Communication

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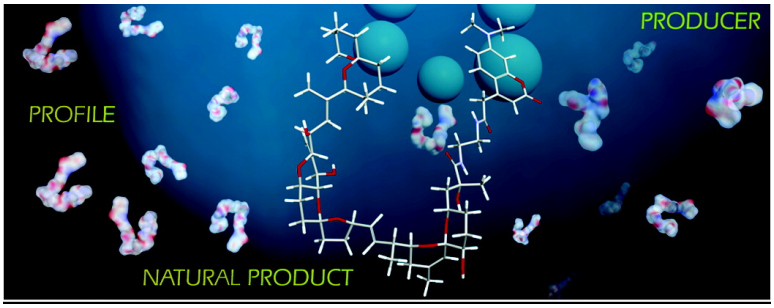

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Fluorescent Profiling of Natural Product Producers

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The biosynthesis of natural products has long been established as a means of regulating social interactions in both aquatic and terrestrial ecologies including aspects of chemical defense, symbiosis, parasitism, and predation.[1a] Recent questions have arisen regarding the relationship between toxin biosynthesis and the ability of the producer organism to avoid autotoxicity.[1b] Here we demonstrate a fluorescent system to profile the connection between biosynthetic production and autotoxicity using complementary producing and nonproducing strains.

Several benthic dinoflagellates of the genus *Prorocentrum* synthesize okadaic acid (**5**), a potent protein phosphatase (PP) inhibitor associated with diarrheic shellfish poisoning and certain harmful algal blooms. For this study, two strains of okadaic acid-producing dinoflagellates in the class Dinophyceae, *P. hoffmannianum* Faust (CCMP683) and *P. lima* Ehrenberg Dodge (CCMP1743), were compared with a related non-okadaic acid-producing strain, *P. micans* Ehrenberg (CCMP692). *P. micans* was chosen as a nontoxic species that is phylogenetically similar to the okadaic acid-producing strains.[1c] The primary goal of this initial investigation was to determine if the exogenous addition of fluorescent natural product analogues may be used to distinguish producer and nonproducing strains based on differences in intracellular response to the labeled toxins.

A set of fluorescent analogues was synthesized to probe the cellular processing of PP inhibitors. Compound **6**, an analogue of okadaic acid (**5**), was synthesized as the analogue to the natural PP inhibitor for *Prorocentrum* sp., while compound **9**, an analogue of microcystin LR (**10**), a metabolite from freshwater *Microcystis* sp., would appear to *Prorocentrum* sp. as a foreign PP inhibitor (Figure 1). Analogues **6** and **9** were prepared using the common fluorescent label 7-dimethylaminocoumarin-4-acetic acid (**1**) by the conversion of **1** to **2** and **3**.[2] From **3**, okadaic amide analogue **6** was prepared in a single step from okadaic acid (**5**).[3] The microcystin analogue **9**, developed through the guidance of SAR studies as reported by Chamberlin,[4] was prepared in three steps from **7** in an overall 21% yield. A third inhibitor, dragmacidin D (**4**),[5] was used in this study as a general blue fluorescent protein phosphatase inhibitor. This inhibitor was ideal, as fluorescence generated from excitation at $\lambda_{ex} = 360$ nm, $\epsilon = 19\,400$ M^{-1} cm^{-1} and emission at $\lambda_{em} = 450$ nm increased 190-fold upon binding to a protein phosphatase (PP1 α-isoform).

The activity of each inhibitor **4**, **6**, and **9** was determined by screening against a set of protein phosphatases (PP1, PP2A1, and PP2A2) using the *p*-nitrophenolphosphate assay.[6] Dragmacidin D (**4**) activity was given by an $IC_{50} = 21.0$ nM to PP1, an $IC_{50} = 3.0$ μM to PP2A1, and an $IC_{50} = 3.9$ μM to PP2A2. The microcystin analogue **9**, while less active than **10**, offered IC_{50} values of 420, 152, and 210 nM for PP1, PP2A1, and PP2A2, respectively. The okadaic acid analogue **6** inhibited PP1, PP2A1, and PP2A2 with IC_{50} values of 1.1, 64, and 79 nM.

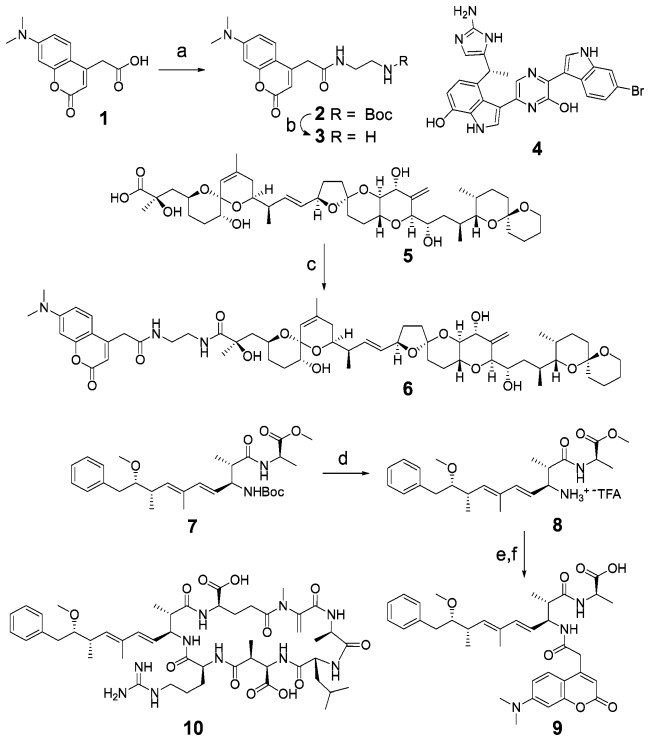

Figure 1. Fluorescent analogue synthesis. (a) ^{t}BocNHCH$_2$CH$_2$NH$_2$, EDC, DMAP, CH_2Cl_2, 85%; (b) TFA,CH_2Cl_2, 92%; (c) **3**, EDC, DMAP, DMF, 61%; (d) TFA, Et_3SiH, CH_2Cl_2; (e) **1**, HATU, collidine; (f) LiOH, H_2O, THF, 21% from **7**.

The uptake and cellular localization of **4**, **6**, and **9** were determined using a three-color fluorescent analysis. The system was designed to combine two channels of autofluorescence in *Prorocentrum* sp., red (excitation at $\lambda_{ex} = 562 \pm 40$ nm, emission at $\lambda_{em} = 624 \pm 40$ nm) and green (excitation at $\lambda_{ex} = 482 \pm 35$ nm, emission at $\lambda_{em} = 536 \pm 40$ nm), with fluorescence from analogues **4**, **6**, and **9** in a blue channel (excitation at $\lambda_{ex} = 377 \pm 50$ nm, emission at $\lambda_{em} = 447 \pm 60$ nm).

A multiwell plate assay was used to determine the uptake of each natural product analogue. As shown in Figure 2, uptake of dragmacidin D (**4**) and microcystin analogue **9** in the okadaic acid-producing strains CCMP683 and CCMP1743 was between 2.6 and 3.8 times greater than that of the okadaic acid analogue **6**. In the nonproducing strain CCMP692, the uptake of analogue **6** was at levels greater than that of **4** or **9**.

In a second series of experiments, uptake studies were conducted in *Microcystis aeruginosa*, class Cyanophyceae, a noxious, bloom-

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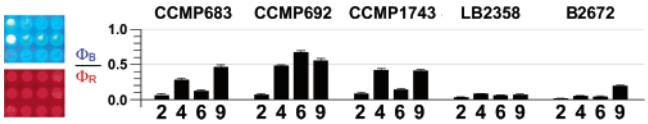

Figure 2. Uptake of fluorescent probes in *Prorocentrum* sp. strains CCMP683, CCMP692, and CCMP 1743 and *Microcystis* sp. strains LB2358 and B2672. (right) Exemplary plate. Glass slides were prepared with probe **2**, **4**, **6**, or **9** dried within 0.1 ± 0.02 mm strips. The slide was covered with an adhesive Teflon mask to provide 30 ± 5 μL wells that contained a lower surface coated with probe, such that the addition of media produced sample with a concentration of 1.0 ± 0.1 μM in the probe. Aliquots of each strain were added to individual wells. After incubation for 10 h at room temperature in the dark, the slides were terminated by coating each plate with a 2 ± 0.5 mm thick sheet of agar. The agar coating served to reduce the movement of the microorganism as well as to extract excess probe from solution. (left) The uptake of each analogue is given by the relative intensities of fluorescence from the probe in the blue (Φ_B) and the autofluorescence in the red (Φ_R). Dragmacidin **6** was used as a control as 98% of the fluorescence from **6** originated from its complex with a PP. This control is used to correlate uptake with PP inhibition.

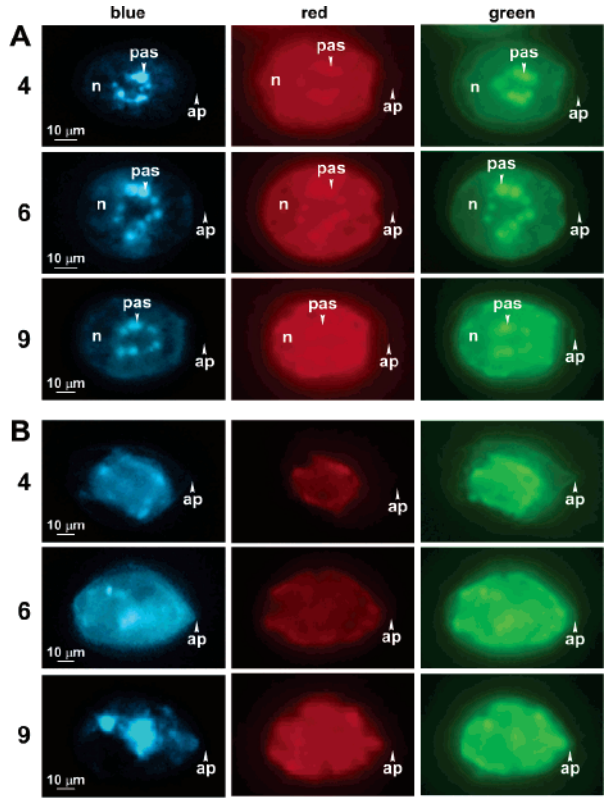

Figure 3. Fluorescent analysis of *Prorocentrum* sp. after exposure to 100 nM **4**, **6**, or **9** for min followed by incubation in fresh media for 10 h. (A) Producer strain CCMP 683. (B) Nonproducer strain CCMP 692. These images were collected under conditions where fluorescence was not obtained in the blue channel upon exposure to 10 μM **2** (see Supporting Information). Structures noted as **n** (nucleus), **pas** (PAS body), and **ap** (apical pore) were identified by counterstaining.[7a]

forming cyanobacterium that produces microcystin LR (**10**). Similarly, a microcystin-producing strain (LB2385) and a closely related nontoxic strain (B2672) were examined. These strains were selected because LB2385 contains the microcystin biosynthetic gene mcyA, whereas B2672 does not.[1d] The uptake specificity was comparable to that in *Prorocentrum* sp. (Figure 2), as indicated by an increase in the uptake of **9** in B2672.

Fluorescence microscopy was used to detail the localization of **2** (control), **4**, **6**, and **9** in *Prorocentrum* sp. Comparable studies in *Microcystis* sp. are provided in the Supporting Information. Ten hours after exposure, blue fluorescence from inhibitors **4**, **6**, and **9** was observed in the PAS bodies (dinoflagellate lysozymes) of the producer strain CCMP683 (Figure 3).[7] While okadaic acid has been observed in the PAS bodies using immunogold labeling, the primary target for okadaic acid observed in these studies was the chloroplast.[7a] While immunogold labeling has been shown to be effective for protein labeling, its use for monitoring natural products is complicated by factors involved in cell preparation. The procedure used by Zhou and Fritz[7a] included fixation with formaldehyde, which can react with okadaic acid (**5**), generating derivatives that may not be observed by a primary antibody elicited against **5**. This was further complicated by washing with ethanol, which can extract **5** from the cell. Fluorescent imaging in live cells offers an improved means to study intracellular specification of natural products by eliminating modifications arising during cell preparation.

In direct contrast, the nonproducer, strain CCMP692, was profoundly modulated by the addition of all three phosphatase inhibitors. Immediate uptake led to nonspecific loading within the cell (see Supporting Information). The primary effect was cell death with 89, 84, and 53% mortality after exposure to 100 nM **4**, **6**, and **9**, respectively. The cellular morphology was dramatic, as indicated by the loss of the intracellular bodies, nucleus, and cytoplasm. The effects of this response were most striking after treatment with dragmacidin D. In contrast to the producer strain, nonproducer CCMP692 failed to avoid the inhibition of PP activity from all three analogues and underwent cell death.

A combination of uptake quantification and cellular imaging provides a tool to distinguish producer and nonproducer strains. While a molecular target for this regulation has not been identified, this study suggests that panels of fluorescent natural product analogues can be used to profile microorganisms for their regulation of autotoxicity and access to biosynthetic activity.

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Supporting Information Available: Synthetic methods, activity analysis, as well as procedures for the cell culturing, imaging, and screening have been provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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