysis—In order to corroborate these data, a large-scale reaction and purification effort were undertaken. Fresh BryT was purified and immediately added to a 10-mg scale reaction of HMG-CoA. Because BryT catalyzes both dehydration and the reverse hydration reaction, and also prefers a single stereoisomer of the rv50:50 mixture present in commercial HMG-CoA, the yields of these reactions are typically modest (rv10%). Representative HPLC traces are shown in Fig. 11. Through careful method development and scale-up, a rapid workup and HPLC purification protocol were established that minimized the amount of time 3MG-CoA product was exposed to room temperature. Even so, the low yields and degradation were persistent challenges.

Eventually, NMR spectra were obtained using samples of sufficient concentration and purity. Initial proton NMR data showed conclusively that a methylene signal is lost and a new methane proton appears, indicating a new double bond. To enable analysis of the double bond installed by BryT, a partial assignment of commercial HMG-CoA starting material was carried out. By comparing this assignment to the 3MG-CoA spectra, the site of the lost methylene could be correlated on HMG-CoA (Fig. 12). This analysis led to the conclusion that the methylene signal disappeared from the α -carbon of 3MG-CoA, indicating that the resulting product was derived from an α/β dehydration. This is consistent with the mass shifts in the deuteration experiments and strongly supports that BryT catalyzes α/β dehydration activity.

4.2 Analysis of Bry β -Branching O-Methylation

Much like the dehydration catalyzed by BryT, mass spectrometry alone cannot confirm the location of a new methyl group by considering mass shift alone. Since BryA *O*-MT was unable to utilize CoA-tethered substrates, scale-up and isolation were not feasible. Thus, we turned again to organic synthesis to address this problem. Diazomethane (DM) is a reagent known to effectively convert carboxylic acids into methyl esters with a high degree of selectivity. Although it is acutely toxic, carcinogenic, and explosive, these risks can be almost completely ameliorated by using a trimethylsilyl-DM (TMS-DM) derivative. The covalently linked TMS is hydrolyzed in situ to generate smaller, safer amounts of DM.

This reaction can be used to probe *O*-methylation by coupling TMS-DM treatment with enzymatic methylation. By treating HMG-ACP with BryT and BryA *O*-MT prior to

chemical treatment with TMS-DM and assessing the outcome by phosphopantetheine ejection, the location of methylation may be tested. If BryA *O*-MT is, in fact, a carboxyl *O*-MT, it will preclude methylation by DM, resulting in a mass shift equivalent to one methylation (14 Da). Enzymatic methylation at any other position will not preclude methyl ester formation, resulting in a mass shift indicative of two methylations (28 Da).

TMS-DM is commercially available as solution in hexanes, which presents a challenge for use with aqueous solutions of biomolecules. Initial experiments resulted in a bimodal distribution of methylation of HMG-ACP, with approximately equal populations of ACP bearing rv120 methylations and 0 methylations, with almost no methylation of the phosphopantetheine ejection ion detected. This is perhaps because the only contact made between HMG-ACP and TMS-DM was at the aqueous-organic interface, resulting in the extensive methylation of the fraction of protein in close proximity to the interface. It is interesting that even in the population of extensively methylated HMG-ACP, there was almost no conversion of HMG-phosphopantetheine to methyl ester. This may be due to sequestration of the phosphopantetheine arm away from solvent, a phenomenon that has been studied in detail in type-II FAS and PKS ACPs (Haushalter et al., 2011; Mercer & Burkart, 2007), but remains poorly studied in type-I PKSs.

Serial dilutions of TMS-DM from hexanes into methanol resolved this problem and altered the methylation pattern. HMG-ACP is soluble in methanol at concentrations up to rv70%, and the addition of dilute TMS-DM to protein that has been prediluted in methanol results in the generation of diverse methylation states of the protein (Fig. 13). Using this method, rv90% of the protein is methylated at least once, with distinct masses corresponding to 1, 2, 3, ... and so on methylations up to rv20. The abundance of these species roughly approximates a Gaussian distribution centered at 11 methylations. Interestingly, the frequency of methylation of the HMG moiety on the phosphopantetheine arm is still much lower than protein as a whole, with roughly 60% of the original HMG-phosphopantetheine ion still intact. This, like the previously described experiments, also implies some sort of sequestration of the phosphopantetheine arm away from solvent.

Most importantly, only one methylation was detected on the phosphopantetheine arm of HMG-ACP that had been treated with BryT and BryA *O*-MT prior to TMS-DM treatment. This indicates that the only site competent for methylation by TMS-DM has been blocked enzymatically by the *O*-MT. Other control samples confirmed that TMS-DM was only able to methylate in one location of the phosphopantetheine arm (Fig. 14). Incubation of HMG-ACP with BryT or no enzyme prior to TMS-DM treatment also resulted in masses corresponding to a single methylation of the phosphopantetheine arm. These results confirm the role of BryA *O*-MT as the carboxyl *O*-MT of the β -branching cassette.

4.2.1 Materials and Equipment

1. ECH₁, *O*-MT, apo-ACP, and Svp were purified as previously described (Gu et al., 2006; Sánchez et al., 2001)
2. *S*-adenosyl-L-methionine *p*-toluenesulfonate (Carbosynth #NA04017)

3. (D/L)-3-Hydroxy-3-methylglutaryl coenzyme A sodium salt hydrate (Sigma #H6132)
4. Magnesium chloride (Fisher #M35–500)
5. (Trimethylsilyl)diazomethane, 2 *M* in hexanes (Sigma #362832)
6. LC/MS methanol (EMD Millipore #MX0475)
7. LC/MS water (Sigma #WX0001)
8. LC/MS formic acid (Fisher #A117–50)
9. LC/MS acetonitrile (EMD Millipore #AX0142)
10. Reversed-phase C4 HPLC column (Waters, Acquity BEH300 C4 col- umn 1.7 μ m, 2.1 $\times$ 50 mm)
11. Q-TOF LC/MS (Agilent G6545A)

4.2.2 Carrier Protein Methylation and LC/MS Analysis

1. Apo-ACP was loaded with HMG-phosphopantetheine using HMG-CoA, Svp, and MgCl_2 using methods previously described (Maloney et al., 2016)
2. (Trimethylsilyl)diazomethane was placed under positive pressure of nitrogen using a syringe needle, and $\sim 200 \mu\text{L}$ was drawn into a 1-mL syringe using a second needle and transferred to a 1.6-mL microcentrifuge tube
3. $200 \mu\text{L}$ of LC/MS methanol was added by pipette and vigorously mixed by pipetting to form a crude emulsion.
4. $200 \mu\text{L}$ of this emulsion was then added to another microcentrifuge tube with $200 \mu\text{L}$ methanol and mixed again.
5. The dilution procedure was repeated four more times for a final TMS-DM concentration of $\sim 31.25 \text{ mM}$ in 1.5% hexanes and 98.5% methanol.
6. Enzymatic methylation reactions and control reactions were prepared with final concentrations as follows: $75 \mu\text{M}$ HMG-BryA module-3 ACP, $30 \mu\text{M}$ BryA O-MT, $40 \mu\text{M}$ BryT, 1 *mM* SAM, 5 *mM* MgCl_2 , 50 *mM* HEPES pH 7.0, and 10% glycerol.
7. Enzymatic methylation reactions were incubated for 30 min at 30°C , then mixed with an equal volume of methanol-diluted TMS-DM. Reactions were mixed gently and allowed to incubate for 5 min at 23°C .
8. TMS-DM treatment was carefully quenched with two volumes of 50% methanol with 10% formic acid. Slow addition of this formic acid solution is important, as any unreacted diazomethane will rapidly evolve nitrogen gas upon acid quenching.
9. Reactions were centrifuged for 30 min at $17,000 \times g$ to remove debris and precipitation.

10. LC/MS analysis was carried out using a 5 min, 0–100% B gradient where A is 95% water, 5% acetonitrile with 0.1% formic acid, and B is 95% acetonitrile, 5% water with 0.1% formic acid.
11. Masses were monitored during protein elution with m/z from 150 to 3000, enabling facile analysis of phosphopantetheine ejection ions as well as deconvolution of intact protein masses.

5. SUMMARY AND CONCLUSIONS

We have successfully confirmed the roles of BryU, BryT, and BryA *O*-MT in the construction of the unique vinyl methylester moieties found in bryostatin-1 and related natural product analogs. These enzymes act in a strict order during the β -branching of the polyketide intermediate, beginning with donation of an acetyl subunit by BryU to β -branching, then dehydration by BryT, followed by *O*-methylation by BryA *O*-MT. We have also confirmed the regiochemistry of both dehydration and *O*-methylation steps using novel chemoenzymatic tools. The generation and processing of γ - d_2 -HMG-CoA revealed α/β (2) position of the double bond resulting from BryT dehydration. Chemical generation of the methyl ester of HMG-and 3MG-ACP_A combined with enzymatic methylation by BryA *O*-MT conclusively demonstrated carboxyl *O*-methylation with a strict dependence upon α/β dehydrated 3MG-CoA. These results support the proposal of a modified model of bryostatin β -branching that includes post- β -branching double bond isomerization prior to spontaneous hemiketalization to generate the unique vinyl methylester pyran rings found in all natural product bryostatins.

In the majority of β -branching cassettes, the double bond configuration of ECH₁ products is obfuscated by downstream modifications. In most cases, this means ECH₂ decarboxylation, which has been shown to be the determinant of product regiochemistry in the cases where it occurs (Geders et al., 2007). In newly discovered pathways, the knowledge that ECH₁ catalyzes α/β dehydration can inform biosynthetic hypotheses about intermediates and biosynthetic strategies.

These results have implications for the future of PKS pathway functional analysis and engineering. Because β -branch acceptor ACPs can be readily identified by a primary sequence motif, one can envision the modification of a normal modular ACP to transform it into a β -branch acceptor. This method would result in the introduction of a novel chemical structure at a specified β -keto position during polyketide synthesis and ultimately a novel polyketide product. In addition, the chemoenzymatic methods developed for these studies may be applied to a number of challenging questions relating to β -branching schemes in other PKS pathways. These methods provide tools to logically determine regiochemistry of several PKS intermediates that remain difficult to prepare and are only available on microscale levels.

ACKNOWLEDGMENTS

NIH Grants R35 GM118101, GM076477, and the Hans W. Vahlteich Professorship (to D.H.S.) are gratefully acknowledged. S.T.S. was supported in part by a Biological Chemistry Ph.D. Graduate Student Award from the

University of Michigan Rackham Graduate School and the Cellular Biotechnology Training Program (T32 GM008353).

REFERENCES

- Blackhall FH, Ranson M, Radford JA, Hancock BW, Soukop M, McGown AT, et al. Cancer Research Campaign Phase I/II Committee, (2001). A phase II trial of bryostatin 1 in patients with non-Hodgkin's lymphoma. *British Journal of Cancer*, 84, 465–469. 10.1054/bjoc.2000.1624. [PubMed: 11263437]
- Buchholz TJ, Rath CM, Lopanik NB, Gardner NP, Håkansson K, & Sherman DH (2010). Polyketide β -branching in bryostatin biosynthesis: Identification of surrogate acetyl-ACP donors for BryR, an HMG-ACP synthase. *Chemistry & Biology*, 17, 1092–1100. 10.1016/j.chembiol.2010.08.008. [PubMed: 21035732]
- Calderone CT, Iwig DF, Dorrestein PC, Kelleher NL, & Walsh CT (2007). Incorporation of nonmethyl branches by isoprenoid-like logic: Multiple β -alkylation events in the biosynthesis of myxovirescin A1. *Chemistry & Biology*, 14, 835–846. 10.1016/j.chembiol.2007.06.008. [PubMed: 17656320]
- Campobasso N, Patel M, Wilding IE, Kallender H, Rosenberg M, & Gwynn MN (2004). *Staphylococcus aureus* 3-hydroxy-3-methylglutaryl-CoA synthase: Crystal structure and mechanism. *Journal of Biological Chemistry*, 279, 44883–44888. 10.1074/jbc.M407882200. [PubMed: 15292254]
- Chang Z, Sitachitta N, Rossi JV, Roberts MA, Platt PM, Jia J, et al. (2004). Biosynthetic pathway and gene cluster analysis of curacin A, an antitubulin natural product from the tropical marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products*, 67, 1356–1367. 10.1021/np0499261. [PubMed: 15332855]
- Davidson SK, Allen SW, Lim GE, Anderson CM, & Haygood MG (2001). Evidence for the biosynthesis of bryostatins by the bacterial symbiont "*Candidatus Endobugula sertula*" of the bryozoan *Bugula neritina*. *Applied and Environmental Microbiology*, 67, 4531–4537. [PubMed: 11571152]
- Davidson SK, & Haygood MG (1999). Identification of sibling species of the bryozoan *Bugula neritina* that produce different anticancer bryostatins and harbor distinct strains of the bacterial symbiont "*Candidatus Endobugula sertula*." *The Biological Bulletin*, 196, 273–280. [PubMed: 10390826]
- DeChristopher BA, Fan AC, Felsher DW, & Wender PA (2012). "Picolog," a synthetically-available bryostatin analog, inhibits growth of MYC-induced lymphoma *in vivo*. *Oncotarget*, 3, 58–66. [PubMed: 22308267]
- Edwards DJ, Marquez BL, Nogle LM, McPhail K, Goeger DE, Roberts MA, et al. (2004). Structure and biosynthesis of the jamaicamides, new mixed polyketide-peptide neurotoxins from the marine cyanobacterium *Lyngbya majuscula*. *Chemistry & Biology*, 11, 817–833. 10.1016/j.chembiol.2004.03.030. [PubMed: 15217615]
- El-Sayed AK, Hothersall J, Cooper SM, Stephens E, Simpson TJ, & Thomas CM (2003). Characterization of the mupirocin biosynthesis gene cluster from *Pseudomonas fluorescens* NCIMB 10586. *Chemistry & Biology*, 10, 419. [PubMed: 12770824]
- Geders TW, Gu L, Mowers JC, Liu H, Gerwick WH, Håkansson K, et al. (2007). Crystal structure of the ECH2 catalytic domain of CurF from *Lyngbya majuscula*: Insights into a decarboxylase involved in polyketide chain branching. *Journal of Biological Chemistry*, 282, 35954–35963. 10.1074/jbc.M703921200. [PubMed: 17928301]
- Gu L, Jia J, Liu H, Håkansson K, Gerwick WH, & Sherman DH (2006). Metabolic coupling of dehydration and decarboxylation in the curacin A pathway: Functional identification of a mechanistically diverse enzyme pair. *Journal of the American Chemical Society*, 128, 9014–9015. 10.1021/ja0626382. [PubMed: 16834357]
- Gu L, Wang B, Kulkarni A, Geders TW, Grindberg RV, Gerwick L, et al. (2009). Metamorphic enzyme assembly in polyketide diversification. *Nature*, 459, 731–735. 10.1038/nature07870. [PubMed: 19494914]

- Gutiérrez C, Serrano-Villar S, Madrid-Elena N, Pérez-Elías MJ, Martín ME, Barbas C, et al. (2016). Bryostatin-1 for latent virus reactivation in HIV-infected patients on antiretroviral therapy. *AIDS*, 30, 1385–1392. 10.1097/QAD.0000000000001064. [PubMed: 26891037]
- Hansen DA, Rath CM, Eisman EB, Narayan ARH, Kittendorf JD, Mortison JD, et al. (2013). Biocatalytic synthesis of pikromycin, methymycin, neomethymycin, novamethymycin, and ketomethymycin. *Journal of the American Chemical Society*, 135, 11232–11238. 10.1021/ja404134f. [PubMed: 23866020]
- Haushalter RW, Filipp FV, Ko K, Yu R, Opella SJ, & Burkart MD (2011). Binding and pKa modulation of a polycyclic substrate analog in a type II polyketide acyl carrier protein. *ACS Chemical Biology*, 6, 413. [PubMed: 21268653]
- Helfrich EJN, & Piel J (2016). Biosynthesis of polyketides by trans-AT polyketide synthases. *Natural Product Reports*, 33, 231–316. 10.1039/C5NP00125K. [PubMed: 26689670]
- Higa T, Tanaka J, Komesu M, & Jefford CW (1992). Miyakolide: A bryostatin-like macrolide from a sponge, *Polyfibrospongia* sp. *Journal of the American Chemical Society*, 114, 7587–7588. 10.1021/ja00045a055.
- Keck GE, Poudel YB, Cummins TJ, Rudra A, & Covell JA (2010). Total synthesis of bryostatin 1. *Journal of the American Chemical Society*, 133, 744–747. [PubMed: 21175177]
- Kedei N, Kraft MB, Keck GE, Herald CL, Melody N, Pettit GR, et al. (2015). Neristatin 1 provides critical insight into bryostatin 1 structure–function relationships. *Journal of Natural Products*, 78, 896–900. 10.1021/acs.jnatprod.5b00094. [PubMed: 25808573]
- Kelsey JS, Cataisson C, Chen J, Herrmann MA, Petersen ME, Baumann DO, et al. (2016). Biological activity of the bryostatin analog merle 23 on mouse epidermal cells and mouse skin. *Molecular Carcinogenesis*, 55, 2183–2195. 10.1002/mc.22460. [PubMed: 26859836]
- Lopanik N, Lindquist N, & Targett N (2004). Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. *Oecologia*, 139, 131–139. 10.1007/s00442-004-1487-5. [PubMed: 14747940]
- Lopanik NB, Shields JA, Buchholz TJ, Rath CM, Hothersall J, Haygood MG, et al. (2008). In vivo and in vitro trans-acylation by BryP, the putative bryostatin pathway acyltransferase derived from an uncultured marine symbiont. *Chemistry & Biology*, 15, 1175–1186. 10.1016/j.chembiol.2008.09.013. [PubMed: 19022178]
- Lu Y, Woo SK, & Krische MJ (2011). Total synthesis of bryostatin 7 via C–C bond-forming hydrogenation. *Journal of the American Chemical Society*, 133, 13876–13879. 10.1021/ja205673e. [PubMed: 21780806]
- Lucke-Wold BP, Logsdon AF, Smith KE, Turner RC, Alkon DL, Tan Z, et al. (2014). Bryostatin-1 restores blood brain barrier integrity following blast-induced traumatic brain injury. *Molecular Neurobiology*, 52, 1119–1134. 10.1007/s12035-014-8902-7. [PubMed: 25301233]
- Maier T, Leibundgut M, & Ban N (2008). The crystal structure of a mammalian fatty acid synthase. *Science*, 321, 1315–1322. 10.1126/science.1161269. [PubMed: 18772430]
- Maloney FP, Gerwick L, Gerwick WH, Sherman DH, & Smith JL (2016). Anatomy of the β -branching enzyme of polyketide biosynthesis and its interaction with an acyl-ACP substrate. *Proceedings of the National Academy of Sciences*, 113(37), 10316–10321. 10.1073/pnas.1607210113.
- Manaviyar S, & Hale KJ (2011). Total synthesis of bryostatin 1: A short route. *Angewandte Chemie International Edition*, 50, 8786–8789. 10.1002/anie.201101562. [PubMed: 21751307]
- Medema MH, Kottmann R, Yilmaz P, Cummings M, Biggins JB, Blin K, et al. (2015). Minimum information about a biosynthetic gene cluster. *Nature Chemical Biology*, 11, 625–631. 10.1038/nchembio.1890. [PubMed: 26284661]
- Mehla R, Bivalkar-Mehla S, Zhang R, Handy I, Albrecht H, Giri S, et al. (2010). Bryostatin modulates latent HIV-1 infection via PKC and AMPK signaling but inhibits acute infection in a receptor independent manner. *PLoS One*, 5, 10.1371/journal.pone.0011160.
- Meluzzi D, Zheng WH, Hensler M, Nizet V, & Dorrestein PC (2008). Top-down mass spectrometry on low-resolution instruments: Characterization of phosphopantetheinylated carrier domains in polyketide and non-ribosomal biosynthetic pathways. *Bioorganic & Medicinal Chemistry Letters*, 18, 3107–3111. 10.1016/j.bmcl.2007.10.104. [PubMed: 18006314]

- Mendola D (2003). Aquaculture of three phyla of marine invertebrates to yield bioactive metabolites: Process developments and economics. *Biomolecular Engineering*, 20, 441–458. [PubMed: 12919831]
- Mercer AC, & Burkart MD (2007). The ubiquitous carrier protein—A window to metabolite biosynthesis. *Natural Product Reports*, 24, 750–773. 10.1039/B603921A. [PubMed: 17653358]
- Miller IJ, Vanee N, Fong SS, Lim-Fong GE, & Kwan JC (2016). Lack of overt genome reduction in the bryostatin-producing bryozoan symbiont, “*Candidatus Endobugula sertula*” *Applied and Environmental Microbiology*, 82(22), 6573–6583. 10.1128/AEM.01800-16. [PubMed: 27590822]
- Miller IJ, Weyna TR, Fong SS, Lim-Fong GE, & Kwan JC (2016). Single sample resolution of rare microbial dark matter in a marine invertebrate metagenome. *Scientific Reports*, 6, 34362 10.1038/srep34362. [PubMed: 27681823]
- Moran NA (2003). Tracing the evolution of gene loss in obligate bacterial symbionts. *Current Opinion in Microbiology*, 6, 512–518. [PubMed: 14572545]
- Newman DJ, & Cragg GM (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products*, 75, 311–335. 10.1021/np200906s. [PubMed: 22316239]
- Ohta S, Uy MM, Yanai M, Ohta E, Hirata T, & Ikegami S (2006). Exiguolide, a new macrolide from the marine sponge *Geodia exigua*. *Tetrahedron letters*, 47(12), 1957–1960.
- Pagliaro L, Daliani D, Amato R, Tu SM, Jones D, Smith T, et al. (2000). A phase II trial of bryostatin-1 for patients with metastatic renal cell carcinoma. *Cancer*, 89, 615–618. [PubMed: 10931461]
- Sánchez C, Du L, Edwards DJ, Toney MD, & Shen B (2001). Cloning and characterization of a phosphopantetheinyl transferase from *Streptomyces verticillus* ATCC15003, the producer of the hybrid peptide–polyketide antitumor drug bleomycin. *Chemistry & Biology*, 8, 725–738. 10.1016/S1074-5521(01)00047-3. [PubMed: 11451672]
- Schäufelberger DE, Koleck MP, Beutler JA, Vatakis AM, Alvarado AB, Andrews P, et al. (1991). The large-scale isolation of bryostatin 1 from *Bugula neritina* following current good manufacturing practices. *Journal of Natural Products*, 54, 1265–1270. 10.1021/np50077a004. [PubMed: 1800630]
- Schofield MM, Jain S, Porat D, Dick GJ, & Sherman DH (2015). Identification and analysis of the bacterial endosymbiont specialized for production of the chemotherapeutic natural product ET-743. *Environmental Microbiology*, 17, 3964–3975. 10.1111/1462-2920.12908. [PubMed: 26013440]
- Schofield MM, & Sherman DH (2013). Meta-omic characterization of prokaryotic gene clusters for natural product biosynthesis. *Current Opinion in Biotechnology*, 24, 1151–1158. 10.1016/j.copbio.2013.05.001. [PubMed: 23731715]
- Schrott LM, Jackson K, Yi P, Dietz F, Johnson GS, Basting TF, et al. (2015). Acute oral bryostatin-1 administration improves learning deficits in the APP/PS1 transgenic mouse model of Alzheimer’s disease. *Current Alzheimer Research*, 12, 22–31. [PubMed: 25523423]
- Sharp KH, Davidson SK, & Haygood MG (2007). Localization of “*Candidatus Endobugula sertula*” and the bryostatins throughout the life cycle of the bryozoan bugula neritina. *International Society for Microbial Ecology Journal*, 1, 693–702. 10.1038/ismej.2007.78.
- Simunovic V, & Müller R (2007). Mutational analysis of the myxovirescin biosynthetic gene cluster reveals novel insights into the functional elaboration of polyketide backbones. *Chembiochem*, 8, 1273–1280. 10.1002/cbic.200700153. [PubMed: 17583882]
- Simunovic V, Zapp J, Rachid S, Krug D, Meiser P, & Müller R (2006). Myxovirescin A biosynthesis is directed by hybrid polyketide synthases/nonribosomal peptide synthetase, 3-hydroxy-3-methylglutaryl-coa synthases, and trans-acting acyltransferases. *Chembiochem*, 7, 1206–1220. 10.1002/cbic.200600075. [PubMed: 16835859]
- Sørensen HP, & Mortensen KK (2005). Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *Journal of Biotechnology*, 115, 113–128. 10.1016/j.jbiotec.2004.08.004. [PubMed: 15607230]
- Staveness D, Abdelnabi R, Near KE, Nakagawa Y, Neyts J, Delang L, et al. (2016). Inhibition of chikungunya virus-induced cell death by salicylate-derived bryostatin analogues provides

- additional evidence for a PKC-independent pathway. *Journal of Natural Products*, 79, 680–684. 10.1021/acs.jnatprod.5b01017. [PubMed: 26900711]
- Stols L, Gu M, Dieckman L, Raffin R, Collart FR, & Donnelly MI (2002). A new vector for high-throughput, ligation-independent cloning encoding a tobacco etch virus protease cleavage site. *Protein Expression and Purification*, 25, 8–15. 10.1006/prep.2001.1603. [PubMed: 12071693]
- Sudek S, Lopanik NB, Waggoner LE, Hildebrand M, Anderson C, Liu H, et al. (2007). Identification of the putative bryostatin polyketide synthase gene cluster from “*Candidatus Endobugula sertula*,” the uncultivated microbial symbiont of the marine bryo- zoan *Bugula neritina*. *Journal of Natural Products*, 70, 67–74. 10.1021/np060361d. [PubMed: 17253852]
- Sun M-K, Hongpaisan J, & Alkon DL (2009). Postschismic PKC activation rescues retrograde and anterograde long-term memory. *Proceedings of the National Academy of Sciences*, 106, 14676–14680. 10.1073/pnas.0907842106.
- Wender PA, Baryza JL, Brenner SE, DeChristopher BA, Loy BA, Schrier AJ, et al. (2011). Design, synthesis, and evaluation of potent bryostatin analogs that modulate PKC translocation selectivity. *Proceedings of the National Academy of Sciences*, 108, 6721–6726. 10.1073/pnas.1015270108.
- Wender PA, Hardman CT, Ho S, Jeffreys MS, Maclaren JK, Quiroz RV, et al. (2017). Scalable synthesis of bryostatin 1 and analogs, adjuvant leads against latent HIV. *Science*, 358, 218–223. 10.1126/science.aan7969. [PubMed: 29026042]
- Wender PA, & Schrier AJ (2011). Total synthesis of bryostatin 9. *Journal of the American Chemical Society*, 133, 9228–9231. 10.1021/ja203034k. [PubMed: 21618969]
- Woollacott RM (1981). Association of bacteria with bryozoan larvae. *Marine Biology*, 65, 155–158. 10.1007/BF00397080.
- Zhang F, He H-Y, Tang M-C, Tang Y-M, Zhou Q, & Tang G-L (2011). Cloning and elucidation of the FR901464 gene cluster revealing a complex acyltransferaseless polyketide synthase using glycerate as starter units. *Journal of the American Chemical Society*, 133, 2452–2462. 10.1021/ja105649g. [PubMed: 21291275]

Fig. 1.
 β -Branching in bryostatin biosynthesis. The β -keto product of BryA module-3 is extended by BryR, an HMGS that uses an acetyl unit carried by BryU, a standalone ACP. BryT dehydrates the HMG-like product, which is then methylated by the *O*-MT domain within BryA module3. The resulting double bond is isomerized prior to pyran ring closure, most likely by the ER-like domain within BryB module-4.

Fig. 2.

Unique β -branching cassettes. Only three polyketide natural products contain β -branches that lack an ECH₂ mediated step: leinamycin, bongkreikic acid, and bryostatin 1 (A). These branches are installed using the same HMGS and ECH₁ steps (B), but downstream modifications diverge from other β -branches.

Fig. 3.
Phylogenetic analysis of BryU.

Fig. 4.
Phylogenetic analysis of BryT.

Purified BryR, ACP_D, and ACP_A SDS-PAGE

Fig. 5.
SDS-PAGE of BryR, BryU, and BryA module-3 ACP.

Fig. 6.

LC/MS confirmation of the roles of BryU, BryT, and BryA *O*-MT. (A) Calculated phosphopantetheine ejection ion masses for the predicted species. (B) Control reaction with acetyl-BryU, acetoacetyl-BryA module-3 ACP. (C) Incubation with BryR generates HMG-BryA module-3 ACP. (D) Addition of BryT to HMG-BryA module-3 ACP generates 3-methylglutaconyl product. (E) Addition of BryA *O*-MT and SAM to BryT reaction generates 3MG methylester product.

Fig. 7.
SDS-PAGE of BryT with and without coexpressed chaperones. Expected mass of BryT with His-tag fusion is 31 kDa

Fig. 8.

Chemoenzymatic preparation of dideuterated 3-hydroxy-3-methylglutaryl-CoA. Deuterated acetic anhydride rapidly converts coenzyme A into trideuterated acetyl-CoA, which can be used as a carbon source for HMG-CoA synthase. After reaction with BryT, the double bond configuration can be inferred by mass.

Fig. 9. LC/MS analysis of deuterated HMG-CoA and 3MG-CoA. Calculated masses for 0, 1, or 2 deuterations at the γ -position of HMG-CoA are shown in (A). These masses were used to generate extracted ion chromatograms for each mass from LC/MS data, shown in (B). The same process was employed to prepare the masses for 3MG-CoA in (C) and the extracted ion chromatograms in (D).

Fig. 10.

Varying reaction times changes deuteration of 3MG-CoA. With a 30-min reaction of γ - d_2 -HMG-CoA with BryT (*left*), the abundance of 2 -3MG-CoA is much higher than that of a 60-min reaction (*right*), likely due to deuterium exchange.

Fig. 11.

Representative HPLC of preparative-scale BryT reaction with HMG-CoA. Baseline separation was achieved at the preparative scale using 10 mg of HMG-CoA starting material and 40 μ M BryT. 3MG-CoA peak was isolated and used in NMR analysis.

Fig. 12.

NMR analysis of HMG- and 3MG-CoA isolated from BryT reactions. Signals listed in (A) were assigned to the chemical structure using commercial HMG-CoA (B). By comparing the 3MG-CoA spectrum (C, *blue*) to HMG-CoA (C, *red*), the lost methylene and gained methine (labeled with *arrows*) signals are apparent. Some impurities are present in the product, most significantly ammonium formate (8.5), water (4.8), and methanol (3.3).

Fig. 13.

Deconvoluted intact protein masses of HMG-BryA module-3 ACP with and without TMS-diazomethane treatment. Untreated protein shows a single primary species at 13.7 kDa (A). Diazomethane treatment results in discrete species with masses corresponding to 1 through 18 methylations of HMG-ACP_A (B).

Fig. 14.

Phosphopantetheine ejection ions of HMG-ACP_A species treated with TMS-diazomethane. Calculated masses of variously methylated phosphopantetheine ejection ions are shown in (A). Control TMS-diazomethane treatment of HMG-ACP_A (B), and HMG-ACP_A pretreated with BryT and BryA *O*-MT prior to TMS-diazomethane treatment (C).