Marine Natural Product Honaucin A Attenuates Inflammation by Activating the Nrf2-ARE Pathway

Samantha J. Mascuch[†], Paul D. Boudreau[†], Tristan M. Carland[‡], N.Tessa Pierce[†], Joshua Olson[§], Mary E. Hensler[§], Hyukjae Choi[⊥] Joseph Campanale[†], Amro Hamdoun[†], Victor Nizet[§,‖], William H. Gerwick[†,‖], Teresa Gaasterland[†,▽], and Lena Gerwick[†,°]

[†] Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093, USA

[‡] Illumina Inc., San Diego, CA 92122, USA

[§] Department of Pediatrics, University of California, San Diego, School of Medicine, La Jolla, CA 92093, USA

[⊥] College of Pharmacy, Yeungnam University, Gyeongsan, 38541, Republic of Korea

[‖] Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093, USA

[▽] Bioinformatics Contact: tgaasterland@ucsd.edu

[°] Lead Contact: lgerwick@ucsd.edu

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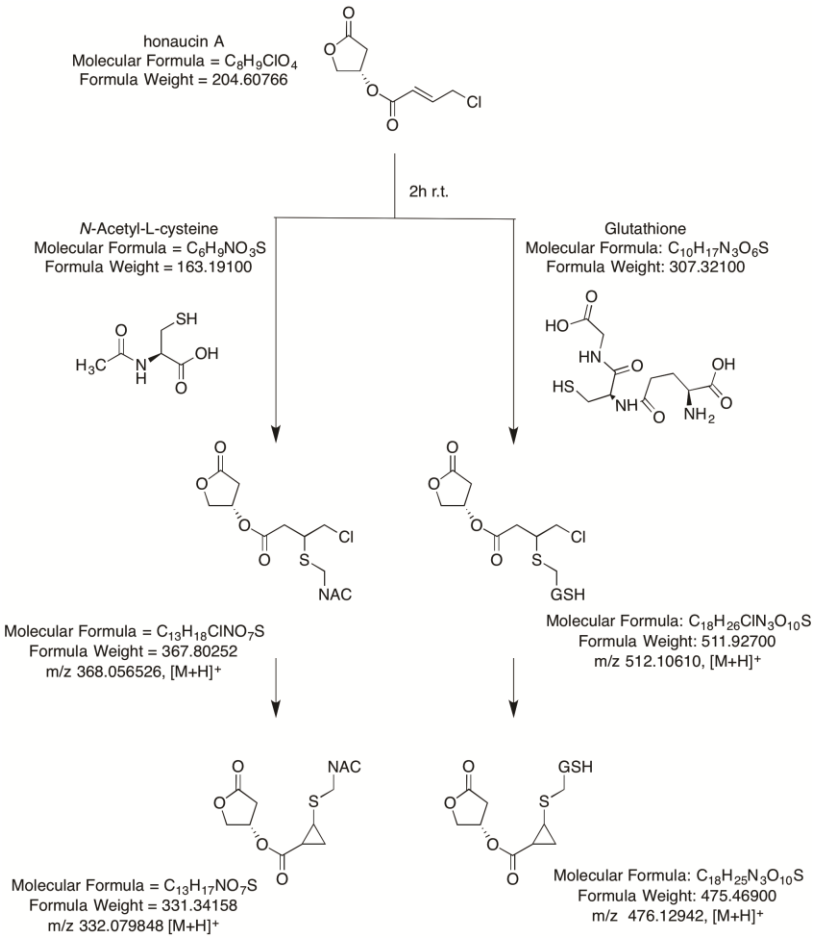
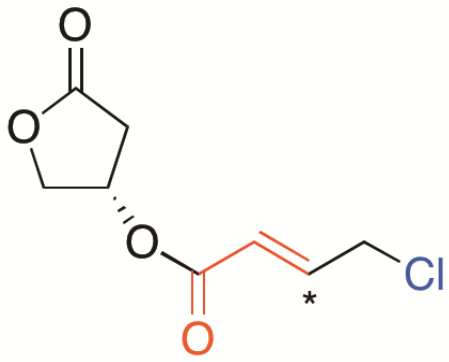
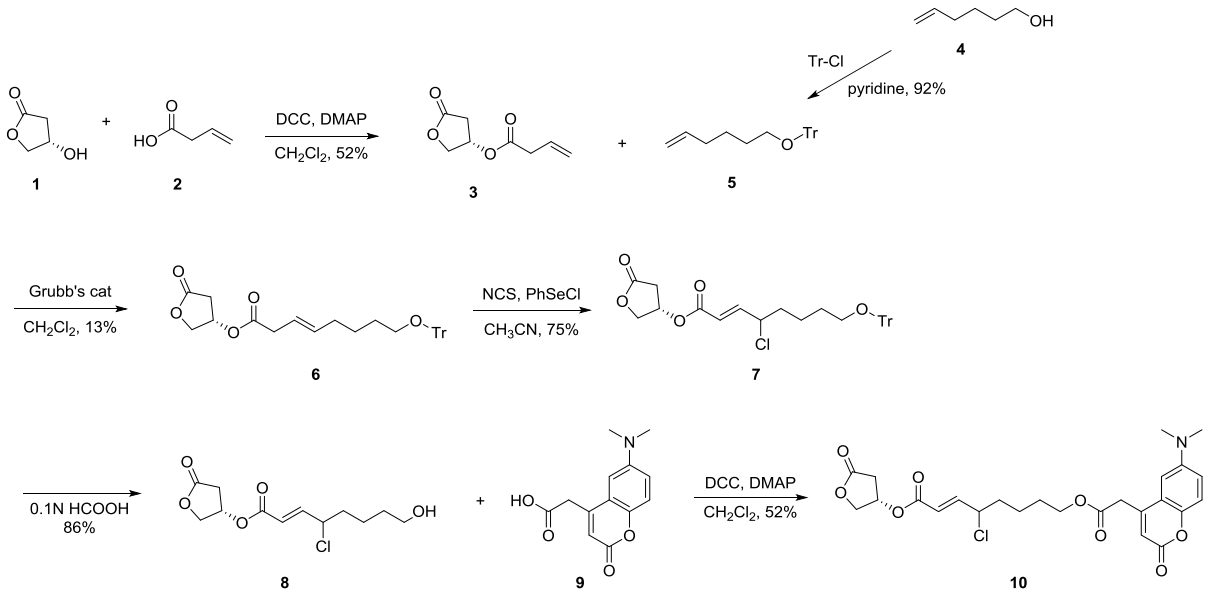


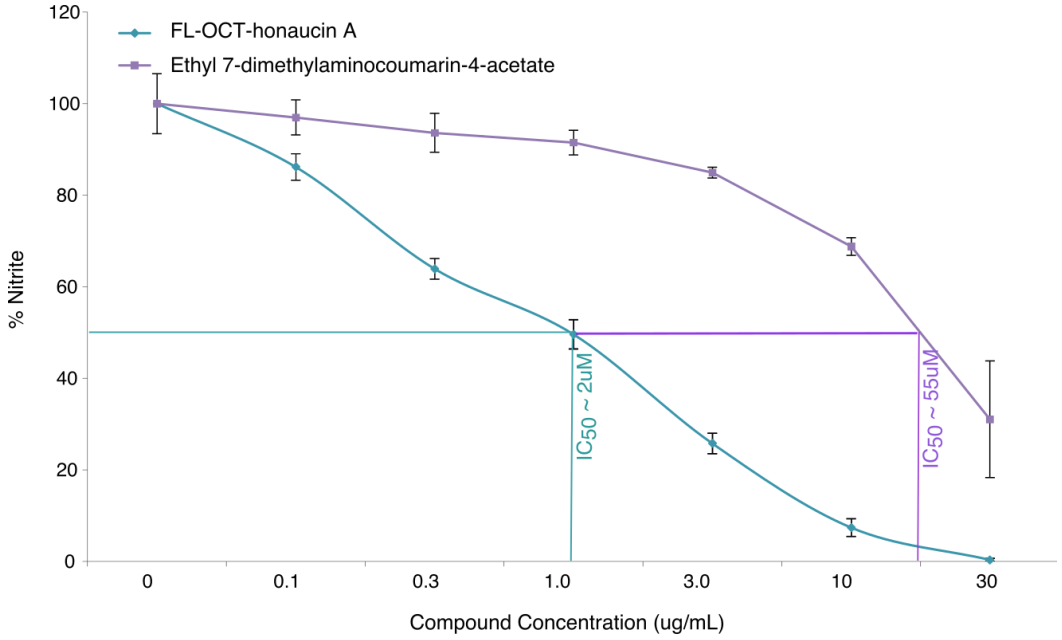
S1. Reaction scheme for honaucin A and N-Acetyl-L-cysteine or reduced glutathione showing predicted adducts.



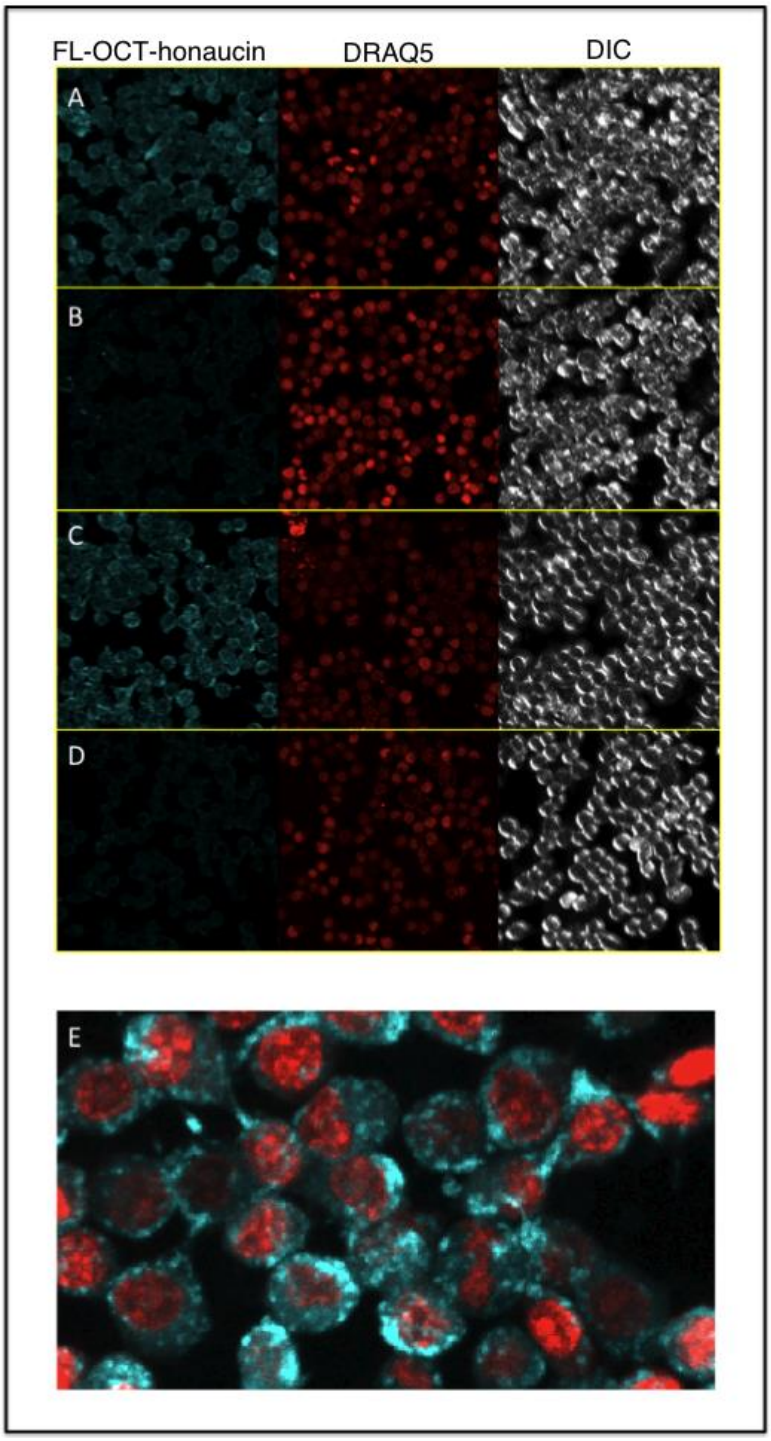
S2. Structure of honaucin A with Michael Acceptor motif (red) and electron withdrawing group (blue) highlighted.



S3. Synthetic scheme for the generation of a fluorescent ethyl 7- dimethylaminocoumarin-4-acetate honaucin probe, Fl-OCT-honaucin A.



S4. Activity data for Fl-OCT-honaucin A and ethyl 7- dimethylaminocoumarin-4-acetate in an assay of nitric oxide inhibition. Error bars represent the standard deviations of 3 replicates.



S5. Confocal images showing the subcellular localization of Fl-OCT-honaucin A and ethyl 7-dimethylaminocoumarin-4-acetate in RAW264.7 cells. Cells were exposed to either honaucin probe or coumarin for 15 minutes (A, B) or one hour (C, D) before being washed with PBS. The probe was better retained than coumarin and showed both a punctate as well as a more diffuse cytosolic distribution. (E) Close-up showing the distribution of the honaucin probe at one hour.

Table S1. Transcript abundances for genes that were significantly differentially regulated in response to honaucin A exposure.

Gene Symbol	Gene Name	Log2 Fold Change (p - value)			
		30 minutes		180 minutes	
		4 µM	12 µM	4 µM	12 µM
Akr1b8	aldo-keto reductase family 1, member B8	-	-	-	1.356 (1.66×10^{-15})
Blvrb	biliverdin reductase B (flavin reductase (NADPH))	-	-	-	1.050 (3.94×10^{-12})
Btg2	B cell translocation gene 2, anti-proliferative	-	-0.676 (2.31×10^{-08})	-	-
Ccnl1	cyclin L1	-	-0.406 (4.41×10^{-05})	-	-
Ccrl2	chemokine (C-C motif) receptor-like 2	-	-1.108 (4.51×10^{-16})	-	-
Cd83	CD83 antigen	-	-0.794 (5.49×10^{-09})	-	-
Cflar	CASP8 and FADD-like apoptosis regulator	-	-0.581 (1.73×10^{-06})	-	-
Cxcl2	chemokine (C-X-C motif) ligand 2	-	-0.494 (1.82×10^{-04})	-	-
Cxcl10	chemokine (C-X-C motif) ligand 10	-	-0.670 (1.59×10^{-06})	-	-
Cybb	cytochrome b-245, beta polypeptide	-	-0.325 (7.24×10^{-05})	-	-
Dusp2	dual specificity phosphatase 2	-	-0.749 (6.39×10^{-08})	-	-
Egr2	early growth response 2	-	-0.635 (3.52×10^{-06})	-	-
Epha2	Eph receptor A2	-	-	-	0.726 (9.35×10^{-06})
Errfi1	ERBB receptor feedback inhibitor 1	-	-0.450 (1.12×10^{-04})	-	-
Esd	esterase D/formylglutathione hydrolase	-	-	-	0.798 (1.05×10^{-04})
Fbxo30	F-box protein 30	-	-	-	0.688 (5.23×10^{-05})
Fgd4	FYVE, RhoGEF and PH domain containing 4	-	-	-	-0.655 (1.13×10^{-04})
Fos	FBJ osteosarcoma oncogene	-	-0.499 (4.42×10^{-06})	-	-
Gabarapl1	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	-	-	-	0.852 (4.69×10^{-05})

Gas7	growth arrest specific 7	-	-	-	-0.699 (8.15×10^{-05})
Gclm	glutamate-cysteine ligase, modifier subunit	-	-	-	1.108 (3.14×10^{-06})
Gpr84	G protein-coupled receptor 84	-	-0.511 (1.29×10^{-04})	-	-
Hmox1	heme oxygenase 1	-	0.890 (4.23×10^{-15})	-	1.818 (1.15×10^{-13})
Il1b	interleukin 1 beta	-	-0.736 (1.32×10^{-7})	-	-
Jag1	jagged 1	-	-	-	1.065 (7.53×10^{-07})
Marcksl1	MARCKS-like 1	-	-0.495 (3.56×10^{-05})	-	-
Mcl1	myeloid cell leukemia sequence 1	-	-0.431 (1.33×10^{-06})	-	-
Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	-	-0.605 (5.52×10^{-06})	-	-
Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta	-	-0.978 (8.54×10^{-27})	-	-
Pid1	phosphotyrosine interaction domain containing 1	-	-	-	-0.775 (2.15×10^{-05})
Ppp1r15a	protein phosphatase 1, regulatory (inhibitor) subunit 15A	-	-0.588 (2.08×10^{-05})	-	-
Prdx1	peroxiredoxin 1	-	-	-	1.802 (9.46×10^{-20})
Ptgir	prostaglandin I receptor (IP)	-	-	-	1.236 (8.15×10^{-08})
Rassf8	Ras association (RalGDS/AF-6) domain -family (N-terminal) member 8	-	-	-	0.941 (1.02×10^{-05})
Rasgef1b	RasGEF domain family, member 1B	0.416 (1.90×10^{-05})	-1.069 (6.87×10^{-18})	-	-
Rusc2	RUN and SH3 domain containing 2	-	-	-	0.984 (1.22×10^{-06})
Sh3bgrl2	SH3 domain binding glutamic acid-rich protein like 2	-	-	-	0.603 (6.27×10^{-05})
Slc11a1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 / Natural resistance-	-	-	-	0.996 (1.90×10^{-07})

	associated macrophage protein 1				
Slc48a1	solute carrier family 48 (heme transporter), member 1 / Heme transporter HRG1	-	-	-	0.887 (4.28×10^{-05})
Socs3	suppressor of cytokine signaling 3	0.526 (6.09×10^{-08})	-1.273 (4.55×10^{-25})	-	-
Sqstm1	sequestosome 1	-	-	-	0.981 (8.77×10^{-05})
Srxn1	sulfiredoxin 1 homolog (S. cerevisiae)	0.492 (1.78×10^{-12})	0.815 (9.68×10^{-16})	1.246 (-2.57×10^{-19})	-
Strn	striatin, calmodulin binding protein	-	-0.369 (2.94×10^{-05})	-	-
Tnf	tumor necrosis factor	-	-0.814 (1.21×10^{-11})	-	-
Tnfaip3	tumor necrosis factor, alpha-induced protein 3	-	-1.278 (7.02×10^{-23})	-	-
Tnfsf9	tumor necrosis factor (ligand) superfamily, member 9	-	-0.541 (1.07×10^{-04})	-	-
Tulp2	tubby-like protein 2	-	-0.642 (3.72×10^{-06})	-	-
Txnrd1	thioredoxin reductase 1	-	-	-	0.983 (2.30×10^{-05})
Ubap1	ubiquitin associated protein 1	-	-	-	0.616 (5.24×10^{-05})
Ube2q2	ubiquitin-conjugating enzyme E2Q (putative) 2	-	-	-	0.666 (1.91×10^{-05})

SUPPORTING EXPERIMENTAL PROCEDURES

Generation of fluorescent ethyl 7- dimethylaminocoumarin-4-acetate honaucin probe
Briefly, 3*S*-Hydroxy butyrolactone (**1**, 1.02 g) and 3-butenoic acid (**2**) were coupled via Steglich esterification in the presence of *N,N*'-dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-(dimethylamino)pyridine (DMAP) to give a compound **3**.[1] An alcohol of hex-5-ene-1-ol (**4**) was treated with trityl chloride to give tritylated compound **5**. Then compounds **3** and **5** were coupled by Grubb's reaction with the Grubbs catalyst 2nd generation.[2] The resulting compound **6** was treated with *N*-chlorosuccinimide under the presence of PhSeCl for allylic chlorination to produce compound **7**.[3] The trityl group of compound **7** was deprotected under mild acidic condition and its primary alcohol (**8**) was coupled with coumarin-4-acetic acid (**9**) via Steglich esterification to give fluorescent honaucin A (**10**).

Specifically, 3-Butenoic acid (**2**, 851 mg) and 1.2 eq. of *N,N*'-dicyclohexylcarbodiimide (DCC, 2.4 g) with a catalytic amount of 4-(dimethylamino)pyridine (DMAP) were dissolved in 10 mL of distilled CH_2Cl_2 and the mixture was stirred for 5 min at room temperature. To the solution, 3*S*-hydroxy butyrolactone (**1**, 1.02 g) was added and the mixture was incubated for 16 h at room temperature. The solution was dried under N_2 and the residue was subjected to silica open column chromatography with a stepped gradient elution (hexanes and ethyl acetate). The fraction eluting with 70% hexanes in ethyl acetate was further purified by RP-HPLC (Phenomenex Luna C18, 250 x 10 mm, 3 mL/min, H_2O:CH_3CN=7:3, 210 nm) to give a purified compound **3** (885 mg, yield 52%). Hex-5-ene-1-ol (**4**, 500 mg) was dissolved in 3 mL of anhydrous pyridine with the addition of trityl chloride (1.394 g). The solution was incubated with stirring for 16 h at room temperature, and the tritylation was quenched with the addition of 1 mL of MeOH. Then the solution was evaporated under reduced pressure and the residue was partitioned between $CHCl_3$ (50 mL) and H_2O (20 mL). The organic layer was washed with brine (20 mL), treated with Na_2SO_4, and evaporated under reduced pressure. The tritylated product (**5**, 1.576 g, 92%) was purified by normal phase Sep-Pak column chromatography. Compounds **3** (425 mg) and **5** (85.6 mg) were coupled by Grubb's reaction with Grubbs catalyst 2nd generation (0.2 eq, 42.5 mg) under distilled CH_2Cl_2 for 16 h with stirring. The reaction mixture was filtered and evaporated under reduced pressure and then subjected to RP-HPLC (Phenomenex Hydro RP, 250 x 10 mm, 3 mL/min, A=90% H_2O in CH_3CN, B=100% CH_3CN, 3:7 for 20 min, 3:7 to 100% CH_3CN for 20 min, 210 nm) to give a pure compound **6** (15.7 mg, yield 13%). Compound **6** (160 mg) was added to a vial containing PhSeCl (16 mg) and anhydrous CH_3CN (150 mL). To a reaction mixture, *N*-chlorosuccinimide (52.3 mg) in anhydrous CH_3CN was added dropwise through a gas tight syringe and the reaction mixture was incubated for 16 h with stirring at room temperature. The reaction mixture was concentrated under N_2 and the residual material was partitioned between Et_2O and H_2O. The Et_2O soluble material was purified by normal phase Sep-Pak chromatography to give pure compound **7** (128 mg, 75%). Compound **7** (51.9 mg) was dissolved in Et_2O and treated with 0.1 *N* formic acid for 4 h to give the detritylated product, compound **8** (23.7 mg, 86%). Coumarin-4-acetic acid (**9**, 4.3 mg) and 3.58 mg of DCC with a catalytic amount of DMAP were dissolved in 3 mL of distilled CH_2Cl_2 and the mixture was stirred for 5 min at room temperature. To the solution, compound **8** (4 mg) was added and the mixture was incubated for 16 h at room temperature. The solution was dried under N_2 and the residue was subjected to silica open column chromatography with a stepped gradient elution (hexanes and ethyl acetate). The fraction eluting with 70% hexanes in ethyl acetate was further purified by RP-HPLC (Phenomenex Luna C18, 250 x 4.6 mm, 1 mL/min, H_2O:CH_3CN=7:3, 210 nm) to give a purified compound **10** (4.5 mg, yield 52%).

Compound **3**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.88 (ddt, *J* = 17.0, 10.4, 7.2 Hz, 1H), 5.45 (m, 1H), 5.21 (dd, *J* = 10.4, 1.1 Hz, 1H), 5.20 (dd, *J* = 17.0, 1.1 Hz, 1H), 4.51 (dd, *J* = 11.2, 4.9 Hz, 1H), 4.37 (d, *J* = 11.2 Hz, 1H), 3.13 (d, *J* = 7.2 Hz, 2H), 2.86 (dd, *J* = 18.6, 6.6 Hz, 1H), 2.62 (d, *J* = 18.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.6, 171.1, 129.3, 119.7, 73.2, 70.2, 39.0, 34.7; HRESITOFMS *m/z* $[M+H]^+$ 171.0657 (calcd for $C_8H_{11}O_4$ 171.0652).

Compound **5**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, *J* = 8.1 Hz, 1H), 7.37 (t, *J* = 7.6 Hz, 1H), 7.30 (t, *J* = 7.3 Hz, 1H), 5.87 (ddt, *J* = 16.9, 10.2, 6.7 Hz, 1H), 5.06 (ddd, *J* = 17.2, 3.5, 1.6 Hz, 1H), 5.04 –

5.00 (m, 1H), 3.15 (t, J = 6.6 Hz, 1H), 2.11 (q, J = 7.2 Hz, 1H), 1.60 – 1.53 (m, 1H); ^{13}C NMR (75 MHz, CDCl3) δ 144.7, 139.0, 128.9, 127.9, 127.0, 114.6, 86.5, 63.6, 33.8, 29.7, 25.8; HREIMS *m/z* $[M]^+$ 342.1981 (calcd for $C_{28}H_{26}O$ 342.1978).

Compound **6**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 (d, J = 7.3 Hz, 6H), 7.30 (t, J = 7.5 Hz, 6H), 7.23 (t, J = 7.3 Hz, 3H), 5.57 (m, 1H), 5.46 (m, 1H), 5.43 (ddd, J = 6.7, 3.2, 1.6 Hz, 1H), 4.50 (dd, J = 11.1, 4.8 Hz, 1H), 4.36 (d, J = 11.1 Hz, 1H), 3.05 (ddd, J = 6.5, 3.4, 2.7 Hz, 2H), 2.85 (dd, J = 18.5, 6.8 Hz, 1H), 2.62 (d, J = 18.4 Hz, 1H), 2.02 (q, J = 7.0 Hz, 2H), 1.63 (m, 2H), 1.47 (dq, J = 15.0, 7.6 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.6, 171.7, 144.7, 135.9, 128.9, 127.9, 127.0, 120.8, 86.5, 73.2, 70.0, 63.5, 38.0, 34.7, 32.5, 29.7, 26.0; HRESITOFMS *m/z* $[M+Na]^+$ 507.2143 (calcd for $C_{31}H_{32}O_5Na$ 507.2142).

Compound **7**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 (d, J = 7.2 Hz, 6H), 7.30 (t, J = 7.5 Hz, 6H), 7.24 (t, J = 7.3 Hz, 3H), 6.93 (dd, J = 15.4, 7.5 Hz, 1H), 6.01 (d, J = 15.4 Hz, 1H), 5.51 (dd, J = 6.5, 5.0 Hz, 1H), 4.54 (dd, J = 11.1, 4.8 Hz, 1H), 4.44 (m, 1H), 4.40 (d, J = 11.0 Hz, 1H), 3.08 (t, J = 6.4 Hz, 2H), 2.89 (dd, J = 18.5, 6.8 Hz, 1H), 2.66 (d, J = 18.5 Hz, 1H), 1.82 (dd, J = 14.7, 7.6 Hz, 2H), 1.65 (ddd, J = 14.1, 8.7, 4.2 Hz, 2H), 1.50 (dt, J = 20.5, 6.8 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.7, 165.2, 148.5, 144.5, 128.8, 128.0, 127.1, 121.3, 86.6, 73.1, 70.3, 63.1, 59.6, 37.4, 34.7, 29.4, 23.2; HRESITOFMS *m/z* $[M+Na]^+$ 541.1753 (calcd for $C_{31}H_{31}ClO_5Na$ 541.1752).

Compound **8**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.96 (dd, J = 15.4, 7.5 Hz, 1H), 6.05 (d, J = 15.4 Hz, 1H), 5.53 (dd, J = 6.5, 5.0 Hz, 1H), 4.55 (dd, J = 11.1, 4.8 Hz, 1H), 4.48 (dd, J = 14.2, 7.2 Hz, 1H), 4.43 (d, J = 11.2 Hz, 1H), 3.68 (t, J = 6.1 Hz, 2H), 2.91 (dd, J = 18.5, 6.7 Hz, 1H), 2.68 (d, J = 18.4 Hz, 1H), 1.91 (dd, J = 14.3, 7.5 Hz, 2H), 1.64-1.50 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.60, 165.15, 148.34, 121.39, 73.15, 70.32, 62.65, 59.62, 37.47, 34.77, 32.09, 22.79; HRESITOFMS *m/z* $[M+Na]^+$ 299.0655 (calcd for $C_{12}H_{17}ClO_5Na$ 299.0657).

Compound **10**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41 (d, J = 8.9 Hz, 1H), 6.91 (dd, J = 15.4, 7.5 Hz, 1H), 6.64 (dd, J = 9.0, 2.5 Hz, 1H), 6.54 (d, J = 2.5 Hz, 1H), 6.05 (s, 1H), 6.02 (dd, J = 15.4, 1.1 Hz, 1H), 5.53 (dd, J = 6.3, 4.9 Hz, 1H), 4.55 (dd, J = 11.2, 4.8 Hz, 1H), 4.44 (d, J = 11.2 Hz, 1H), 4.37 (dd, J = 13.6, 6.6 Hz, 1H), 4.13 (t, J = 6.4 Hz, 2H), 3.69 (s, 2H), 3.07 (s, 6H), 2.91 (dd, J = 18.5, 6.7 Hz, 1H), 2.69 (d, J = 18.4 Hz, 1H), 1.80 (dt, J = 10.7, 4.3 Hz, 2H), 1.65 (m, 2H), 1.53 – 1.42 (m, 1H), 1.42 – 1.33 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.6, 169.3, 165.1, 161.9, 156.2, 153.2, 148.5, 148.0, 125.5, 121.5, 111.0, 109.3, 108.7, 98.6, 73.2, 70.4, 65.2, 59.4, 40.4, 38.6, 37.1, 34.8, 28.0, 22.8; HRESITOFMS *m/z* $[M+Na]^+$ 528.1394 (calcd for $C_{25}H_{28}ClNO_8Na$ 528.1396).

Detection of NO Production in Murine Macrophages treated with Fl-OCT honaucin A (Villa et al. 2010)[4]

A 96-well plate was seeded with RAW 264.7 cells at a density of 5 x 10^4 cells/180 µL medium/well in Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin/streptomycin. The plate was incubated overnight at 37 °C with 5% CO_2 in order to achieve a confluent cellular monolayer. Fl-OCT honaucin A, consisting of honaucin A conjugated to ethyl 7- dimethylaminocoumarin-4-acetate, as well as ethyl 7- dimethylaminocoumarin-4-acetate itself were serially diluted in 30% EtOH:PBS and each dilution was added to the plate in triplicate wells (10 µL/well) to give a final concentration series of 30, 10, 3.0, 1.0, 0.3 and 0.1 µg/mL. The plate with compounds was incubated for 1 hr at 37 °C with 5% CO_2 prior to the addition of 10 µL/well bacterial endotoxin yielding a final concentration of 3.0 µg/mL. Controls included the positive control dimethylsulfoxide (5%), an LPS-free control, and a compound-free LPS only control. Plates were incubated overnight 1 hr at 37 °C with 5% CO_2 and the amount of nitric oxide in each well was determined the following morning by measurement of the NO breakdown product nitrite via Griess reaction.[5] Briefly, the amount of nitrite in each sample was compared to a prepared nitrite standard curve (0-100 µM). 50 µL 1% sulfanilamide in 5% phosphoric acid was added to 50 uL of supernatant from each well of the overnight assay plate, as well as to the prepared nitrite standard curve, and incubated in the dark at room temperature for 10 minutes. Following this incubation, 0.1% N-1-napthylethylenediamine dihydrochloride in water (50 µL, Ricca Chemical Company LLC, Pocomoke City, MD) was added to the plate and the incubation was repeated. The absorbance of the

wells was measured at 570 nm and sample nitrite concentration was calculated by regression using the nitrite standard curve. Average nitrite and standard deviation for each sample treatment was reported. An MTT assay of cell viability ensured that cell mortality was not observed at the IC_{50} values of the compounds.

Cell tracking with a fluorescent honaucin A probe
RAW 264.7 macrophages were seeded at a density of $1x10^6$ cells per milliliter in MatTek (Ashland, MA, USA) 35 mm glass-bottom dishes and allowed to achieve confluency in a CO_2 incubator overnight. Either coumarin or the coumarin-conjugated honaucin A probe (2 μM) was introduced to the cells. Cells were incubated for 15 minutes or 1 hour at 37 °C with 5% CO_2 and then washed 5 times with phosphate buffered saline. The nuclear stain DRAQ5 was then added and the cells were incubated for an additional 10 minutes. A Zeiss LSM-700 laser scanning confocal microscope (Jena, Germany) running Zen 2010 software (Zeiss, revision 5.5) was used to visualize compound localization in the cells (40x magnification). This was done by visualizing 390 nm Z-sections that were ultimately compiled into a maximum intensity projection using ImageJ (National Institutes of Health, Bethesda, MD, USA). Settings were maintained between treatments so that differences in fluorescence were comparable.

ACKNOWLEDGEMENT
Fluorescent compound (**9**) was gifted by J.J. La Clair in the Chemistry Department at University of California, San Diego.

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