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Biosynthetic *O*-Methylation Protects Cladoniamides from Self-destruction

Yi-Ling Du, Tong Ding, and Katherine S. Ryan*

Department of Chemistry, University of British Columbia, Vancouver, B.C. Canada

ksryan@chem.ubc.ca

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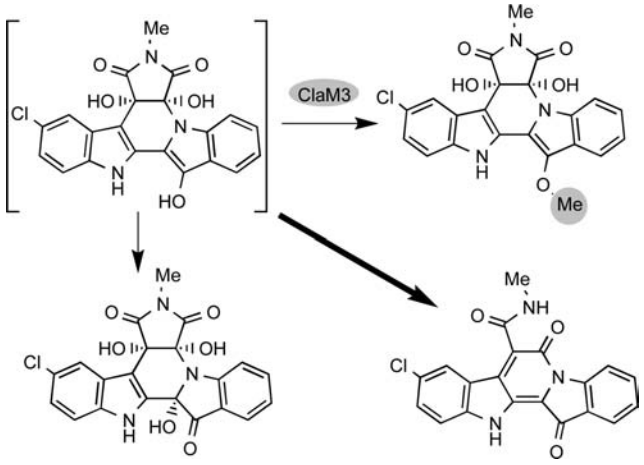
ABSTRACT



Bisindole cladoniamides, nanomolar inhibitors of colon cancer cell line HCT-116, contain a rare, indolotryptoline substructure. In this report, the structures of xenocladoniamides A–E (9–13) are described. Compounds 9–13 are generated from a cladoniamide heterologous production system where *O*-methyltransferase gene *claM3* has been inactivated. The results suggest that *O*-methylation, installed by enzyme ClaM3, is critical to maintaining the structural integrity of the indolotryptoline scaffold. Xenocladoniamides D and E are modestly cytotoxic against colon cancer cell line HCT-116.

Cladoniamides A–G (**1**–**7**)[1] (Figure 1) are unique bisindole structures, containing rare, indolotryptoline substructures. These molecules are isolated from *Streptomyces uncialis*, a strain found on the surface of the lichen *Cladonia uncialis*, isolated in British Columbia. Cladoniamide A has been shown to be a nanomolar inhibitor of colon cancer cell line HCT-116,[2] and our studies show that cladoniamide B also has potent cytotoxic activity against HCT-116 (IC_{50} = 10 ng/mL) (Table S6, Supporting Information (SI)). Total syntheses of cladoniamides A, G, and BE-54017 (**8**), the *N*-methyl derivative of cladoniamide A, have recently been reported.[3]

Previously, we published the cladoniamide (*cla*) biosynthetic gene cluster (Figure S1, SI).[4] The *cla* cluster was compared to the highly related BE-5017 (*abe*) cluster.[2] Both gene clusters contain indolocarbazole genes,[5] along with genes for unique flavin oxygenases and methyltransferases. In the *abe* pathway, functions of many of the enzymes were deduced from metabolic profiling of transposon mutants. These results demonstrate that BE-54017 (and, by extension, cladoniamide) derives from an indolocarbazole. A biosynthetic proposal for conversion of an indolocarbazole to an indolotryptoline (originally hypothesized by Andersen and colleagues[1]) is consistent with the gene cluster data and transposon mutagenesis results to date (Figure S2, SI).[1,2,4]

Given the potent cytotoxicity of the cladoniamides, we sought to generate a heterologous production platform for the routine generation of these molecules. The original producer, *Streptomyces uncialis*, is slow-growing

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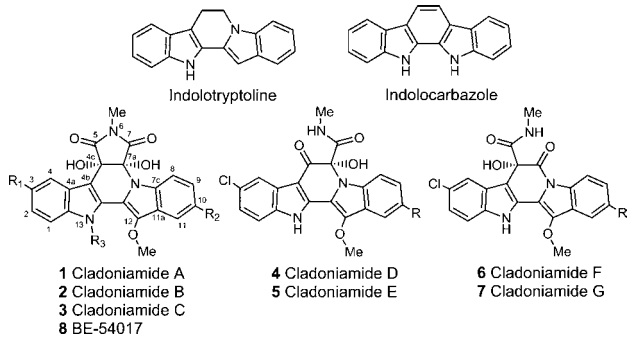

Figure 1. Cladoniamides and BE-54017. R_1 = Cl, R_2 = R_3 = H for **1**; R_1 = R_2 = Cl, R_3 = H for **2**; R_1 = R_2 = R_3 = H for **3**; R = H for **4** and **6**; R = Cl for **5** and **7**; R_1 = Cl, R_2 = H, R_3 = Me for **8**.

and yields of cladoniamides are generally low. Thus, we exported the cladoniamide (*cla*) gene cluster[4] to both *Streptomyces albus* J1074[6] and *Streptomyces coelicolor* M1146.[7] Both strains have simple metabolic profiles and are faster-growing strains.

Exporting the *cla* gene cluster to *Streptomyces albus* J1074 gave robust production of cladoniamides in liquid medium (Figure 2). Indeed, we observed high-level production of cladoniamides from this heterologous production strain (~8.7 mg/L of **1**) versus *Streptomyces uncialis* (~1.2 mg/L of **1**[1]). The relative distribution of metabolites approximately mirrors that observed in the native cladoniamide producer *Streptomyces uncialis* (Figure S3, SI). *Streptomyces albus* J1074 + *cla* thus represents an ideal platform for routine production of the major cladoniamides A and B. We also exported the *cla* cluster to *Streptomyces coelicolor* M1146. Again, we demonstrated production of cladoniamides. Here, however, the distribution of cladoniamides was altered, with relative increase in production of "ring-opened" cladoniamides **4**, **5**, and **6** (Figure 2).

Interestingly, this *Streptomyces coelicolor* M1146 + *cla* strain produces higher levels of purple-colored metabolites, which are not readily soluble in methanol (and are thus not seen in the HPLC traces in Figure 2) and were not previously observed from other *cla*-containing strains. The first compound (**9**) (Figure 3), obtained as a purple powder, gave an $[M + H]^+$ ion at m/z 378.0652 in HRESIMS, corresponding to the molecular formula $C_{20}H_{12}N_3O_3Cl$ (calcd for $C_{20}H_{13}N_3O_3Cl$ at 378.0645). Analysis of the 1D and 2D NMR spectra (Figure S4–S7, Table S3, SI) revealed that they were similar to those of **6**, as both have three isolated proton spin system (H-1/H-2/H-4, H-6/H-14, H-8/H-9/H-10/H-11) and an HMBC correlation of an *N*-methyl singlet to only one carbonyl carbon with resonance at δ 163.65 (C-5), indicating that **9** has a similar *N*-methylamide fragment as **6**. The absence of a methoxy

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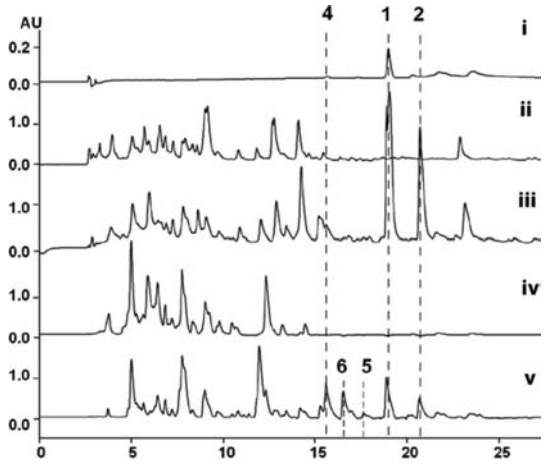

Figure 2. Heterologous expression of cladoniamides in *S. albus* J1074 and *S. coelicolor* M1146. (i) **1** standard, (ii–v) ethyl acetate extracted, methanol-solubilized metabolites from (ii) *S. albus* J1074 + pYLD6 (empty vector), (iii) *S. albus* J1074 + *cla*, (iv) *S. coelicolor* M1146, and (v) *S. coelicolor* M1146 + *cla*. Detection wavelength = 211 nm. No cladoniamides are observed in traces (ii) and (iv).

singlet and the presence of a new HMBC correlation between H-11 to a carbonyl carbon resonance at δ 180.48 in **9** suggested the methoxy carbon (δ 138.8, C-12) in **6** was oxidized to a keto-carbonyl carbon (δ 180.48, C-12) (Figure 4). Comparison of the ^{1}H and ^{13}C NMR of **9** with those of **6** also revealed that the hydroxyl sp^3 carbon (δ 76.2, C-4c) in **6** was replaced by a sp^2 hybridized carbon (δ 128.44, C-4c). The rest of NMR data as well as HRESIMS data are in complete agreement with the assigned structure. The second compound, **10**, was characterized as the C-10 chloro analogue of **9** based on the HRESIMS (**10**: $[M - H]^-$ ion at m/z 410.0104, calcd for $C_{20}H_{10}N_3O_3Cl_2$, 410.0099), ^{1}H NMR data (Table S3, SI), UV–vis spectra, and the isotope pattern observed in the MS spectra. Both **9**

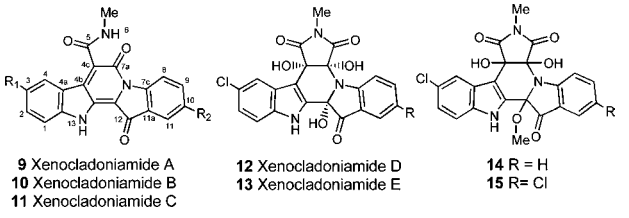

Figure 3. Xenocladoniamides and derivatives. R_1 = Cl, R_2 = H for **9**; R_1 = R_2 = Cl for **10**; R_1 = R_2 = H for **11**; R = H for **12** and **14**; R = Cl for **13** and **15**.

and **10** lack the signature indolotryptoline bonding pattern associated with the parent cladoniamides. Thus, we name them xenocladoniamides A (**9**) and B (**10**) (Figure 3).

We were intrigued by the biosynthetic origins of these molecules. In particular, these structures lack the

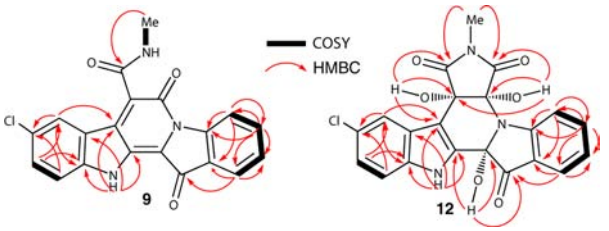

Figure 4. COSY and HMBC correlations observed in **9** and **12**.

C12-*O*-methylation seen across the **1**–**7** series. *O*-methyltransferase gene *claM3* is a likely candidate to install the C12-*O*-methyl group.[4,2] We postulated that genetic elimination of *claM3* might give rise to xenocladoniamides A and B and possibly other structures. We thus generated *S. albus* J1074 + *cla* (Δ*claM3*). From this strain, we observed dominant production of **9** (Figure 5). We also observed **11**, which we characterize as the nonchloro derivative of **9** and name xenocladoniamide C. Structural assignment of **11** is based on the HRESIMS (**11**: $[M + H]^+$ ion at *m*/*z* 344.0130, calcd for $C_{20}H_{14}N_3O_3$, 344.0135), ^1H NMR and COSY data (Figure S4, Figure S8, Table S3, SI), and UV–vis spectra (Figure S21, SI).

The total yield of **9**–**11** in the Δ*claM3* deletion mutant is 11.4 mg/L, compared to 0.5 mg/L in *S. albus* J1074 + *cla* (Figure S9, SI). Based on total yields of cladoniamides in *S. albus* J1074 + *cla*, it appears that a majority of cladoniamide-like metabolites funnel toward **9**-**11** in the absence of *claM3*. Analysis of the methanol-soluble fraction from extracts of this Δ*claM3* deletion mutant further revealed two additional, minor compounds (Figure S10, SI), which we structurally characterized as **12** and **13**. Compound **12**, obtained as yellow powder, was assigned a molecular formula of $C_{21}H_{14}N_3O_6Cl$ through HRESIMS (*m*/*z* 438.0498, $[M - H]^-$, calcd for $C_{21}H_{13}N_3O_6Cl$ at 438.0493). Comparison of the NMR spectra (Table S4–S5, Figure S11, Figure S13–S16, SI) with that of **1** revealed the presence of an additional exchangeable proton (δ 7.33, OH-12a) and the absence of a methoxy singlet (δ_H/δ_C 4.11/61.8, C-12). Further, a new HMBC correlation was observed between H-11 (δ 7.50) and a carbonyl carbon (δ 196.40, C-12), which suggested the methoxy carbon (δ 136.7, C-12) in **1** was oxidized to a keto-carbonyl carbon (δ 196.40, C-12) in **12** (Figure 4). The OH (12a) resonance at δ 7.33 was assigned based on the observation of HMBC correlations between OH (12a) and C-12/C-12a/C-12b. Stereochemical assignment of **12** is based on the tROESY correlation across C4c–OH, C7a–OH and C12a–OH in **12** (Figure S17–S18, SI), and the assumption that stereochemistry at C4c and C7a is unchanged from the parent cladoniamide A (**1**). Compound **13** was identified as the C-10 chloro analogue of **12** based on ^1H NMR data and MS analysis (**13**: $[M - H]^-$ ion at *m*/*z* 472.0104 through HRESIMS, calcd for $C_{21}H_{12}N_3O_6Cl_2$, 472.0103). Like **9**–**11**, compounds **12** and **13** have altered structures compared to indolotryptolines but are clearly

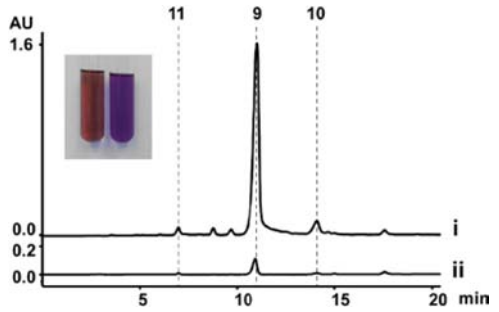

Figure 5. *O*-Methyltransferase gene *claM3* is critical to stability of cladoniamides. Ethyl acetate extracted, methanol-washed, and DMSO-dissolved metabolites for (i) *S. albus* J1074 + *cla* (Δ*claM3*) and (ii) *S. albus* J1074 + *cla*. Detection wavelength = 254 nm. Inset: Ethyl acetate extract from *S. albus* J1074 + *cla* (left) and *S. albus* J1074 + *cla* (Δ*claM3*) (right).

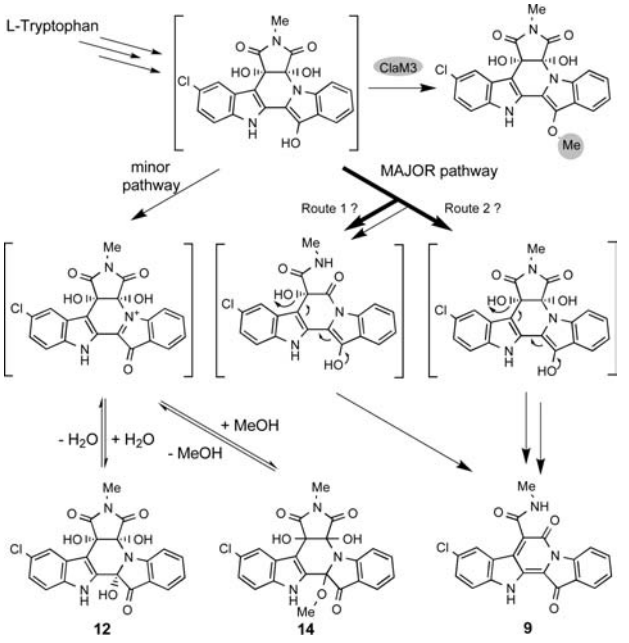

Figure 6. Proposed decomposition route for cladoniamide precursors in the absence of ClaM3. Autoxidation is likely to occur in all proposed pathways. In particular, an oxidation gives the proposed intermediate in the minor pathway, followed by attack by water (or methanol) to give **12** (or **14**). In the major pathway (Route 1), *N*-methylsuccinimide ring-opening occurs first, which may involve amide hydrolysis, followed by oxidative decarboxylation, as proposed by Andersen and colleagues[1] (Figure S2, SI). Dehydration then occurs, possibly driven by formation of a conjugated pi system in **9**. In Route 2, dehydration occurs first, followed by *N*-methylsuccinimide ring destruction only from the "right" side of the molecule. Route 2 would explain the observation that **9** is the major compound in the Δ*claM3* strain, and derivatives opened from the "left" side of the molecule are not observed from this strain. Note that **4**–**7** are minor compounds in *Streptomyces uncialis*; by contrast, **9** is the major compound in the engineered Δ*claM3* strain.

related to the parent cladoniamides; thus, we name them xenocladoniamide D (**12**) and E (**13**).

While manipulating **12** and **13** in methanol, we observed methylated derivatives **14** and **15**, respectively. Compounds **12** and **14** can be easily interconverted through a change of solvent from acetonitrile/H_2O (4:1) to methanol, or vice versa. Characterization of **14** by 1D and 2D NMR spectroscopy (Table S4−S5, Figure S12−S13, Figure S19−S20, SI), revealed that **14** differs from **12** by the presence of a methoxy group (δ_H/δ_C 3.29/51.22) at C-12a, instead of OH (12a) in **12**, an assignment which is also supported by the LC-MS data for **14** (*m*/*z* 476, $[M + Na]^+$). Compound **15** was identified as the C-10 chloro analogue of **14** based on ^1H NMR data (Table S4, Figure S12, SI) and MS analysis ($[M + Na]^+$ ion at *m*/*z* 510 through LC-MS).

Biological evaluation of the new molecules demonstrated that **12** and **13** have IC_{50} values of 3.0 μg/mL and 2.5 μg/mL, respectively, against colon cancer cell line HCT-116 (Table S6, SI).

From the Δ*claM3* mutant we did not observe any compounds consistent with non-*O*-methylated cladoniamides or earlier putative non-*O*-methylated intermediates. Instead, it appears that **9**−**13** derive spontaneously from non-*O*-methylated cladoniamide precursors. We propose that **9**−**13** are generated through the decomposition pathways shown in Figure 6. By contrast, cladoniamides **1**−**3** are apparently protected from decomposition through the effect of the methyoxy group at the C12 position. ClaM3 then plays a critical role in the cladoniamide biosynthetic pathway. By installation of a methyl group at the C12-hydroxyl, ClaM3 prevents spontaneous decomposition of the cladoniamide scaffold to shunt products **9**−**13**. However, enzymatic methylation is not a perfect solution to protection of the indolotryptoline scaffold. Using chemical standards available from this work, we are now able to detect **9**−**11** at trace levels in the original producer, *Streptomyces uncialis*. The presence of trace **9**−**11** suggests that the decomposition routes compete with enzymatic methylation in the native producer.

Biological *O*-methylation is widespread as a natural product tailoring modification.[8] Prior to our study, a small number of natural product biosynthetic intermediates have been shown to undergo spontaneous chemical transformations with the loss of *O*-methylation. For instance, in aflatoxin biosynthesis, xanthone demethylsterigmatocystin undergoes off-pathway isomerization in the absence of *O*-methyltransfer by AflO,[9] and enzymatic demethylation of thebaine enables spontaneous conversion of the product to the morphine precursor codeinone.[10] We show here that in cladoniamide biosynthesis, removal of *O*-methylation via a genetic approach gives elimination of the cladoniamides with a concomitant increase in production of xenocladoniamides A−E (**9**−**13**). *O*-Methyltransferase ClaM3 may thus represent an integrated biosynthetic strategy to protect a reactive scaffold from self-destruction, and genetic elimination of *O*-methylation is a facile route to access new, biologically active structures.

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Supporting Information Available. Experimental methods, Tables S1−S6, Figures S1−S21. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.