

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

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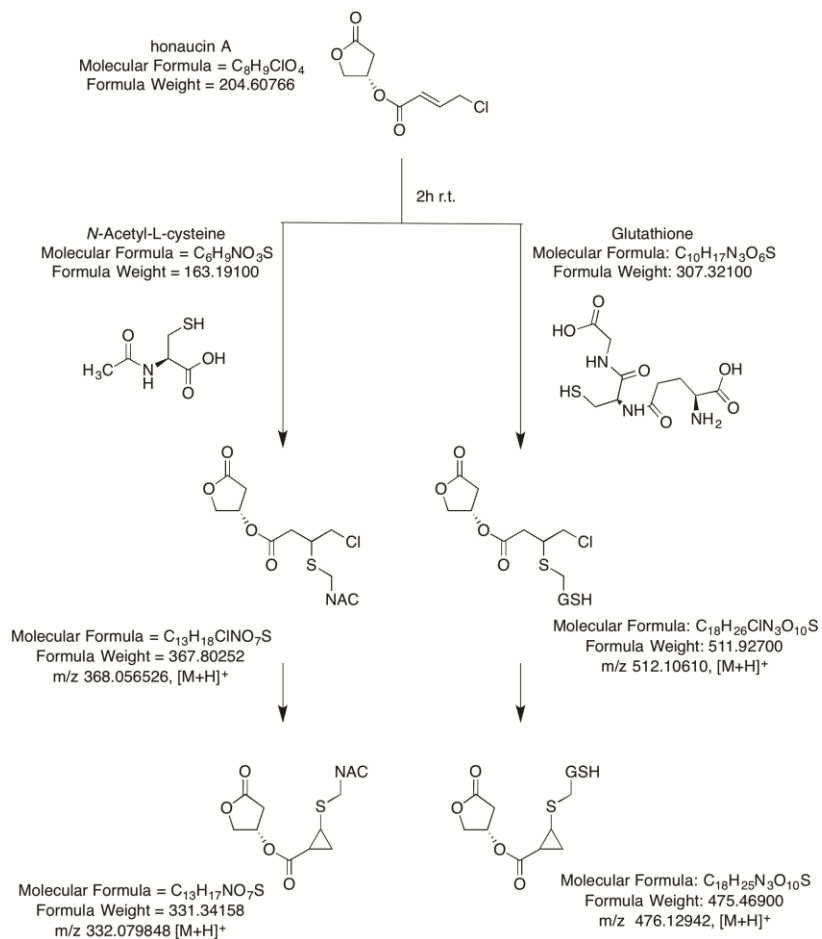
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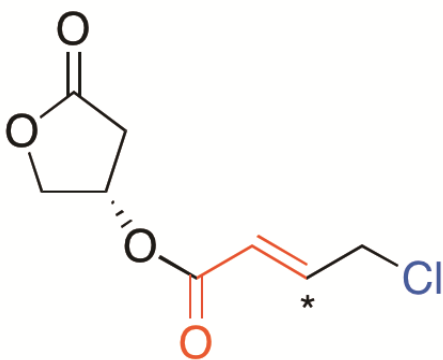
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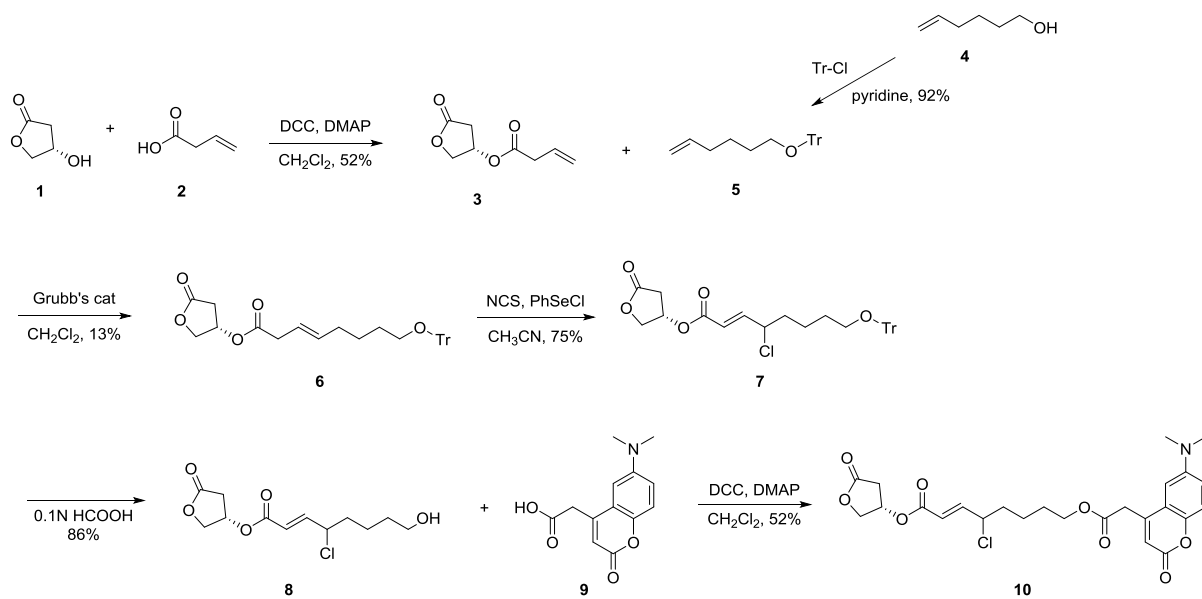
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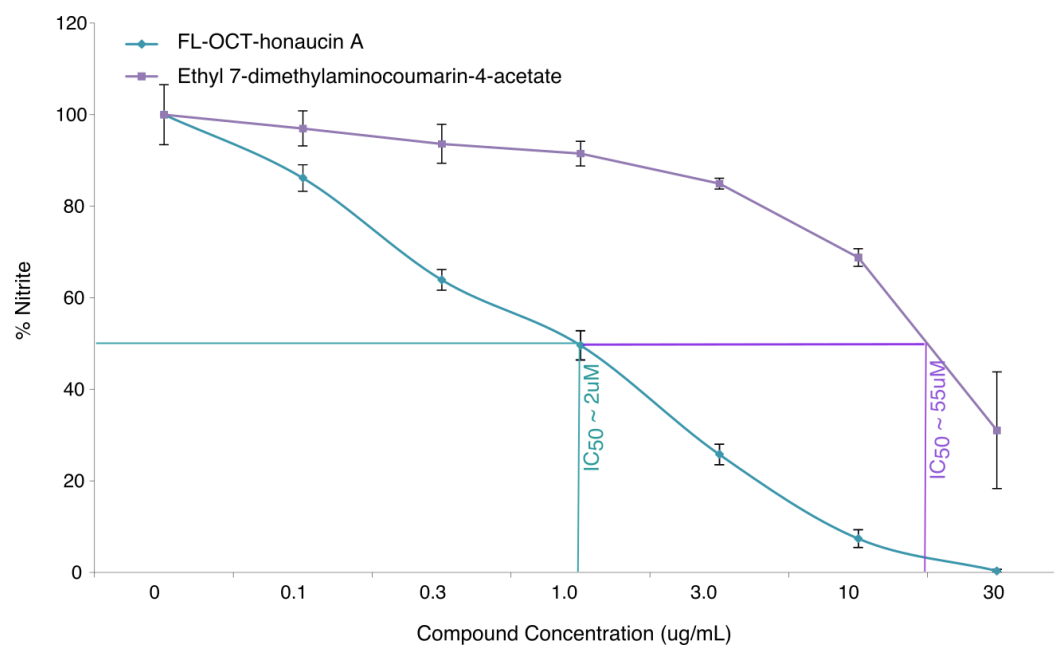
S1. Reaction scheme for honaucin A and *N*-Acetyl-L-cysteine or reduced glutathione showing predicted adducts.



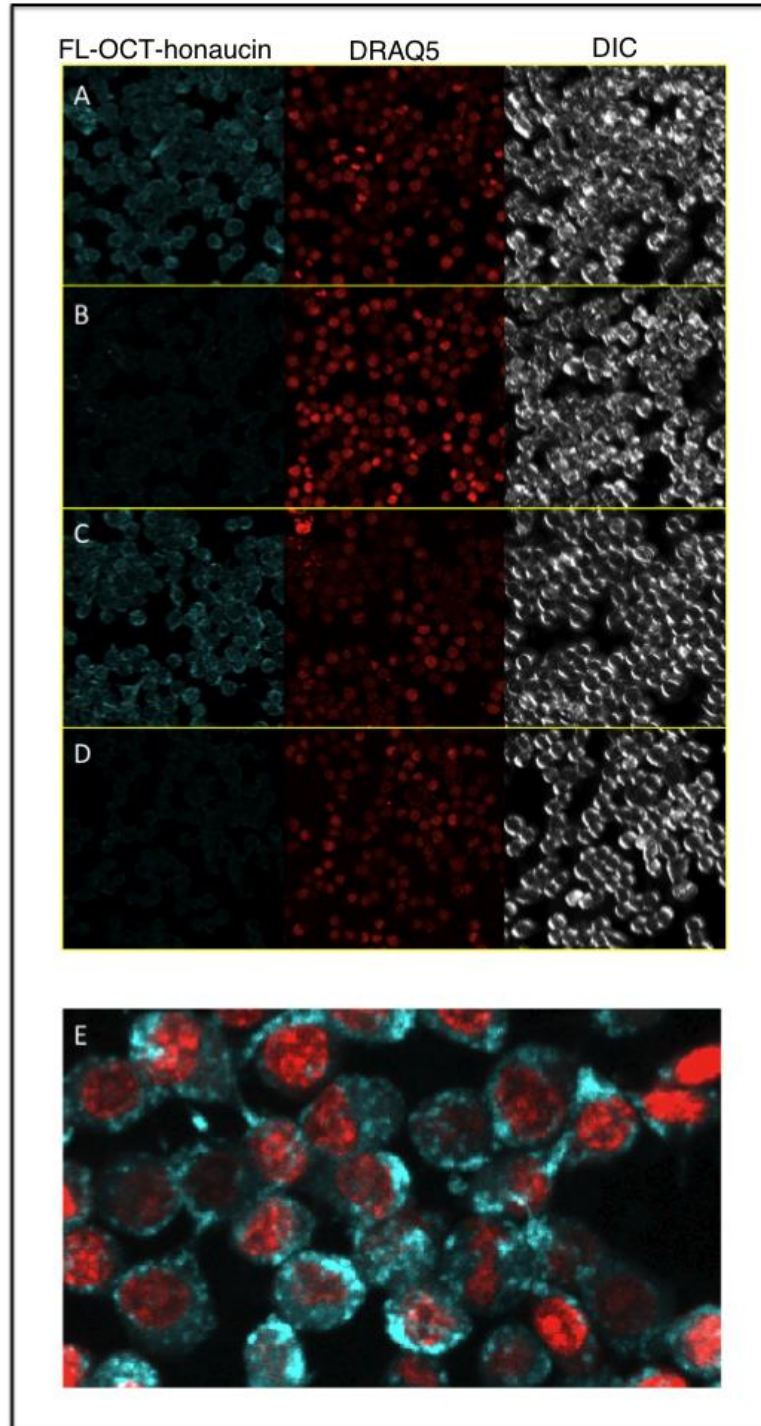
S2. Structure of honaucin A with Michael Acceptor motif (red) and electron withdrawing group (blue) highlighted.



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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 μ M	12 μ M	4 μ M	12 μ M
<i>Akr1b8</i>	aldo-keto reductase family 1, member B8	-	-	-	1.356 (1.66 x 10 ⁻¹⁵)
<i>Blvrb</i>	biliverdin reductase B (flavin reductase (NADPH))	-	-	-	1.050 (3.94 x 10 ⁻¹²)
<i>Btg2</i>	B cell translocation gene 2, anti-proliferative	-	-0.676 (2.31 x 10 ⁻⁰⁸)	-	-
<i>Ccn1l</i>	cyclin L1	-	-0.406 (4.41 x 10 ⁻⁰⁵)	-	-
<i>Ccr12</i>	chemokine (C-C motif) receptor-like 2	-	-1.108 (4.51 x 10 ⁻¹⁶)	-	-
<i>Cd83</i>	CD83 antigen	-	-0.794 (5.49 x 10 ⁻⁰⁹)	-	-
<i>Cflar</i>	CASP8 and FADD-like apoptosis regulator	-	-0.581 (1.73 x 10 ⁻⁰⁶)	-	-
<i>Cxcl2</i>	chemokine (C-X-C motif) ligand 2	-	-0.494 (1.82 x 10 ⁻⁰⁴)	-	-
<i>Cxcl10</i>	chemokine (C-X-C motif) ligand 10	-	-0.670 (1.59 x 10 ⁻⁰⁶)	-	-
<i>Cybb</i>	cytochrome b-245, beta polypeptide	-	-0.325 (7.24 x 10 ⁻⁰⁵)	-	-
<i>Dusp2</i>	dual specificity phosphatase 2	-	-0.749 (6.39 x 10 ⁻⁰⁸)	-	-
<i>Egr2</i>	early growth response 2	-	-0.635 (3.52 x 10 ⁻⁰⁶)	-	-
<i>Epha2</i>	Eph receptor A2	-	-	-	0.726 (9.35 x 10 ⁻⁰⁶)
<i>Errfi1</i>	ERBB receptor feedback inhibitor 1 esterase	-	-0.450 (1.12 x 10 ⁻⁰⁴)	-	-
<i>Esd</i>	D/formylglutathione hydrolase	-	-	-	0.798 (1.05 x 10 ⁻⁰⁴)
<i>Fbxo30</i>	F-box protein 30	-	-	-	0.688 (5.23 x 10 ⁻⁰⁵)
<i>Fgd4</i>	FYVE, RhoGEF and PH domain containing 4	-	-	-	-0.655 (1.13 x 10 ⁻⁰⁴)
<i>Fos</i>	FBJ osteosarcoma oncogene	-	-0.499 (4.42 x 10 ⁻⁰⁶)	-	-
<i>Gabarapl1</i>	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	-	-	-	0.852 (4.69 x 10 ⁻⁰⁵)

<i>Gas7</i>	growth arrest specific 7	-	-	-	-0.699 (8.15 x 10 ⁻⁰⁵)
<i>Gclm</i>	glutamate-cysteine ligase, modifier subunit	-	-	-	1.108 (3.14 x 10 ⁻⁰⁶)
<i>Gpr84</i>	G protein-coupled receptor 84	-	-0.511 (1.29 x 10 ⁻⁰⁴)	-	-
<i>Hmox1</i>	heme oxygenase 1	-	0.890 (4.23 x 10 ⁻¹⁵)	-	1.818 (1.15 x 10 ⁻¹³)
<i>Il1b</i>	interleukin 1 beta	-	-0.736 (1.32 x 10 ⁻⁷)	-	-
<i>Jag1</i>	jagged 1	-	-	-	1.065 (7.53 x 10 ⁻⁰⁷)
<i>Marcks11</i>	MARCKS-like 1	-	-0.495 (3.56 x 10 ⁻⁰⁵)	-	-
<i>Mcl1</i>	myeloid cell leukemia sequence 1	-	-0.431 (1.33 x 10 ⁻⁰⁶)	-	-
<i>Nfkbia</i>	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	-	-0.605 (5.52 x 10 ⁻⁰⁶)	-	-
<i>Nfkbiz</i>	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta	-	-0.978 (8.54 x 10 ⁻²⁷)	-	-
<i>Pid1</i>	phosphotyrosine interaction domain containing 1	-	-	-	-0.775 (2.15 x 10 ⁻⁰⁵)
<i>Ppp1r15a</i>	protein phosphatase 1, regulatory (inhibitor) subunit 15A	-	-0.588 (2.08 x 10 ⁻⁰⁵)	-	-
<i>Prdx1</i>	peroxiredoxin 1	-	-	-	1.802 (9.46 x 10 ⁻²⁰)
<i>Ptgir</i>	prostaglandin I receptor (IP)	-	-	-	1.236 (8.15 x 10 ⁻⁰⁸)
<i>Rassf8</i>	Ras association (RalGDS/AF-6) domain -family (N- terminal) member 8	-	-	-	0.941 (1.02 x 10 ⁻⁰⁵)
<i>Rasgef1b</i>	RasGEF domain family, member 1B	0.416 (1.90 x 10 ⁻⁰⁵)	-1.069 (6.87 x 10 ⁻¹⁸)	-	-
<i>Rusc2</i>	RUN and SH3 domain containing 2	-	-	-	0.984 (1.22 x 10 ⁻⁰⁶)
<i>Sh3bgrl2</i>	SH3 domain binding glutamic acid-rich protein like 2	-	-	-	0.603 (6.27 x 10 ⁻⁰⁵)
<i>Slc11a1</i>	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 / Natural resistance-	-	-	-	0.996 (1.90 x 10 ⁻⁰⁷)

<i>Slc48a1</i>	associated macrophage protein 1 solute carrier family 48 (heme transporter), member 1 / Heme transporter HRG1	-	-	-	0.887 (4.28 x 10 ⁻⁰⁵)
<i>Socs3</i>	suppressor of cytokine signaling 3	0.526 (6.09 x 10 ⁻⁰⁸)	-1.273 (4.55 x 10 ⁻²⁵)	-	-
<i>Sqstm1</i>	sequestosome 1	-	-	-	0.981 (8.77 x 10 ⁻⁰⁵)
<i>Srxn1</i>	sulfiredoxin 1 homolog (S. cerevisiae)	0.492 (1.78 x 10 ⁻¹²)	0.815 (9.68 x 10 ⁻¹⁶)	1.246 (- 2.57 x 10 ⁻¹⁹)	-
<i>Strn</i>	striatin, calmodulin binding protein	-	-0.369 (2.94 x 10 ⁻⁰⁵)	-	-
<i>Tnf</i>	tumor necrosis factor	-	-0.814 (1.21 x 10 ⁻¹¹)	-	-
<i>Tnfaip3</i>	tumor necrosis factor, alpha-induced protein 3	-	-1.278 (7.02 x 10 ⁻²³)	-	-
<i>Tnfsf9</i>	tumor necrosis factor (ligand) superfamily, member 9	-	-0.541 (1.07 x 10 ⁻⁰⁴)	-	-
<i>Tulp2</i>	tubby-like protein 2	-	-0.642 (3.72 x 10 ⁻⁰⁶)	-	-
<i>Txnrd1</i>	thioredoxin reductase 1	-	-	-	0.983 (2.30 x 10 ⁻⁰⁵)
<i>Ubap1</i>	ubiquitin associated protein 1	-	-	-	0.616 (5.24 x 10 ⁻⁰⁵)
<i>Ube2q2</i>	ubiquitin-conjugating enzyme E2Q (putative) 2	-	-	-	0.666 (1.91 x 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-butenic 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 CH₂Cl₂ 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 N₂ 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, H₂O:CH₃CN=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 CHCl₃ (50 mL) and H₂O (20 mL). The organic layer was washed with brine (20 mL), treated with Na₂SO₄, 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 CH₂Cl₂ 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% H₂O in CH₃CN, B=100% CH₃CN, 3:7 for 20 min, 3:7 to 100% CH₃CN 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 CH₃CN (150 mL). To a reaction mixture, *N*-chlorosuccinimide (52.3 mg) in anhydrous CH₃CN 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 N₂ and the residual material was partitioned between Et₂O and H₂O. The Et₂O 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 Et₂O 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 CH₂Cl₂ 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 N₂ 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, H₂O:CH₃CN=7:3, 210 nm) to give a purified compound **10** (4.5 mg, yield 52%).

Compound **3**: ¹H NMR (500 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₈H₁₁O₄ 171.0652).

Compound **5**: ¹H NMR (500 MHz, CDCl₃) 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, CDCl_3) δ 144.7, 139.0, 128.9, 127.9, 127.0, 114.6, 86.5, 63.6, 33.8, 29.7, 25.8; HRESIMS m/z $[\text{M}]^+$ 342.1981 (calcd for $\text{C}_{28}\text{H}_{26}\text{O}$ 342.1978).

Compound 6: ^1H 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 $[\text{M}+\text{Na}]^+$ 507.2143 (calcd for $\text{C}_{31}\text{H}_{32}\text{O}_5\text{Na}$ 507.2142).

Compound 7: ^1H 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 $[\text{M}+\text{Na}]^+$ 541.1753 (calcd for $\text{C}_{31}\text{H}_{31}\text{ClO}_5\text{Na}$ 541.1752).

Compound 8: ^1H 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 $[\text{M}+\text{Na}]^+$ 299.0655 (calcd for $\text{C}_{12}\text{H}_{17}\text{ClO}_5\text{Na}$ 299.0657).

Compound 10: ^1H 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 $[\text{M}+\text{Na}]^+$ 528.1394 (calcd for $\text{C}_{25}\text{H}_{28}\text{ClNO}_8\text{Na}$ 528.1396).

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

A 96-well plate was seeded with RAW 264.7 cells at a density of 5×10^4 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 $^\circ\text{C}$ with 5% CO_2 in order to achieve a confluent cellular monolayer. FI-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 $\mu\text{g}/\text{mL}$. The plate with compounds was incubated for 1 hr at 37 $^\circ\text{C}$ with 5% CO_2 prior to the addition of 10 μL /well bacterial endotoxin yielding a final concentration of 3.0 $\mu\text{g}/\text{mL}$. 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 $^\circ\text{C}$ 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 μL 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-naphthylethylenediamine 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 IC₅₀ values of the compounds.

Cell tracking with a fluorescent honaucin A probe

RAW 264.7 macrophages were seeded at a density of 1x10⁶ cells per milliliter in MatTek (Ashland, MA, USA) 35 mm glass-bottom dishes and allowed to achieve confluency in a CO₂ 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% CO₂ 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

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