

Characterization of the Initial Enzymatic Steps of Barbamide Biosynthesis

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Barbamide is a mixed polypeptide–polyketide natural product that contains an unusual trichloromethyl group. The origin of the trichloromethyl group was previously shown to be through chlorination of the pro-*R* methyl group of L-leucine. Trichloroleucine is subsequently decarboxylated and oxidized to trichloroisovaleric acid and then extended with an acetate unit to form the initial seven carbons of barbamide. In this study we used a combination of biosynthetic feeding experiments and enzymatic analysis to characterize the initial steps required for formation of trichloroleucine and its chain-shortened product, trichloroisovaleric acid. Results from isotope-labeled feeding experiments showed that both dichloroleucine and trichloroleucine are readily incorporated into barbamide; however, monochloroleucine is not. This suggests that halogenation of the pro-*R* methyl group of leucine occurs as two discrete reactions, with the first involving incorporation of at least two halogen atoms and the second converting dichloroleucine to trichloroleucine. Additionally, the initial tandem dichlorination must occur before substrate can be further processed by the remaining *bar* pathway enzymes. In vitro analysis of the first five open reading frames (ORFs; *barA*, *barB1*, *barB2*, *barC*, *barD*) of the barbamide gene cluster has yielded new insights into the processing of leucine to form the trichloroisovaleryl-derived unit in the final product.

Over the past 30 years, cyanobacteria have emerged as extraordinarily prolific producers of biologically active secondary metabolites.^{1,2} Several compounds deriving from cyanobacteria are currently in clinical trials for the treatment of human cancer, including analogues of dolastatin 10, dolastatin 15, and cryptophycin A.^{3–6} A number of other metabolites have been identified as promising preclinical lead compounds for cancer treatment, including curacin A and scytophycin A.^{1,6} Numerous cyanobacterial metabolites have been reported to possess other biological effects as well, including antifungal, antibacterial, and anti-HIV properties.^{1,2}

The metabolic and biomedical potential of marine cyanobacteria is well exemplified by a Curaçao collection of *Lyngbya majuscula*, which yielded nine biologically active compounds that are predicted to be the products of at least five distinct biosynthetic gene clusters.^{7–12} In addition to having an exceptional capacity to generate diverse natural product frameworks by a rich utilization of interdigitated non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) pathways, marine cyanobacterial compounds show evidence of many unique tailoring modifications, including multiple N-, O-, and C-methylations, oxidations, heterocyclic ring formation, and an exceptional ability to create diverse halogen-containing functionalities. Included in this latter group are vinylic and acetylenic bromides, as found in phormidolide¹³ and jamaicamide A,¹⁴ and vinyl chlorides and trichloromethyl groups, as present in malyngamide I¹⁵ and barbamide (1).¹¹

Previously, we have reported on the isolation and characterization of the gene cluster encoding barbamide (1) biosynthesis (*bar*).¹⁶ The genetic architecture and catalytic domain organization of the *bar* cluster is generally collinear and contains 12 open reading frames (ORFs). However, barbamide is produced by a mixed PKS/NRPS system with several unusual features, which include (1) a

peptidyl carrier (PCP) and adenylation (A) domain of one NRPS module being encoded on separate ORFs, (2) a PKS module that is encoded on two separate ORFs, (3) an oxidative decarboxylation of a cysteine-derived residue at the end of the assembly to form a terminal thiazole ring, and (4) the chlorination, α -oxidation, and decarboxylation of leucine to form a trichloroisovaleric acid-derived moiety in the final product. This latter chlorinated residue is subsequently ketide extended, and the enolized form of the β -keto acid is *O*-methylated before extension with additional amino acid residues.

The trichloroisovalerate-derived moiety found in barbamide (1) is very rare in natural products chemistry and provides a unique opportunity to dissect and understand the biochemical mechanism(s) responsible for its formation. Stable isotope feeding experiments using [2-¹³C]leucine (3) or [2-¹³C]5,5,5-trichloroleucine (4) suggest that L-leucine or an α -keto derivative of L-leucine is the actual substrate for chlorination (Figure 1).¹⁷ Although none of the ORFs in the *bar* cluster were initially identified as sharing homology with known halogenases, it became evident through BLAST analysis that *barB1* and *barB2* encoded for proteins showing homology to oxidoreductases that share high sequence homology with the syringomycin protein, SyrB2, and the coronamic acid protein, CmaB.^{16,18} Enzymes within this family have an obligate requirement for iron(II) and molecular oxygen, and all but two also require 2-oxoglutarate as a cofactor.^{19,20} Alignment with the phytanoyl-CoA hydrolase (PAHX) protein, SyrB2, and CmaB shows that BarB1 and BarB2 retain the conserved HXD motif required for coordination with the iron(II).²¹ Recently, the SyrB2 and CmaB proteins were demonstrated to be a new class of 2-oxoglutarate, iron-dependent halogenases.^{22,23} In the case of syringomycin biosynthesis, SyrB2 was shown to carry out the monochlorination of the methyl group of L-Thr bound as a thioester to the SyrB1 protein.²² In the case of coronamic acid, the CmaB protein has been shown to chlorinate the gamma-position of L-alloisoleucine.²³ This chlorinated intermediate is then suitably activated for cyclopropyl ring formation with consequent loss of chlorine (i.e., a cryptic chlorination). Each of these enzymes requires molecular oxygen, iron, 2-oxoglutarate, and chloride ion for activity.^{22,23} In the current

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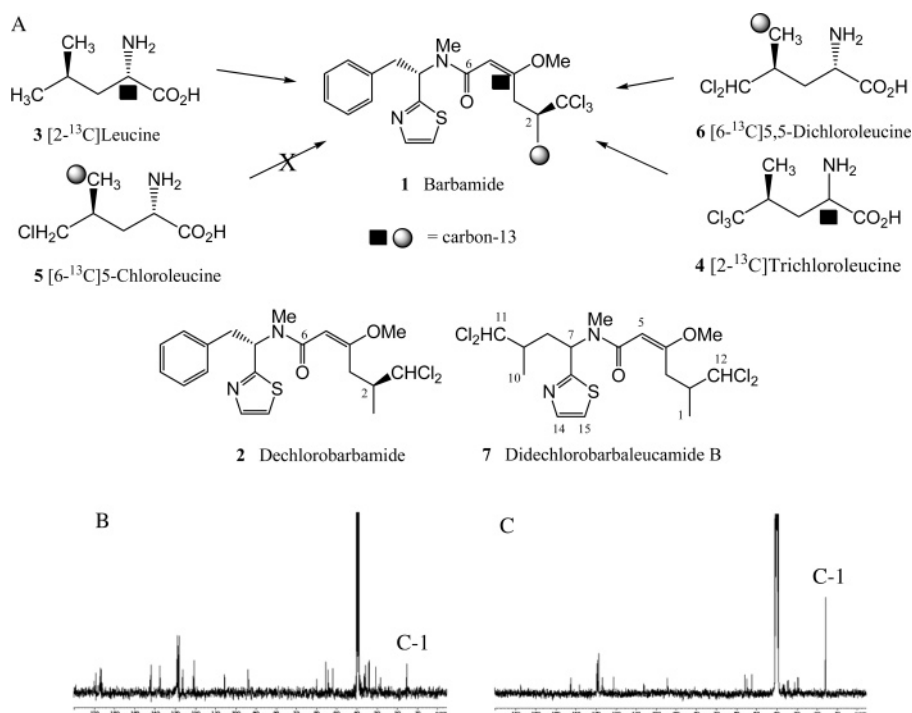


Figure 1. Incorporation of biosynthetic precursors into barbamide. (A) Summary of leucine-derived precursors that are incorporated into barbamide and structures of dechlorobarbamide (**2**) and didechlorobarbaleucamide B (**7**). Comparison of the ¹³C NMR spectra for (B) natural abundance barbamide and (C) barbamide produced during supplementation with [6-¹³C](2S,4S)-5,5-dichloroleucine.

study, we have employed a combination of biosynthetic feeding experiments and recombinant technologies to characterize the initial steps of barbamide biosynthesis.

Results and Discussion

Biosynthetic Feeding Experiments. Previous isotope-labeled precursor feeding experiments have demonstrated that both leucine and trichloroleucine can be incorporated into the barbamide (**1**) biosynthetic pathway;¹⁶ however, monochloroleucine was not incorporated.²⁴ These results suggest that multiple halogenations of leucine or a leucine-derived intermediate occur as an initial event and that this is a prerequisite for further processing of the leucine-derived substrate. Since a dichlorinated form of barbamide, dechlorobarbamide (**2**), has also been isolated from the producing organism,¹⁷ we hypothesized that dichloroleucine would be incorporated into the pathway and at least produce compound **2**. However, it was uncertain whether dichloroleucine could be a substrate for further chlorination and thus lead to barbamide (**1**), or if dichloroleucine is only incorporated into dechlorobarbamide (**2**).

Laboratory cultures of *L. majuscula* were incubated with [6-¹³C]-(2S,4S)-dichloroleucine (**6**) for 7 days and harvested, and lipid natural products were extracted by standard methods. Barbamide and related metabolites were subsequently isolated by successive chromatography via solid-phase extraction and two rounds of HPLC. Isolated barbamide (0.5 mg) was analyzed by ESIMS and high-field ¹H and ¹³C NMR and showed a strongly enriched (604% over natural abundance) ¹³C NMR signal for the C-1 methyl group at δ 15.2 (Figure 1; Supporting Information). This experiment clearly revealed that the dichloromethyl group of dichloroleucine is indeed a recognized and acceptable substrate for additional chlorination. However, dechlorobarbamide, while observed by DAD-HPLC and LC-MS, was produced in too small quantity in this experiment for NMR analysis (see Supporting Information data S3). Nevertheless, these UV and MS data, taken in conjunction with retention times for authentic dechlorobarbamide, strongly support the identity of this material. However, the complicated

molecular ion cluster for **2**, as well as for the other chlorinated metabolites from these experiments, prevented calculation of ¹³C incorporation rates from ESIMS data alone because ¹³C-enriched standards were not available, and therefore the TIC averages measured for each metabolite showed considerable variability in isotomeric composition. Incorporation of dichloroleucine (**6**) but not monochloroleucine (**5**) into barbamide (**1**) suggests either that the halogenation of leucine occurs initially as a tandem reaction that incorporates two chlorine atoms or that only dichlorinated or trichlorinated intermediates are recognized and processed into the remaining steps of the barbamide/dechlorobarbamide biosynthetic pathway. This is fully consistent with the recently published work by Walsh et al., who have worked in vitro with the barbamide halogenases BarB1 and BarB2 as prepared from synthetic genes,²⁵ and demonstrates that these halogenation reactions occur in vivo in the tandem stepwise manner predicted from the in vitro studies.

Intriguingly, a new compound (**7**) was produced in small yield (0.1 mg) from the [6-¹³C](2S,4S)-dichloroleucine feeding experiment and purified by recycling HPLC. By LC-MS analysis, this new material was consistent with a didechloro analogue of barbaleucamide B, a compound previously obtained from a Philippine collection of the sponge *Dysidea* sp.²⁶ [We have recently shown that the metabolic capacity to produce halogenated natural products in *Dysidea* resides within its symbiotic cyanobacterium *Oscillatoria spongeliae*.²¹] The ¹H NMR spectrum for **7** clearly resembled that of barbamide except for the conspicuous absence of aromatic signals for the monosubstituted phenyl group. Additionally, it possessed new resonances for an additional methyl doublet (δ 1.40) as well as several methylene and methine signals (δ 2.10–3.10, Supporting Information data S5). Finally, HRFABMS data were consistent with a formula of C₁₇H₂₄³⁵Cl₄N₂O₂S for **7** (Supporting Information data S4). Most significantly, the ¹³C NMR spectrum for this minor metabolite exhibited two ¹³C-enriched methyl signals at the appropriate chemical shifts for the two leucine-derived fragments. However, no natural abundance signals were detected in the ¹³C NMR spectrum of **7**, consistent with a very high level of ¹³C-enrichment from exogenous [6-¹³C](2S,4S)-dichloroleucine (see Supporting Information data S5). Hence, it appears that the

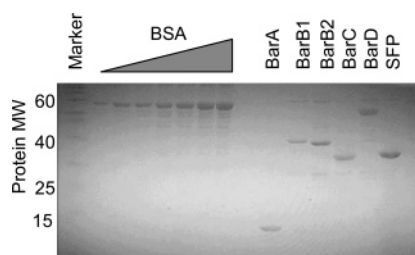


Figure 2. His-fusion proteins of BarA, BarB1, BarB2, BarC, and BarD and the SFP protein were purified using Ni^{2+} agarose affinity chromatography and visualized using a 15% SDS-PAGE stained with Coomassie. Bovine serum albumin (BSA) was used as a standard (0.5, 1, 2, 4, 6, 8, and 10 μg).

adenylation domain of BarG, which normally specifies for L-phenylalanine,¹¹ has considerable substrate tolerance and can accept halogenated leucine derivatives when they are available in abundance.

Recombinant Protein Expression. To reconstitute the initial steps involved in the biosynthesis of the barbamide starter unit, we recombinantly expressed the first five open reading frames of the pathway (*barA*, *barB1*, *barB2*, *barC*, *barD*). We predicted that the BarD protein was required to activate leucine and load it onto the small PCP protein, BarA. BarB1 and BarB2 were envisioned to catalyze the multiple chlorinations of the BarA-leucyl intermediate. Release and recovery of the halogenated product was then proposed using the type II thioesterase protein, BarC. Thus, the five genes encoding BarA–BarD were subcloned in frame with a 6XHis fusion tag into the pET20b expression vector and expressed in the BL21Δ(DE3) protein expression strain of *E. coli*. All of the expressed proteins were soluble and could be readily purified using nickel agarose chromatography (Figure 2).

Thioester Formation Assay. Previous experiments using the ATP/PPi conversion assay have demonstrated that BarD is capable of activating leucine to its aminoacyl adenylate.¹⁶ To determine if BarD is also competent to transfer the activated form of leucine to BarA and thus form a thioester linkage, we incubated the BarA protein at 5 μM with *Bacillus subtilis* phosphopantetheinyl transferase (SFP), the BarD protein, and [UL-¹⁴C]leucine. Incorporation of [UL-¹⁴C]leucine into BarA was visualized using SDS-PAGE followed by autoradiography. Results demonstrated that leucine was incorporated into the BarA protein and that this reaction was dependent on the presence of both BarD and ATP (Figure 3). In addition, this set of experiments demonstrated that the thioesterase, BarC, was able to release the bound ¹⁴C-labeled intermediate (Figure 3).

2-Oxoglutarate Conversion Assay. The activity of iron-dependent, 2-oxoglutarate-dependent enzymes can be monitored by the conversion of 2-oxoglutarate to succinate. Thus, to determine if BarB1 and BarB2 can utilize 2-oxoglutarate as a cofactor, we incubated BarB1 and BarB2 with [1-¹⁴C]2-oxoglutarate and measured the release of radiolabeled CO₂. The rate of 2-oxoglutarate turnover increased with increasing concentrations of the proposed substrate, leucyl-BarA (Figure 4). However, it has previously been demonstrated that many iron-dependent, 2-oxoglutarate oxidoreductases can mediate the modest, but significant, conversion of the cofactor 2-oxoglutarate to succinate even in the absence of substrate.^{27,28} In agreement with these observations, an increased release of CO₂ from [1-¹⁴C]2-oxoglutarate was also seen in the absence of leucyl-BarA, albeit at a reduced rate over the former experiments (Figure 4). These results indicate that BarB1 and BarB2 are indeed 2-oxoglutarate, iron-dependent enzymes that are most likely involved in catalyzing chlorination of leucine, as shown in Figure 5.²⁹

Additional incubations of BarA appropriately charged with leucine were reacted with the BarB1 and BarB2 constructs for 60

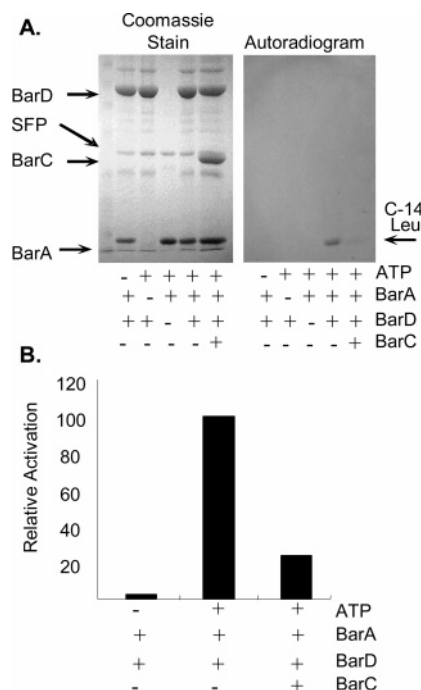


Figure 3. To demonstrate the formation of a thioester bond between BarA and leucine, BarA, BarD, and the SFP protein were incubated with 0.5 mM CoASH and 1 mM TCEP for 45 min at 37 °C to load phosphopantetheine onto BarA. Thioester formation activity was initiated by the addition of 5 mM ATP in the presence of 1 μCi [UL-¹⁴C]leucine. The reaction was incubated at 37 °C for 30 min and terminated by the addition of 10% TCA and 400 mg of BSA. Precipitated proteins were pelleted by centrifugation and washed three times with 10% TCA. Incorporation of radiolabeled leucine was determined by (A) SDS-PAGE-Coomassie staining with subsequent autoradiography and (B) liquid scintillation counting.

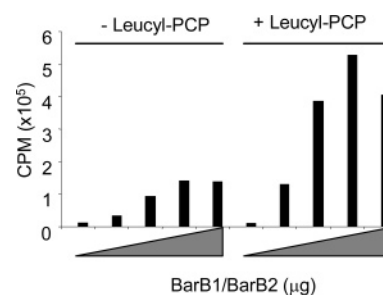


Figure 4. To demonstrate that BarB1/BarB2 mediate conversion of 2-oxoglutarate to succinate, the release of [¹⁴C]CO₂ from 2-oxoglutarate was measured with increasing BarB1/BarB2 protein concentrations (0, 5, 10, 15, and 20 μg) in the presence or absence of the substrate, leucyl-BarA.

min at 30 °C and then subjected to treatment with 5 μM of the BarC thioesterase. Amino acids in the crude lysate were separated from the protein slurry using a Microcon centrifugal device with a MW cutoff of 3000, derivatized using dansyl chloride and analyzed by ESIMS. However, despite three attempts, including one with isolation of the BarB1 and BarB2 proteins in the absence of oxygen (it has been shown that the purification of 2-oxoglutarate, iron-dependent enzymes in the presence of oxygen can limit or completely inhibit the activity of the recombinant protein^{22,23,30}), we were unable to detect a halogenated product. Because the assay is a single-turnover reaction that requires the activity of six reconstituted recombinant proteins (BarA, BarB1, BarB2, BarC, BarD, and the SFP protein), it has inherent low detectability. Furthermore, turnover is also dependent on the efficiency of leucine incorporation into BarA. While some refinement of the reagents

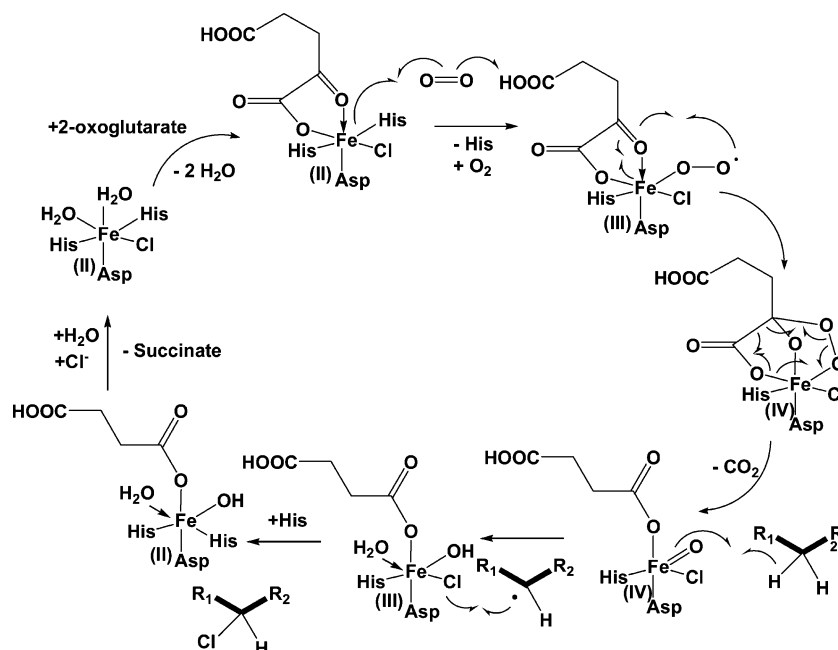


Figure 5. Barbamide halogenation is believed to occur via a modified 2-oxoglutarate, Fe(II)-dependent dioxygenase mechanism.²⁹ The catalytic scheme is based on the crystal structure and mechanistic data from the BarB1/BarB2 homologue human phytanoyl-CoA hydroxylase (PAHX).³⁷ We propose that coordination of chlorine with iron will displace either a water molecule or one of the enzyme coordination points (e.g., histidine).

involved in forming leucyl-BarA was accomplished, this is the only step in the overall transformation for which it was possible to perform any optimization. Further experiments are planned with a genetically modified strain of *E. coli* capable of overexpressing the first five ORFs of the *bar* pathway in addition to the *B. subtilis* SFP protein. Alternatively, it may be that our inability to observe a halogenated product arises because leucyl-BarA is not the optimal substrate for the halogenase reaction (however, Walsh et al. were recently able to demonstrate chlorination of this substrate using BarB2 isolated in the absence of O₂).²⁵

Adenylation Domain Activity. During the biosynthesis of barbamide, it is clear that several tailoring modifications to the leucine starter unit must occur, including halogenation, oxidation, and decarboxylation, to convert leucine to the trichloroisovaleryl-derived moiety found in barbamide. Our previous adenylation domain experiments suggested that the first step in leucine modification is the conversion of leucine to the trichlorinated intermediate. BarD was shown to activate both leucine and trichloroleucine, whereas BarE demonstrated moderate activity only toward trichloroleucine. These initial results suggested that a leucyl-BarA intermediate was the likely substrate for the halogenase enzymes, BarB1 and BarB2. However, the scope of these initial experiments did not rule out the possibility that the oxidation and/or decarboxylation of leucine may precede halogenation of the leucine-derived substrate.

To address this issue, additional experiments were conducted using isovaleric acid, trichloroisovaleric acid, and the α -keto derivatives of leucine and trichloroleucine as substrates for the BarD and BarE adenylation domains. The results from these experiments supported our previous findings that BarD can activate both leucine and trichloroleucine with high affinity (Figure 6A). However, BarE demonstrated only modest activity toward trichloroleucine in comparison with the α -keto derivatives of leucine and/or trichloroleucine (Figure 6B). Neither of the enzymes demonstrated significant activity toward isovaleric acid or the trichloroisovaleric acid substrates (Figure 6). Together, these data suggest that leucine is converted to the α -keto derivative prior to its transfer to BarE and that decarboxylation occurs after incorporation of the α -keto intermediate into BarE. A candidate enzyme from the barbamide

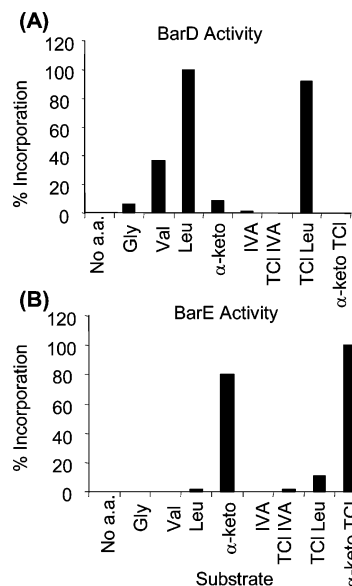


Figure 6. ATP/P_i exchange activity of (A) BarD and (B) the BarE adenylation domain were evaluated using the following substrates: no amino acid (No a.a.), glycine (Gly), valine (Val), leucine (Leu), α -keto derivative of leucine (α -keto), isovaleric acid (IVA), trichloroisovaleric acid (TCI/IVA), trichloroleucine (TCI/Leu), and the α -keto derivative of trichloroleucine (α -keto TCI). Results are representative of three independent experiments.

gene cluster predicted to convert leucine to the α -keto derivative is BarJ, as it shares high homology with L-amino acid oxidases. However, several attempts at recombinant expression of BarJ yielded an unstable protein product that is cleaved into two major fragments. We are currently developing an alternate expression system using the freshwater cyanobacterium *Nostoc punctiforme* to try to overcome these and related heterologous expression problems. While these experiments lend insight into the timing of leucine oxidation, the precise timing of halogenation remains unclear.

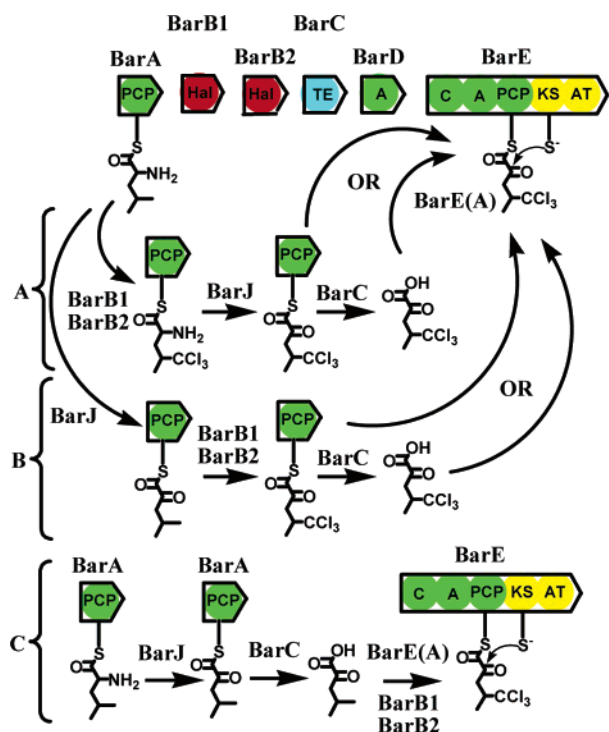


Figure 7. Three possible mechanisms for the modification of leucine during barbamide biosynthesis: (A) halogenation of leucyl-BarA precedes α -oxidation, (B) α -oxidation of leucyl-BarA precedes halogenation, or (C) the leucyl-BarA intermediate is α -oxidized prior to transfer onto BarE, where it undergoes halogenation.

We propose three possible routes to the formation of a trichloro intermediate, as depicted in Figure 7. In the first two hypotheses (Figure 7A and 7B), both the halogenation and oxidation of leucine are proposed to occur while leucine is bound as a thioester intermediate to BarA. In pathway A, the halogenation of leucine is proposed to precede oxidation, whereas in pathway B oxidation occurs prior to halogenation. In pathway C, an alternate hypothesis is proposed where leucyl-BarA is oxidized to the α -keto acid derivative prior to being incorporated into BarE. The BarE intermediate is then proposed to be the substrate for the halogenation reaction (Figure 7C).

Conclusions. Barbamide biosynthesis is mediated by a mixed NPRS–PKS biosynthetic pathway that incorporates a unique trichloroisovaleryl starter unit. To elucidate the reactions that lead to the formation of this unique starter unit, we used a combination of biosynthetic feeding experiments and enzymatic analysis. Biosynthetic feeding experiments demonstrate the incorporation of dichloroleucine as well as trichloroleucine into the barbamide structure; monochloroleucine is not incorporated.²⁴ These results suggest that the barbamide halogenases act in tandem to incorporate at least two halogen atoms in the first reaction and a third in a second reaction, or that only di- or trichlorinated intermediates can be recognized by enzymes downstream in the barbamide biosynthetic pathway. Similar conclusions were recently reached by the Walsh group working with the cloned and overexpressed BarB1 and BarB2 proteins.²⁵ Because our studies reported herein were conducted with the native barbamide-producing strain of *L. majuscula*, it confirms that the deduced sequence of tandem halogenations observed in these latter in vitro studies is relevant to natural metabolic transformations within the cyanobacterium.

Initial characterization of the putative halogenases, BarB1 and BarB2, demonstrates that they share high homology with a superfamily of 2-oxoglutarate-dependent oxygenases. Results from the current study also demonstrate that BarB1 and BarB2 can utilize 2-oxoglutarate as a cofactor. Recent discovery that the SyrB2 and

CmaB proteins function as halogenase enzymes involved in syringomycin and coronamic acid biosynthesis, respectively, lends strong support that BarB1 and BarB2 are indeed representative of a new class of halogenase proteins belonging to the 2-oxoglutarate superfamily.^{22,23} Further mechanistic studies with these novel tailoring enzymes will reveal the timing and optimal substrate intermediates for the halogenation reactions, offering a unprecedented opportunity to explore the utility of this new class of halogenases in biotechnological applications.

Experimental Section

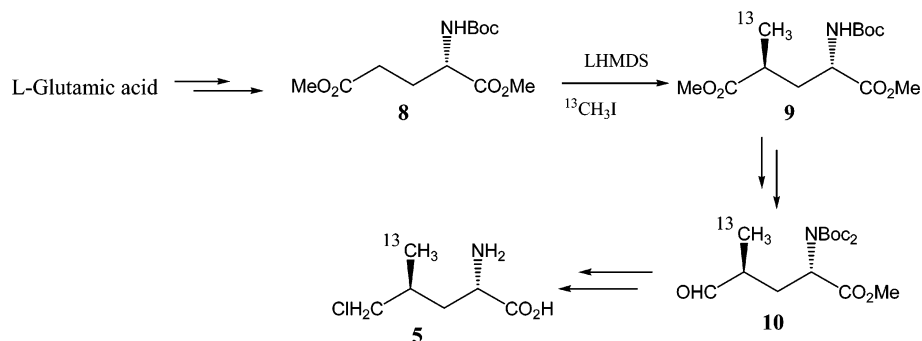
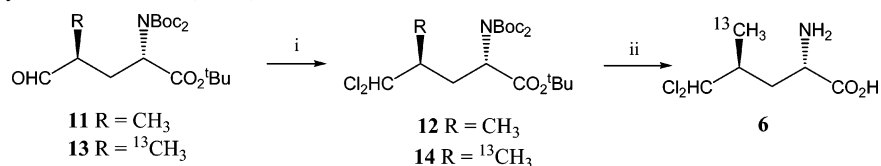
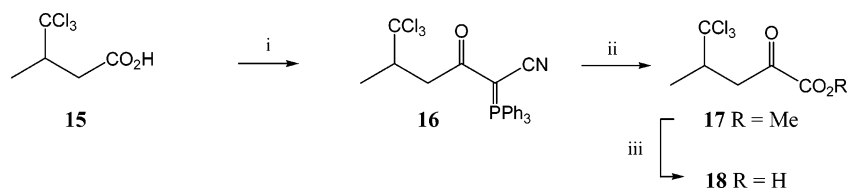
General Experimental Procedures. NMR data were obtained on a Bruker DRX300 in DMSO- d_6 using the solvent as an internal reference (δ_C 39.51). ^{13}C NMR (75 MHz) experiments on both natural abundance and labeled barbamide samples were performed using an inverse-gated pulse program (zgig) with a preparation delay of 500 ms. Low-resolution MS data were acquired on a ThermoFinnigan LCQ Advantage Max spectrometer. High-resolution MS data were acquired on a VG micromass 3DS/2RS MS9 double-focusing mass spectrometer. HPLC analyses were carried out using a Shimadzu SPD-10A dual-wavelength UV–vis detector with LC610 pumps or a Waters 996 photodiode array detector with Waters 515 pumps.

Synthesis of [6- ^{13}C](2S,4S)-5-Monochloroleucine (5), [6- ^{13}C](2S,4S)-5,5-Dichloroleucine (6), and 4-Trichloromethyl-2-oxopentanoic Acid (18). [6- ^{13}C](2S,4S)-5-Chloroleucine (5) was prepared from L-glutamic acid.²⁴ The key step was a stereoselective alkylation³¹ of the protected glutamate (8) using $^{13}\text{CH}_3\text{I}$ to give 9, which was transformed to the target compound 5 in a further five steps and 40% yield. A similar strategy was used to introduce the isotopic label into [6- ^{13}C](2S,4S)-5,5-dichloroleucine (6). Rodriguez and co-workers³² have used a modification of Takeda's procedure³³ involving hydrazine monohydrate in anhydrous MeOH to form the hydrazone followed by CuCl_2 and Et_3N for the conversion of unlabeled aldehyde 11 to the geminal dichloride 12 in 40% yield. These conditions were used to transform [6- ^{13}C]aldehyde 13 to 14, which, after deprotection in refluxing 6 N HCl, gave the required [6- ^{13}C](2S,4S)-dichloroleucine (6) as a white solid. 4-Trichloromethyl-2-oxopentanoic acid (18) was prepared from the known³⁴ 3-trichloromethylbutanoic acid (15). The one carbon homologation of acid 15 was achieved via ozonolysis of the β -keto-cyanophosphorane 16 using conditions analogous to those reported for the synthesis of [5- ^{13}C]L-leucine.³⁵ Hydrolysis of methyl ester 17 under mild conditions using 1 equiv of aqueous 2 N NaOH gave the novel α -keto acid 18 as a yellow oil.

[6- ^{13}C](2S,4S)-5,5-Dichloroleucine (6): mp 208–210 °C; [α]_D –24 (c 1, D₂O), (lit.³² [α]_D unlabeled material –23 (c 0.16, 1 N HCl); ^1H NMR (400 MHz, D₂O) 1.2 (3H, dd, J 12.8, 6, $^{13}\text{CH}_3$), 1.9 (1H, ddd, J 15, 9, 5, 3-HH), 2.13 (1H, ddd, J 15, 10, 5, 3-HH), 2.35 (1H, m, 4-H), 3.8 (1H, dd, J 10, 5, 2-H), 6.11 (1H, t, J 3, 5-H); ^{13}C NMR (100 MHz, D₂O) 14.4 ($^{13}\text{CH}_3$ enriched), 33.7 (C-3), 40.0 (C-4, d, J 19.2), 52.8 (C-2), 78.3 (C-5), 174.5 (C-1); m/z (CI) 201 (MH^+ , 23%), 155 (65), 74 (100); $^{13}\text{C}_5\text{H}_{12}\text{O}_2\text{N}^{35}\text{Cl}_2$ requires 201.0277, found 201.0278.

4-Trichloromethyl-2-oxopentanoic acid (18): ^1H NMR (300 MHz, CDCl_3) 1.36 (3H, d, J 6.5, 5-H₃), 3.12 (1H, dd, J 19, 9, 3-HH), 3.26 (1H, m, 4-H), 3.60 (1H, dd, J 19, 2.5, 3-HH), 7.57 (1H, br s, CO_2H); ^{13}C NMR (75 MHz, CDCl_3) 17.0 (C-5), 42.3 (C-3), 50.9 (C-4), 104.2 (CCl_3), 160.5 (C-1), 192.6 (C-2); m/z (CI) 186.9500 ($\text{MH}^+ - \text{CO}_2\text{H}$, $\text{C}_5\text{H}_6\text{OCl}_3$ requires 186.9484), 233/235/237/239 (MH^+ , 8/10/5/3), 187/189/191/193 (34/34/22/6), 151/153/155 (60/46/15), 123 (100).

Biosynthetic Feeding Experiments. Three 1 L flasks were seeded with 4 g of *L. majuscula* strain 3L in 500 mL of BG-11 medium.³⁶ The cultures were established and allowed to grow for 5 days prior to the start of feedings. Each flask was fed a total of 20 mg of [6- ^{13}C](2S,4S)-5,5-dichloroleucine (6) in three 6.6 mg doses on days 1, 4, and 8. The cultures were harvested on day 10 by removing the filaments from the culture medium, pooling, blotting briefly on Whatman filter paper, and storing at –20 °C until the samples were extracted. The wet weight of the algae prior to freezing was 18.15 g. Primary extraction was performed by repetitive steeping (4 \times) in 2:1 CH_2Cl_2 /MeOH. Water was removed from the CH_2Cl_2 layer in a separatory funnel, which was then filtered over Whatman #1 paper, and dried using rotary evaporation to yield 98 mg of crude extract. Initial fractionation utilized a stepped gradient of EtOAc in hexanes (5% to 100%) and a Varian Mega Bond Elute SI column (1.5 \times 2 cm; normal phase) to yield seven fractions.

Scheme 1. Chemical Synthesis of [6-¹³C](2*S*,4*S*)-5-Chloroleucine **5****Scheme 2.** Chemical Synthesis of [6-¹³C](2*S*,4*S*)-5,5-Dichloroleucine **6**^a^a Reagents: (i) NH₂NH₂·H₂O, MeOH, Et₃N, CuCl₂; (ii) 6 N HCl.**Scheme 3.** Chemical Synthesis of 4-Trichloromethyl-2-oxopentanoic Acid **18**^a^a Reagents: (i) Ph₃PCHCN, EDCI, DMAP; (ii) O₃, MeOH, CH₂Cl₂; (iii) NaOH.

Normal-phase TLC analysis (1:1 EtOAc/hexanes) revealed UV-active materials in fractions 4 and 5 (50% and 70% EtOAc, respectively). These two fractions were pooled and further purified using a Varian Mega Bond Elute C18 cartridge and a stepped gradient of 60%, 80%, and 100% MeOH. Normal-phase TLC (1:1 EtOAc/hexanes) indicated that the 80% and 100% fractions contained a UV-active compound consistent with barbamide. These fractions were pooled and further purified using exhaustive reversed-phase HPLC (YMC-ODS-AQ-323, 250 × 10 mm; 3 mL/min, 80% aqueous MeOH; Waters Symmetry, 250 × 4.6 mm, 1 mL/min, 70% aqueous CH₃CN) to yield 0.5 mg of barbamide. In addition, purification of barbamide (**1**) by a second round of HPLC led to the concomitant isolation of the new metabolite didechlorobarbaleucamide B (**7**, 0.1 mg). Dechlorobarbamide (**2**) was detected by LC-MS in the fraction preceding that containing barbamide from the initial round of HPLC.

Didechlorobarbaleucamide B (7): colorless oil; ¹H NMR (DMSO-*d*₆, 300 MHz) δ 7.74 (2H, m, H-14/H-15), 6.22 (2H, m, H-11/H-12), 5.50 (1H, s, H-5), 5.35 (1H, m, H-7), 3.66 (3H, s, OCH₃), 3.10–2.10 (6H, m, H-2, H-9, H₂-3, H₂-8), 2.82 (3H, s, N-CH₃) 1.39 (3H, d, *J* = 6.4 Hz, H₃-10), 0.96 (3H, d, *J* = 6.0 Hz, H₃-1); ¹³C NMR partial data (DMSO-*d*₆, 75 MHz) δ 15.9 (CH₃, C-1), 15.3 (CH₃, C-10); TOF MS/MS 463.0 ES+ *m/z* 247/246/245/244 (18/100/19/35), 211/210/209/208 (6/74/27/78), 182/181/180/179 (41/9/44/7); HRESIMS obsd *M*⁺ *m/z* 460.0300 (calcd for C₁₇H₂₄³⁵Cl₄N₂O₅S, 460.0313).

Recombinant Protein Expression. The genes *barA*, *barB1*, *barB2*, and *barC* were each subcloned and recombinantly expressed in frame with the 6XHis fusion tag into the pET20b expression vector. Full-length *barA*, *barB1*, *barB2*, and *barC* were each PCR amplified and subcloned into the pET20b expression vector at the NdeI/XhoI restriction sites in frame with the C-terminal 6XHis fusion tag. Full-length *barD* and the *barE* adenylation domain were subcloned into pET20b as previously described.¹⁶ The *Bacillus subtilis* SFP gene, kindly provided by C. Walsh (Harvard), was PCR amplified from the pUC-SFP vector and subcloned in frame with the 6XHis tag into the pET29 expression vector using the following primers: SFP-F1, 5'-GGAATTCATATGAAGATTACGGAATTTATATG-3'; SFP-R1, 5'-CCGCTCGAGTAAAGCTCTTCGTACGAGAC-3'.

Recombinant proteins BarA, BarB1, BarB2, BarC, BarD, and BarE (adenylation domain) were expressed and purified from BL21Δ(DE3)

cells as follows. One liter of LB culture was inoculated with a 1:100 dilution of an overnight culture of *B. subtilis* and grown in a 3 L Fernbach flask at 18 °C overnight. The *E. coli* were pelleted by centrifugation and resuspended in 10 mL of lysis buffer (20 mM Tris, pH 8.0 with 500 mM NaCl and 20 mM imidazole) per liter of culture. The lysate was sonicated five times at 70 W for 10 s/burst. The cellular debris was removed by centrifugation and the protein isolated by nickel agarose chromatography as previously described.¹⁶ Protein purity and concentration were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining.

Thioester Formation Assay. The assay was carried out in 100 μL total volume containing 75 mM Tris, pH 8.0, 1 mM TCEP, 10 mM MgCl₂, 5 mM ATP, 0.5 mM CoASH, 550 nM SFP protein, 5 μM BarD, 5 μM BarA, and 800 μM [UL-¹⁴C]leucine. The reaction was initiated by the addition of 5 mM ATP. Prior to the initiation of the reaction, the sample was incubated in the absence of ATP and leucine for 45 min at 37 °C to convert the apo PCP domain in BarA to the homoenzyme (phosphopantetheine containing). Once the reaction was initiated by the addition of ATP, it was incubated for an additional 30 min at 37 °C. The reaction was quenched by the addition of 0.8 mL of 10% TCA containing 375 μg of BSA. The precipitated proteins were pelleted by centrifugation at 12600g and washed three times with 10% TCA to remove any free [UL-¹⁴C]leucine from the assay mixture. After the final wash, the protein pellet was resuspended in 150 μL of 1 M Tris, diluted with scintillation fluid (3.5 mL), and counted for radioactivity. Alternatively, the samples were resuspended in 20 μL of 1× Laemmli sample buffer and analyzed by SDS-PAGE, Coomassie staining, and autoradiography.

2-Oxoglutarate Conversion Assay. To measure basal levels of 2-oxoglutarate activity in the absence of substrate, increasing concentrations of BarB1 and BarB2 were incubated with [1-¹⁴C]2-oxoglutarate in 50 mM Tris, pH 7.5, 1 mM FeSO₄, 100 μM TCEP, 10 mM ascorbate, 4 mM ATP, and 50 mM (NH₄)₂SO₄. The reaction mixture was initiated by the addition of the [1-¹⁴C]2-oxoglutarate in an open 1.5 mL Eppendorf tube. The tube was then incubated open, suspended over 200 μL of hyamine hydroxide in a sealed 50 mL conical tube. Hyamine hydroxide absorbs [¹⁴C]CO₂ that is released during the conversion of [1-¹⁴C]2-oxoglutarate to succinate. To measure the effect of leucyl-BarA on the conversion of [1-¹⁴C]2-oxoglutarate to [¹⁴C]CO₂ and

succinate, we generated the BarA-leucyl intermediate as above in the thioester formation assay, using 2 mM unlabeled leucine instead of the [UL-¹⁴C]leucine. A total of 100 μ L of this reaction mixture was added to the 2-oxoglutarate protocol given above for a final volume of 200 μ L. All of the reactions were incubated for 2 h at 28 °C and stopped by the addition of 800 μ L of 10% TCA. The hyamine hydroxide was transferred to 3 mL of scintillation cocktail and analyzed by liquid scintillation counting (LSC).

Adenylation Domain Exchange Assay. BarD and the BarE adenylation domains were prepared as previously described and assayed in the ATP–PPi exchange assay. Briefly, the assay was initiated by the addition of 0.3 μ Ci of [³²P] pyrophosphate to 2 μ M protein, 2 mM ATP, and 2 mM substrate (glycine, leucine, trichloroleucine, valine, isovaleric acid, trichloroisovaleric acid, or the α -keto derivatives of leucine and trichloroleucine), in 75 mM Tris, pH 8.0, with 100 mM NaCl, 10 mM MgCl₂, and 5 mM DTT. The samples were incubated at 30 °C for 2 h and the reactions quenched by the addition of 0.5 mL of Stop Buffer (1.2 wt %/vol activated charcoal, 0.1 M tetrasodium pyrophosphate, and 0.35 M HClO₄). Trapped ATP was washed three times using 0.1 M tetrasodium pyrophosphate in 0.35 M HClO₄. The charcoal pellet was resuspended in 0.5 mL of H₂O, added to 3.5 mL of scintillation fluid, and analyzed by LSC.

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Supporting Information Available: Panels of selected carbon atom intensities in ¹³C-enriched barbamide, and LC-MS data for ¹³C-labeled barbamide and didechlorobarbaleucamide B. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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