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Heterologous Production of 4-*O*-Demethylbarbamide, a Marine Cyanobacterial Natural Product

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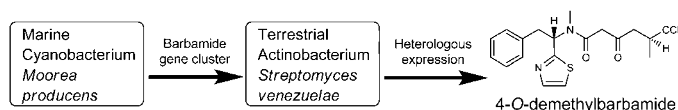
ABSTRACT



Heterologous expression of the barbamide biosynthetic gene cluster, obtained from the marine cyanobacterium *Moorea producens*, in the terrestrial actinobacterium *Streptomyces venezuelae*, resulted in the production of a new barbamide congener 4-*O*-demethylbarbamide, demonstrating the potential of this approach for investigating the assembly and tailoring of complex marine natural products.

Marine invertebrates and bacteria are an extraordinarily rich source of novel bioactive secondary metabolites.[1] Several marine natural products and their derivatives are now used as clinical therapeutics, and many others have shown potential for the treatment of cancer, inflammation, pain, and other diseases.[2] However, general impediments to the development of many of these exciting molecules are the limited quantities obtained from Nature and the difficulty in culturing the source organisms, including invertebrates, algae, or associated symbiotic microorganisms. Moreover, total chemical synthesis of structurally complex metabolites is economically impracticable in many cases. Therefore, to harness the therapeutic potential of these natural product lead compounds, reliable methods must be developed for their resupply. Cloning and heterologous expression of the biosynthetic gene clusters represents one such solution that can also facilitate the generation of insightful analog structures through metabolic engineering. To date, the majority of biosynthetic studies of marine secondary metabolites have been limited to those isolated from marine actino- and cyanobacteria.[3] Most genetic and enzymatic studies of cyanobacterial secondary metabolites have been conducted with the marine genus *Moorea producens* (previously classified as *Lyngbya majuscula*),[4] a prolific producer of structurally diverse natural products[5] including the cancer cell cytotoxin curacin A[6] and the molluscicidal agent barbamide (**1**; Figure 1).[7] A draft genome was recently reported for the *M. producens* 3L strain,[8] which revealed the genetic basis for its unique

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(1) Salomon, C. E.; Magarvey, N. A.; Sherman, D. H. *Nat. Prod. Rep.* **2004**, *21*, 105.
(2) Gerwick, W. H.; Moore, B. S. *Chem. Biol.* **2012**, *19*, 85.
(3) Lane, A. L.; Moore, B. S. *Nat. Prod. Rep.* **2011**, *28*, 411.

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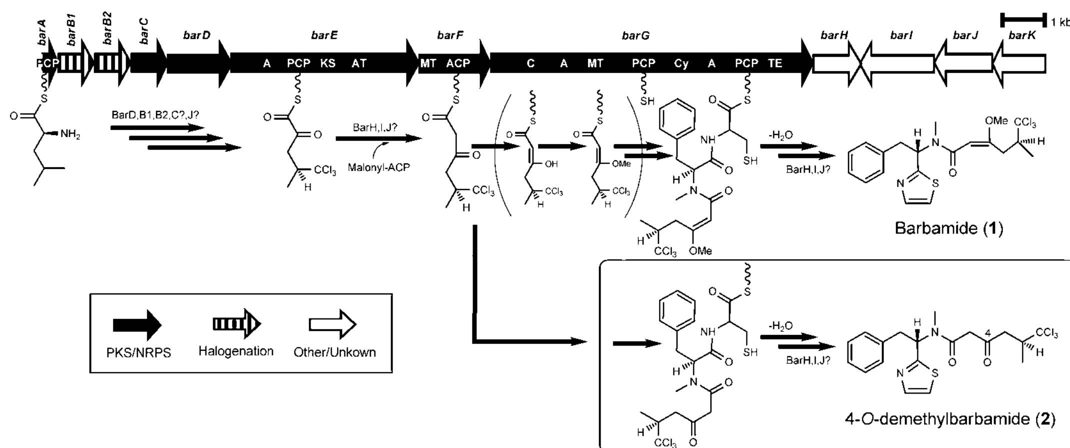

Figure 1. Barbamide gene cluster and the proposed biosynthetic pathways for 4-*O*-demethylbarbamide (**2**) and barbamide (**1**).[11] A, adenylation domain; ACP, acyl carrier protein; AT, acyltransferase domain; C, condensation domain; Cy, cyclization domain; KS, ketosynthase domain; MT, methyltransferase domain; PCP, peptidyl carrier protein; TE, thioesterase domain.

spectrum of natural products. However, an inability to genetically manipulate this filamentous cyanobacterium along with its extremely slow growth rate (doubling time ≥ 6 days)[9] motivated us to develop a robust heterologous expression platform for cyanobacterial secondary metabolite gene clusters.

Although cyanobactin ribosomal peptides from cyanobacteria were heterologously produced and engineered successfully in *Escherichia coli,*[10] no polyketides or hybrid polyketide-nonribosomal peptides from marine cyanobacteria have been produced in a heterologous host to date. Barbamide (**1**) attracted our attention because its biosynthetic gene cluster encoding for a NRPS/PKS (nonribosomal peptide synthetase/polyketide synthase) hybrid was the first identified from a marine cyanobacterium,[11] and its assembly involves several unique biochemical features such as trichlorination, a one-carbon truncation during chain extension, formation of an *E*-double bond, and thiazole ring formation (Figure 1; see the detailed biosynthetic pathway in Figure S1, Supporting Information (SI)). Currently, only the trichlorination of leucine has been biochemically characterized.[12] The 26 kb barbamide gene cluster contains 12 ORFs, designated *barA*−*barK* (GenBank accession no. AF516145).[11]

Streptomyces spp. are known to produce an enormous range of secondary metabolites. Thus, this genus has been used as a heterologous expression host for a variety of secondary metabolites including polyketides and nonribosomal peptides.[13] An advantage of *Streptomyces* for the heterologous expression of PKS or NRPS pathways is its unique capability to accommodate these large genes compared to other heterologous hosts, such as *E. coli.*[13] Recently, heterologous expression was applied in *Streptomyces coelicolor* M512 for the ~11 kb biosynthetic gene cluster of the indole alkaloid lyngbyatoxin A from *M. producens.*[9] Despite large differences in the %GC content and codon usage between cyano- and actinobacteria, two nonmodular proteins, a cytochrome P450 monooxygenase LtxB and a reverse prenyltransferase LtxC were successfully expressed, thus showing the potential of *Streptomyces* as a useful heterologous host. Yet, premature transcriptional termination of the *ltxA* gene product, a large bimodular NRPS, was observed and thus neither lyngbyatoxin A nor its derivatives were produced. Among *Streptomyces*, pikromycin-producing *S. venezuelae* has been developed as a promising host for the production of polyketides, hybrid polyketide-nonribosomal peptides, and aminoglycosides due to its rapid growth and relative ease of genetic manipulation.[14−16] Here, we demonstrate heterologous expression of the entire barbamide

(4) Engene, N.; Rottacker, E. C.; Kastovsky, J.; Byrum, T.; Choi, H.; Ellisman, M. H.; Komárek, J.; Gerwick, W. H. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 1171.

(5) Jones, A. C.; Monroe, E. A.; Eisman, E. B.; Gerwick, L.; Sherman, D. H.; Gerwick, W. H. *Nat. Prod. Rep.* **2010**, *27*, 1048.

(6) Gerwick, W. H.; Proteau, P. J.; Nagle, D. G.; Hamel, E.; Blokhin, A.; Slate, D. L. *J. Org. Chem.* **1994**, *59*, 1243.

(7) Orjala, J.; Gerwick, W. H. *J. Nat. Prod.* **1996**, *59*, 427.

(8) Jones, A. C.; Monroe, E. A.; Podell, S.; Hess, W. R.; Klages, S.; Esquenazi, E.; Niessen, S.; Hoover, H.; Rothmann, M.; Lasken, R. S.; Yates, J.; Reinhardt, R.; Kube, M.; Burkart, M.; Allen, E. E.; Dorrestein, P. C.; Gerwick, W. H.; Gerwick, L. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 8815.

(9) Jones, A. C.; Ottilie, S.; Eustáquio, A. S.; Edwards, D. J.; Gerwick, L.; Moore, B. S.; Gerwick, W. H. *FEBS J.* **2012**, *279*, 1243.

(10) (a) Schmidt, E. W.; Nelson, J. T.; Rasko, D. A.; Sudek, S.; Eisen, J. A.; Haygood, M. G.; Ravel, J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7315. (b) Tianero, M. D.; Donia, M. S.; Young, T. S.; Schultz, P. G.; Schmidt, E. W. *J. Am. Chem. Soc.* **2012**, *134*, 418.

(11) Chang, Z.; Flatt, P.; Gerwick, W. H.; Nguyen, V. A.; Willis, C. L.; Sherman, D. H. *Gene* **2002**, *296*, 235.

(12) Galonić, D. P.; Vaillancourt, F. H.; Walsh, C. T. *J. Am. Chem. Soc.* **2006**, *128*, 3900.

(13) Zhang, H.; Boghigian, B. A.; Armando, J.; Pfeifer, B. A. *Nat. Prod. Rep.* **2011**, *28*, 125.

(14) Park, S. R.; Han, A. R.; Ban, Y. H.; Yoo, Y. J.; Kim, E. J.; Yoon, Y. J. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1227.

(15) Park, S. R.; Park, J. W.; Jung, W. S.; Han, A. R.; Ban, Y. H.; Kim, E. J.; Sohng, J. K.; Sim, S. J.; Yoon, Y. J. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 109.

(16) Park, J. W.; Park, S. R.; Nepal, K. K.; Han, A. R.; Ban, Y. H.; Yoo, Y. J.; Kim, E. J.; Kim, E. M.; Kim, D.; Sohng, J. K.; Yoon, Y. J. *Nat. Chem. Biol.* **2011**, *7*, 843.

biosynthetic gene cluster in an engineered strain of *S. venezuelae* DHS 2001 from which the pikromycin PKS gene cluster has been deleted.[17] These efforts resulted in the production of 4-*O*-demethylbarbamide (**2**; Figure 1), a new barbamide derivative that only lacks an *O*-methyl group relative to the parent structure but shows several-fold improved molluscicidal activity compared to **1**. To our knowledge, this is the first successful functional heterologous expression of a marine cyanobacterial NRPS/PKS gene cluster in a genetically amenable terrestrial host.

As a starting point, the barbamide biosynthetic genes *barA*−*barK* were cloned into a replicative *E. coli*−*Streptomyces* shuttle vector pDHS702,[18] containing the *pikAI* promoter of the pikromycin PKS from cosmid PLM49[11] in a series of subcloning steps (Figure S2, Tables S1 and S2, SI). The resulting expression plasmid pYJ1614 was designed to express the *barA*−*barH* genes under the control of the *pikAI* promoter and to transcribe *barI*−*barK* genes convergently from a native promoter in the barbamide cluster. The native intergenic regions and ribosome-binding site of each gene were maintained in this construct. The engineered plasmid was introduced into *S. venezuelae* DHS2001 to provide the *S. venezuelae* YJ348 strain. HPLC-ESI-MS/MS analysis of the organic extract of YJ348 grown on R2YE[19] solid medium at 30 °C for 6 days revealed a peak that was consistent with a barbamide (**1**) derivative, although **1** was not detected (Figure S3, SI). Analysis of the extracts of the cultured recombinant *S. venezuelae* YJ348 strain revealed a new peak that eluted at 42.8 min with m/z = 448. This product was only detected in the YJ348 strain and not from extracts obtained from *S. venezuelae* DHS2001 possessing an empty vector. The m/z 448 molecular ion cluster showed the characteristic isotope signature for three Cl atoms, and the MS/MS spectrum deriving from it yielded fragment ions at m/z 231, 219, 188, and 134. The presence of fragments at m/z 219, 188, and 134 in common with those of **1**, as well as the production of a fragment at m/z 231 corresponding to the loss of 14 Da from the diagnostic fragment of **1** at m/z 245 (Figure S4, SI), suggested that it was a close structural analog of **1**. These mass differences relative to **1** suggested that the new compound was deficient in a methyl group located between the C-1 and C-4 positions. Based on the proposed barbamide biosynthetic pathway and precursor incorporation studies,[20] elimination of the C-1 methyl group deriving from the Leu starter unit seemed unlikely. Thus, the new barbamide derivative detected in the extract of this engineered YJ348 strain was predicted to be 4-*O*-demethylbarbamide (**2**).

Interestingly, we found this same peak as a minor compound in partially purified *M. producens* fractions by HPLC-ESI-MS analysis, suggesting that the demethyl analog is a naturally occurring derivative of **1** (Figure S5, SI). Hence, the new analog was purified in a larger scale from an *M. producens* extract by reversed-phase HPLC, and its chemical structure was fully elucidated by detailed spectroscopic analyses (Figures S6−S11, SI). The ^{1}H NMR spectrum of the *O*-demethyl derivative of barbamide was complicated by the presence of four interconverting isomers (**2a**−**2d**), present in an equilibrium ratio of *ca.* 17:7:2:1. However, 1D and 2D NMR methods were used to assign almost all shifts of the four isomers (**2a**−**2d**, Figure 2 and Table S3). In **2a**, HMBC correlations from H-2/H$_2$-3/H$_2$-5 to C-4 (δ_C 201.7) allowed assignment of a ketone at the C-4 position, a key point of departure from the structure of **1**. Additionally, a NOESY correlation between the *N*CH$_3$ group and H$_2$-5 revealed that this major compound was the *trans* amide isomer. The ^{1}H NMR signals of the second isomer (**2b**) were almost identical to those of **2a**, except that the chemical shifts of H-7 and C-7 of **2b** were located at δ_H 5.59 and δ_C 59.8, suggesting that this was the *cis* amide conformer. Consistent with this proposal (i.e., the *cis* amide isomer with a 4-keto functionality), a NOESY correlation between the *N*CH$_3$ and H$_2$-5 was not observed. The NMR signals of the third isomer (**2c**) were slightly different from those of **2a**. In the ^{1}H NMR, H-7 of **2c** was observed at δ_H 6.27, indicating a *trans* amide isomer as found in **2a**. However, the peak for H-5 in **2c** was found as a broad singlet at δ_H 5.47 and the ^{13}C NMR shift of C-5 of **2c** was located as δ_C 88.8, both of which are inconsistent with a 4-keto functionality. Furthermore, the H-5 singlet proton showed HMBC correlations with two deshielded quaternary carbons (C-4 at δ_C 174.4; C-6 at δ_C 171.8). These data indicated that **2c** was present in the 4,5-enolic form (i.e., **2a** and **2c** are tautomers). The geometry of the $\Delta^{4,5}$ double bond was established as *Z* by observation of a NOESY correlation between H-5 and H$_2$-3. Finally, the chemical shifts for H-5 and C-5 of minor isomer **2d** were determined as δ_H 5.83 and δ_C 88.2, respectively, and HMBC correlations from H-5 to C-4 and C-6 again indicated the presence of a $\Delta^{4,5}$ double

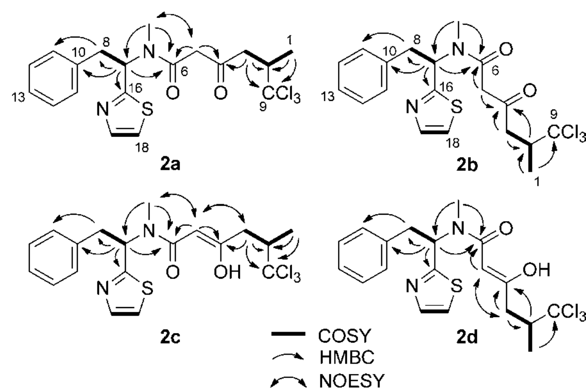

Figure 2. Simultaneous structure elucidation of four isomers of 4-*O*-demethylbarbamide (**2a**−**2d**) based on key COSY, HMBC, and NOESY correlations.

(17) Jung, W. S.; Lee, S. K.; Hong, J. S.; Park, S. R.; Jeong, S. J.; Han, A. R.; Sohng, J. K.; Kim, B. G.; Choi, C. Y.; Sherman, D. H.; Yoon, Y. J. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 763.
(18) Xue, Y.; Sherman, D. H. *Metab. Eng.* **2001**, *3*, 15.
(19) Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics*; John Innes Centre: Norwich, U.K., 2000.
(20) (a) Flatt, P. M.; O'Connell, S. J.; McPhail, K. L.; Zeller, G.; Willis, C. L.; Sherman, D. H.; Gerwick, W. H. *J. Nat. Prod.* **2006**, *69*, 938. (b) Sitachitta, N.; Rossi, J.; Roberts, M. A.; Gerwick, W. H.; Fletcher, M. D.; Willis, C. L. *J. Am. Chem. Soc.* **1998**, *120*, 7131.

bond. The configuration of this olefin was determined as *Z* by observation of a NOESY correlation between H-5 and H_2-3. However, no NOESY correlation was observed between the NCH_3 and H-5 in **2d**; combined with the chemical shifts of H-7 and C-7 at δ_H 6.00 and δ_C 58.0, respectively, these data indicated isomer **2d** possesses a *cis* amide bond. A comparison of the specific rotation of barbamide with this new barbamide analog, present as a mixture of these four isomers, allowed assignment of the absolute configuration as 2*S*, 7*S*.

Compound **2** was found to be a potent molluscicidal agent against the snail *Biomphalaria glabrata* (LD_{50} 3.9 μM, 95% CI 2.7−5.5 μM, Figure S12, SI) and is several-fold more potent than **1** (revised LD_{100} 22 μM).[7] Indeed, we had recognized that there was a more potent molluscicide in the extract of this cyanobacterium but had been unable to complete its structure elucidation as **2** due to the small quantity and multiple isomers in solution.

It is plausible that **2** is a biosynthetic congener of **1**, which results from skipping of the BarF-catalyzed *O*-methylation reaction (Figures 1 and S1, SI). However, it is at present unclear why **2** is generated as the sole product in the heterologous host. Semiquantitative RT-PCR analysis of the transcript levels in the engineered YJ348 strain demonstrated that most of the *bar* genes are transcribed, including *barF* that encodes for the *O*-methyltransferase (Figure S13, SI). Therefore, it is possible that the *barF* transcript is unstable in *S. venezuelae* and fails to be translated. Alternatively, other gene product(s) located outside of the barbamide gene cluster might facilitate the unfavorable energetics involved in forming the *E* enol substrate which appears to be required for the *O*-methyl transfer reaction to form **1**. The detection of *barJ* transcripts suggests that the native *Moorea* promoter can be recognized in *Streptomyces*, as previously reported,[9] although its expression level appears to be lower as compared to genes transcribed from the *Streptomyces* promoter. Therefore, placement of a *Streptomyces* promoter in front of the *barI*−*barK* operon could increase production of **2**. However, truly significant enhancement of production would not be expected because even the expression levels of those cyanobacterial genes driven by the *pikAI* promoter were low compared to *Streptomyces* PKS genes heterologously expressed in *S. venezuelae*,[17] probably due to codon usage bias between cyanobacteria and actinobacteria.

In the absence of a gene disruption system for *M. producens*, identification of the barbamide gene cluster was supported by ATP-PP_i exchange assay results using the BarE NRPS adenylation domain as well as the halogenase activity of both BarB1 and BarB2 using recombinant enzymes expressed in *E. coli*.[8,9] The production of **2** by heterologous expression of the 26 kb barbamide gene cluster (*barA*−*barK*) unequivocally confirms the identity of this gene cluster. Also, our results demonstrate that the *barA*−*barK* genes constitute the minimal gene set required for formation of **2**, which includes the unusual one-carbon truncation. Yet, significant challenges remain in the heterologous production of **2** in *S. venezuelae*, most dramatically the very low production yields (< 1 μg/L) which may be due to suboptimal codon usage in the cyanobacteria DNA compared to the ‘high %GC content’ actinobacteria. Our previous studies demonstrated that heterologous expression of tylosin PKS genes derived from *S. fradiae* produced ~0.5 mg/L of tylactone using the same host and vector system employed in this study.[17] Yet, heterologous expression of epothilone NRPS-PKS genes obtained from the myxobacterium *Sorangium cellulosum* produced very low levels of epothilone (< 1 μg/L).[15] Synthesis and expression of codon-optimized *bar* biosynthetic genes corresponding to the *S. venezuelae* codon bias would likely improve production of **2**, as observed for the production of plant-derived phenylpropanoids in *S. venezuelae*.[21]

The results presented herein provide an important precedent and approach for the biosynthetic study of marine natural products derived from genetically intractable organisms, such as sheath-containing filamentous cyanobacteria. In the current work, we demonstrated heterologous production and identification of a new barbamide derivative **2**, which had not been previously isolated from the natural producer. This new compound is several-fold more potent than **1** as a molluscicide and, given its lack of other toxicities,[7] may be a superb candidate for treating snail-infested waterways which pose health risks for human populations. Moreover, we report here the first functional heterologous expression of a complete marine cyanobacterial NRPS/PKS gene cluster using a readily manipulable antibiotic-producing *Streptomyces* species, suggesting new opportunities for the large-scale production of valuable marine natural products. Continued advances using cloning-independent next-generation sequencing-based metagenomic approaches[22] will certainly provide numerous biosynthetic gene clusters from unculturable symbionts or environmental metagenomic DNA. Heterologous gene expression technologies will thus play a critical role in understanding their products and advancing their utility in drug discovery programs.

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Supporting Information Available. Experimental and biosynthetic details, spectroscopic data for **2**, RT-PCR, and molluscicidal assay. This material is available free of charge via the Internet at http://pubs.acs.org.

(21) Park, S. R.; Yoon, J. A.; Paik, J. H.; Park, J. W.; Jung, W. S.; Ban, Y. H.; Kim, E. J.; Yoo, Y. J.; Han, A. R.; Yoon, Y. J. *J. Biotechnol.* **2009**, *141*, 181.

(22) Rath, C. M.; Janto, B.; Earl, J.; Ahmed, A.; Hu, F. Z.; Hiller, L.; Dahlgren, M.; Kreft, R.; Yu, F.; Wolff, J. J.; Kweon, H. K.; Christiansen, M. A.; Hakansson, K.; Williams, R. M.; Ehrlich, G. D.; Sherman, D. H. *ACS Chem. Biol.* **2011**, *6*, 1244.

The authors declare no competing financial interest.