#### Metabolic probes for imaging endosymbiotic bacteria within toxic dinoflagellates

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## **General Methods.**

CP probe **1** was prepared according to published methods.<sup>\$1</sup> Control **2** was synthesized as described in the Chemical Synthesis section (below). TE probe **3** was prepared using a published procedure.<sup>\$2</sup> All compounds were purified to  $\ge$  99% purity (HPLC). LC–MS and LC–MS/MS analyses were conducted on a type MGIII Shiseido C–8 column (Tokyo, Japan) using Electrospray Ionization (ESI) on a ThermoFinnigan LCQ DECA– MS (Waltham, MA). Commercial okadaic acid (Calbiochem, La Jolla, CA) was used as an internal standard. Fluorescent microscopy was conducted on a Nixon Eclipse TE300 using irradiation from mercury vapor lamp (Nikon) as published.<sup>9</sup> Blue fluorescence was collected using a dichroic filter set with excitation filtered at 377 nm BP 50 and emission filtered at 477 BP 60 (FF409, Semrock). Red fluorescence was collected using a dichroic filter set with excitation filtered at 562 nm BP 40 and emission filter at 624 BP 40 (FF506, Semrock). Confocal fluorescent images were collected on a Leica DMI6000 inverted confocal microscope (Wetzlar, Germany) with a Yokogawa spinning disk confocal head (Tokyo, Japan), Orca ER high resolution black and white cooled CCD camera (6.45  $\mu$ m pixel<sup>-1</sup> at 1X) (Hamamatsu, Sewickley, PA), plan apochromat 40x and 1.25 na or 63x and 1.4 na objective, and a Melles Griot Argon/Krypton 100 mW air–cooled laser for excitation at 488, 568, and 647 nm excitations (Carlsbad, CA).

### **Chemical Synthesis**

*N*–(6–acetamidohexyl)–7–dimethylaminocoumarin–4–acetamide (2). A dried sample of 6–aminohexyl–7 dimethylaminocoumarin–4–acetamide (150 mg, 0.45 mmol) was dissolved in pyridine (4 ml) and treated with

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acetic anhydride (56 µl, 0.59 mmol) and catalytic amount of 4–dimethylaminopyridine. After stirring overnight, the solvent was evaporated *in vacuo* to yield a yellow oil that was purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>: MeOH, 9:1) to afford a yellow solid **2** (160 mg, 95%). <sup>1</sup>H–NMR (400 MHz, CDCl<sub>3</sub>: CD<sub>3</sub>OD 9.5:0.5):  $\delta$  1.31 (m, 4H), 1.46 (m, 4H), 1.91 (s, 3H), 3.06 (s, 6H), 3.09 (t, *J* = 6.8 Hz, 2H), 3.16 (t, *J* = 6.8 Hz, 2H), 3.66 (s, 2H), 6.04 (s, 1H), 6.56 (d, *J* = 2.4 Hz, 1H), 6.62 (dd, *J* = 9.2, 2.4 Hz, 1H), 7.57 (d, *J* = 9.2 Hz, 1H). <sup>13</sup>C–NMR (100 MHz, CDCl<sub>3</sub>:CD<sub>3</sub>OD 9.5:0.5):  $\delta$  22.6, 26.1, 28.9, 29.5, 39.2, 39.5, 39.8, 40.1, 98.0, 108.6, 109.4, 109.5, 126.0, 151.3, 153.4, 155.9, 163.1, 169.0, 171.6. FT/IR: 3316, 3262, 3079, 2920, 2850, 1725, 1625, 1620, 1515, 1500, 1430 cm<sup>-1</sup>. MS (ESI): [M+H]<sup>+</sup> 388.21. HRMS (FAB) (*m*/*z*): calcd. for C<sub>21</sub>H<sub>29</sub>N<sub>3</sub>O<sub>4</sub>Na 410.2050, found 410.2054.

#### **Biological Methods**

Culturing protocols. Prorocentrum lima (S2-195-5) was provided as a generous gift from Prof. Yuzuru Shimizu (Department of Pharmacology and Environmental Health Science, University of Rhode Island). Prorocentrum micans (CCMP692), an okadaic acid non-producer was obtained from the Provosoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). Strains were grown in f/2 - Si Guillard medium<sup>S3</sup> in 250 ml culture flasks, at 22° C, under a light to dark cycle of 16:8 h with an intensity of 54 µmol photons m<sup>-2</sup>s<sup>-1</sup> from cool white fluorescent lighting (Phillips, USA). All cultures were handled aseptically to prevent bacterial contamination and cross-contamination between cultures. Cultures were sampled in early stationary phase at 18 days to obtain the highest cell concentration for *in vivo* labeling studies  $(10^5 - 10^6 \text{ cells L}^3)$ <sup>1</sup>). Antibiotic treatment was conducted by treating 125 ml cultures at the early stationary phase with a cocktail containing 100 IU mL<sup>-1</sup> penincillin (Cellgro, Manassas, VA), 100 µg mL<sup>-1</sup> streptomycin (Cellgro, Manassas, VA) and 160 ug mL<sup>-1</sup> kanamycin (EMD, Gibbstown, NJ).<sup>S4</sup> After 3 days of treatment, the culture was considered axenic, as bacteria were not found when plated on a modified marine agar (ZM/10) prepared with seawater (75% aged, filtered through a 0.22 µm pore and autoclaved), 0.05% bactopeptone (Difco, Franklin Lakes, NJ), 0.01% yeast extract (Difco, Franklin Lakes, NJ), and 1.5% agar (Difco, Franklin Lakes, NJ), supplemented following autoclaving with sterile vitamin elements as used in f/2 medium.<sup>23</sup>

*In vivo* labeling studies. Our studies began by screening for appropriate dose and exposure time. After conducting a series of time course experiments, we identified optimal labeling when treating 1 mL of seawater containing  $10^5-10^6$  dinoflagellates for 90 min with 250  $\mu$ M probe 1, 50 nM probe 2 or 250  $\mu$ M control 3 from a DMSO stock (the final concentration of DMSO was 0.025%). After treatment, the dinoflagellates were harvested by centrifugation at 7,000 rpm for 5 min, and the cell pellet was washed three times with 1 mL of autoclaved seawater to remove excess probe.

**Fluorescent microscopy**. The dinoflagellates were imaged live. Time course imaging was collected at 100x and 400x on a Nixon Eclipse TE300 and confocal fluorescent imaging on a Leica DMI6000 inverted confocal microscope (Wetzlar, Germany).

**Blue mussel analyses**. Blue mussels, *Mytilus edulis*, wild caught in the Mexican Pacific coast, were purchased from Bristol Farms (La Jolla, CA) washed and acclimated to autoclaved seawater for 3 days. Mussels were infected by treating individual specimens in 250 mL of seawater with  $10^5$ – $10^6$  dinoflagellate cells L<sup>-1</sup>. After 5 h, the mussels were collected and dissected. Their digestive glands were extracted, chopped, and vortexed in 5 mL of autoclaved seawater. The supernatant was collected, centrifuged at 7,000 rpm for 5 min. The pellet was resuspended in 1 mL of seawater and treated with 0.5 mM of probe **1** by addition of 0.5  $\mu$ l of a 1 M stock of **1** in DMSO (providing a final concentration of 0.05% DMSO). After 90 min, the sample was pelted by centrifugation at 7,000 rpm for 5 min and washed with autoclaved seawater (3 x 1 mL). Imaging was conducted on a conventional fluorescence microscope (Nikon TE 3000) or confocal microscope (Leica DMI6000).

Toxin analysis by LC–MS and LC–MS/MS. The production of okadaic acid was determined for each strains examined. A 50 mL sample of each culture, in early stationary phase, was centrifuged at 7,000 rpm for 5 min, and the pellet was collected, resuspended in 5 mL of  $CH_2Cl_2$ : MeOH (1:1) and sonicated for 45 min at 30° C in a Fisher Scientific FS30 ultrasonic bath (Pittsburgh, PA). Extracts were then prepared by filtration and removal of the solvent on a rotary evaporator. LC–MS analyses were conducted using established conditions.<sup>S5–S6</sup> Using

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an okadaic acid standard (Calbiochem, La Jolla, CA), we obtained detection limit of 0.1 ng mL<sup>-1</sup> with a LC retention time of 9.77 min (m/z 821.82 for M<sup>+</sup>NH<sub>4</sub>) on a 2mm x 100mm, 3 um, Type MGIII Shiseido C–8 column (Tokyo, Japan) when passing at a flow rate of 200 µl min<sup>-1</sup> with ESI<sup>+</sup> detection on a ThermoFinnigan LCQ DECA–MS (Waltham, MA). Evidence for this peak was further confirmed by its fragmentation pattern of [M+Na]<sup>+</sup> at m/z 827 with ions at m/z 723.5, 705.5, 679.3, 607.2, 595.3, 571.2, 483.5, 383.9 and 274.6. Okadaic acid was not found in extracts of the non–producing organism *P. micans*. In addition, treating *P. lima* with a cocktail of antibiotics led to a 35 fold reduction in production of okadaic acid as given by 0.20 ng mL<sup>-1</sup> of okadaic acid in the antibiotic treated cultures *versus* 7.34 ng mL<sup>-1</sup> of okadaic acid in cultures of untreated *P. lima*.

## References

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Images depicting colonies of dinoflagellates treated with the probes and control stains have been provided in Fig. S1, below. This figure also depicts the uptake and localization in strains treated with antibiotics and non-producing strains (*P. micans*).



**Fig. S1** *In vivo* labeling strategy with images depicting comparative analyses of CP and TE domains. Dinoflagellates, *P. lima*, antibiotic–treated *P. lima*, and the non–okadaic acid producing strain *P. micans*, were grown to  $10^5$ – $10^6$  cells L<sup>-1</sup> in seawater. A 1 mL aliquot of each strain was treated with 250  $\mu$ M probe 1, 250  $\mu$ M probe 2 or 50 nM of probe 3. After incubating for 90 min, the cells were harvested by centrifugation and washed using fresh seawater (3 x 1 mL) to remove the excess probe. Imaging was conducted at using conventional fluorescence microscope (Nikon TE 3000). Bars denote 30 microns.

acid in the dinoflagellate extracts.

Fig. S2 Plots depicting the LCEMS analysis of an okadaic acid standard.



# Fig. S3 Plots depicting the LCEMS analysis of a *Prorocentrum lima* culture extract.



Fig. S4 Plots depicting the LCEMS analysis of an extract from *Prorocentrum lima* culture treated for 3 days with antibiotics.









Fig. S7 A plot depicting the LCEMS/MS analysis at m/z 723.3 from a Prorocentrum lima culture extract.

