Supporting Information for "Unbiased Screening of Marine Sponge Extracts for Anti-Inflammatory Agents Combined with Chemical Genomics Identifies Girolline as an Inhibitor of Protein Synthesis."

Shan-Yu Fung,[1] Vladimir Sofiyev,[2] Julia Schneiderman,[1] Aaron F. Hirschfeld,[1] Rachel E. Victor,[1] Kate Woods,[2] Jeff S. Piotrowski,[4] Raamesh Deshpande,[5] Sheena C. Li,[6] Nicole J. de Voogd,[3] Chad L. Myers,[5] Charlie Boone,[6] Raymond J. Andersen,[2] Stuart E. Turvey[1,*]

[1]Department of Pediatrics, British Columbia Children's Hospital and Child & Family Research Institute, Vancouver, British Columbia, V5Z 4H4, Canada

[2]Department of Chemistry, University of British Columbia, Vancouver, British Columbia, V6T 1Z1, Canada

[3]Netherlands Centre for Biodiversity Naturalis, P.O. Box 9517, 2300 RA, Leiden, The Netherlands

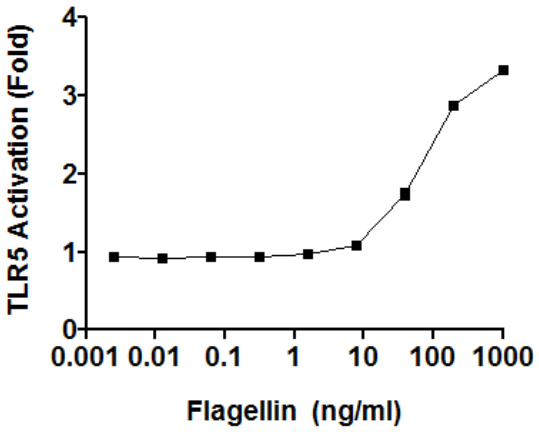
[4]Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI, USA

[5]Department of Computer Science and Engineering, University of Minnesota – Twin Cities, Mineapolis, MN, USA

[6]Department of Molecular Genetics, Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, ON, Canada

Supporting Figures

a



b

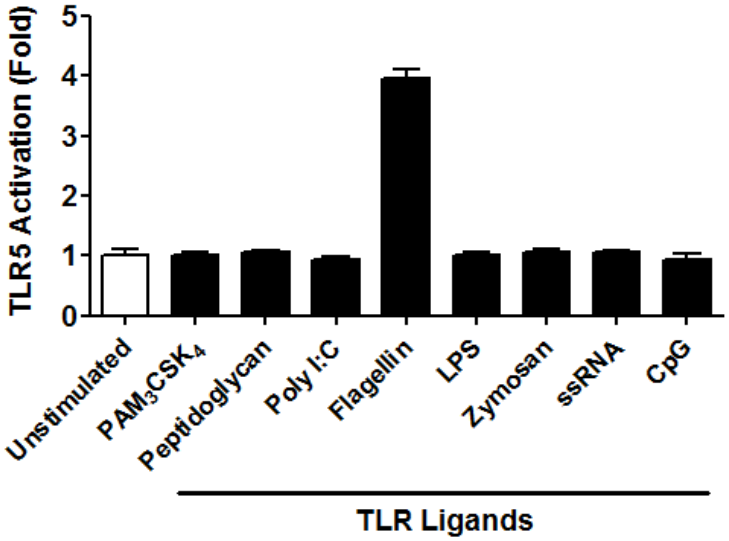
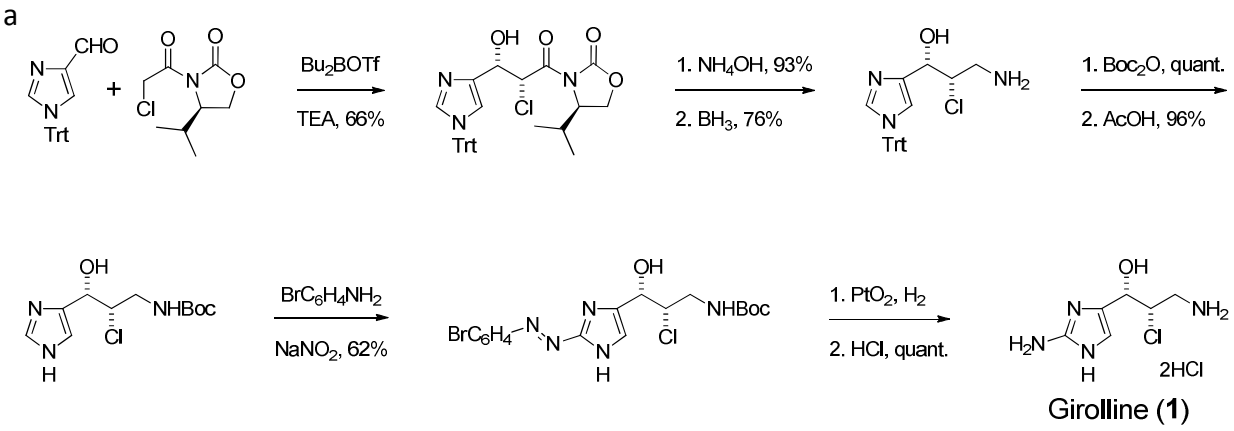


Figure S1. The sensitivity and specificity CHO-TLR5 cells toward flagellin stimulation. (a) TLR5 activation upon stimulation with flagellin at different concentrations. (0.002-1000 ng ml^{-1}). (b) Responses of CHO-TLR5 cells to various TLR ligands. Cells only responded to flagellin stimulation in comparison to the unstimulated control. TLR1/2: Pam_3CSK_4 and peptidoglycan; TLR3: Poly I:C; TLR4: LPS; TLR5: flagellin; TLR2/6: Zymosan; TLR7/8: ssRNA; TLR9: CpG.



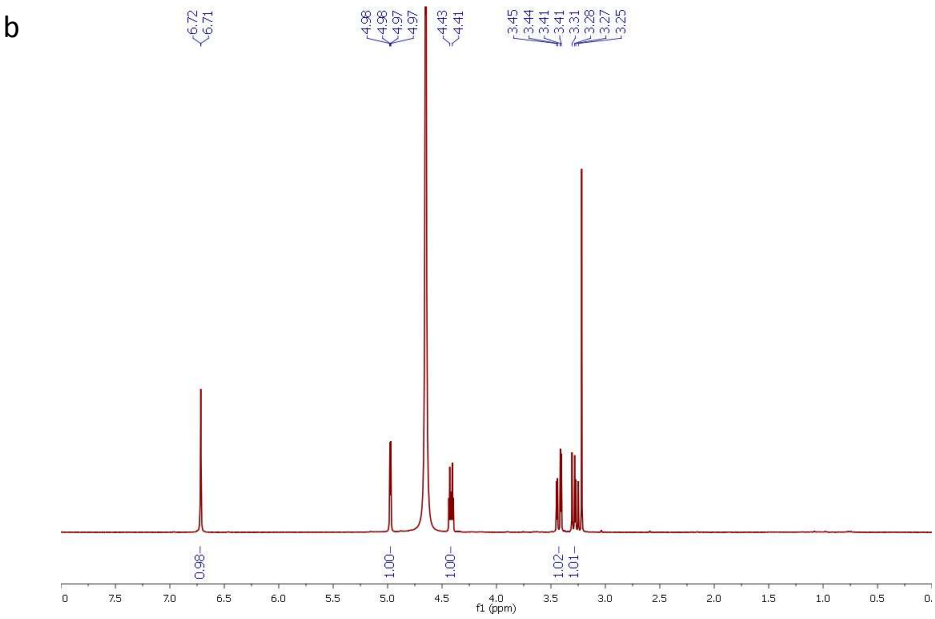


Figure S2. Chemical synthesis of girolline (**1**). (a) Synthesis procedure. (b) Structural characterization by NMR spectroscopy.

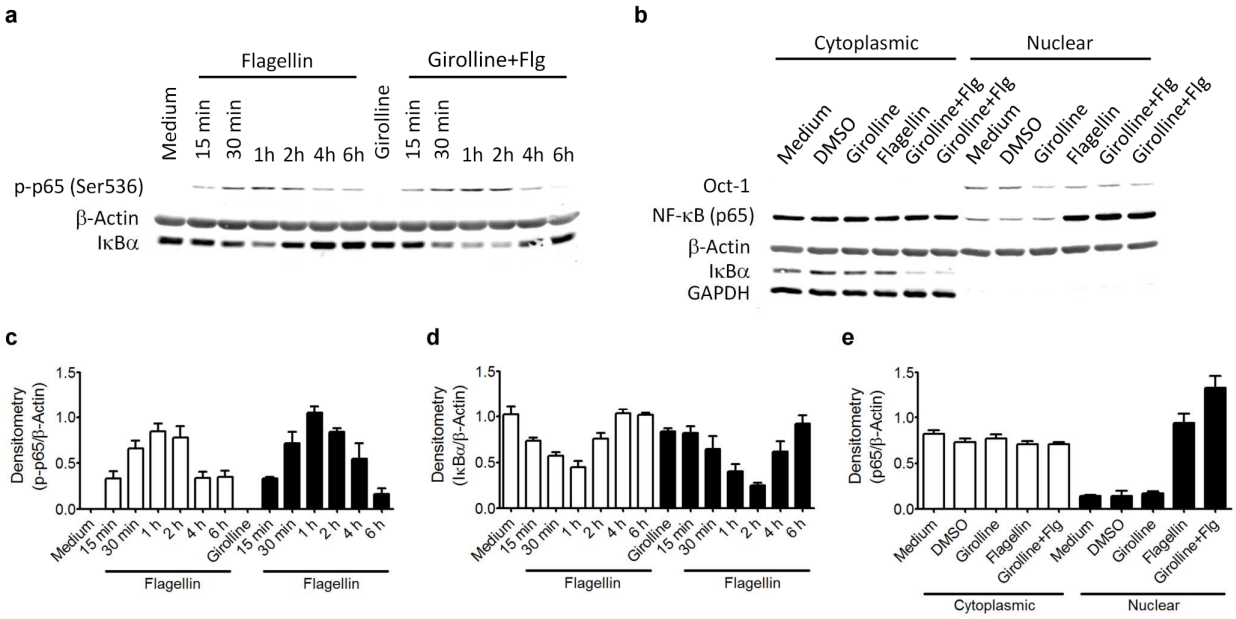


Figure S3. Impact of girolline on NF-κB signaling. (a) Changes of IκBa and phosporylated NF-κB subunit p65 (Ser536) over time with/without girolline pre-treatment. (b) p65 in cytoplasm and nucleus after 2 h flagellin stimulation with/without girolline. GAPDH and Oct-1 were blotted as cytoplasmic and nuclear controls, respectively. Densitometry analysis of p-p65 (c), IκBa (d) and p65 (e); n = 3. Flg: flagellin.

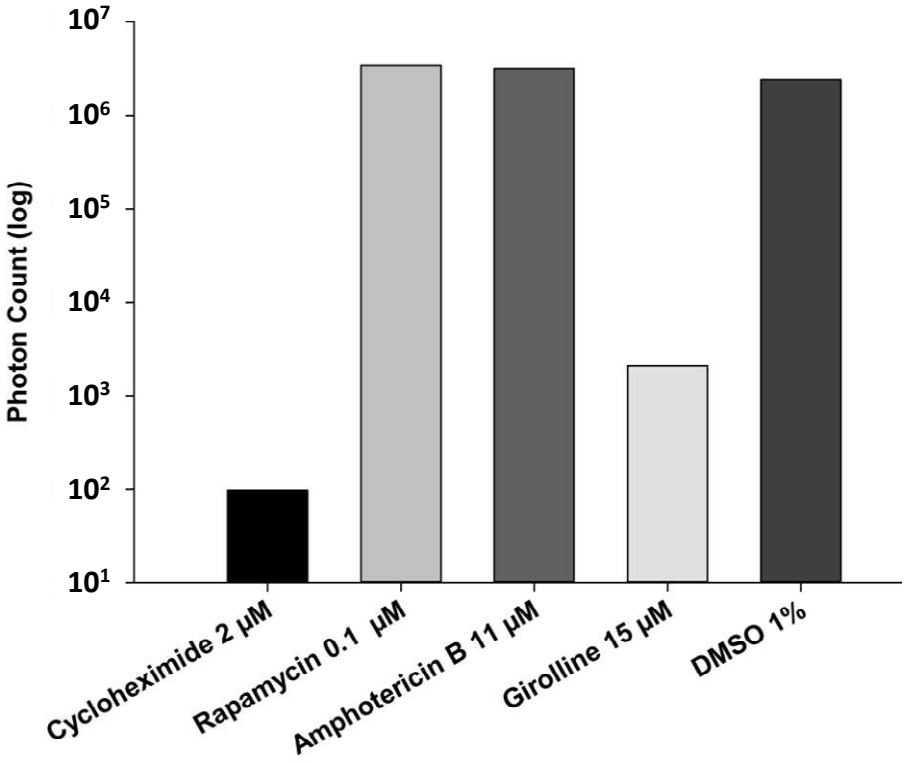


Figure S4. Girolline inhibits mammalian translation. Compared to the solvent control and other compounds, only girolline and the known translation inhibitor cycloheximide inhibited translation of the luciferase reporter (average of 2 replicates). Girolline 15 μM = 2.86 μg ml^{-1}.

Supporting Tables

Table S1. Inhibitory activity of girolline derivatives on TLR5 signaling

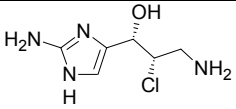
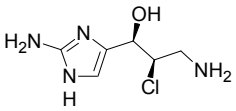
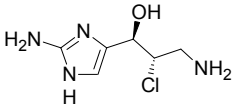
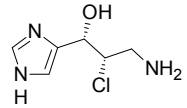
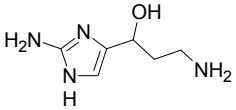
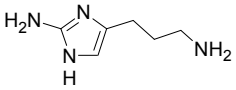
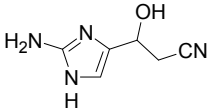
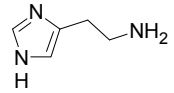
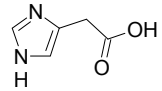
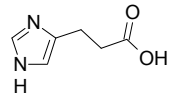
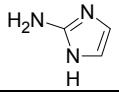
Name	Chemical Structure	IC50 (ng ml^{-1})
Girolline (**1**)		~2
Diastereomer (**2**)		~0.2
Enantiomer (**3**)		~300
Des-amino girolline (**4**)		~5000
Des-chloro girolline (**5**)		~10000
Des-chlorohydroxy girolline (**6**)		No activity
Nitrile (**S4**)		No activity
Histamine (**S5**)		No activity
Imidazole-4-acetic acid (**S6**)		No activity
Imidazole-4-propanoic acid (**S7**)		No activity
2-amino imidazole (**S8**)		No activity

Table S2. List of stimulants used in this study

TLR Ligands	Concentration (ng ml^{-1})	Targets
Pam_3CSK_4	10	TLR2
Poly I:C	100	TLR3
LPS (from *E. coli* K-12)	1	TLR4
Flagellin (from *Salmonella typhimurium*)	100	TLR5
R848	1000	TLR7
TNFα	500	TNFR
IL-1β	500	IL1R

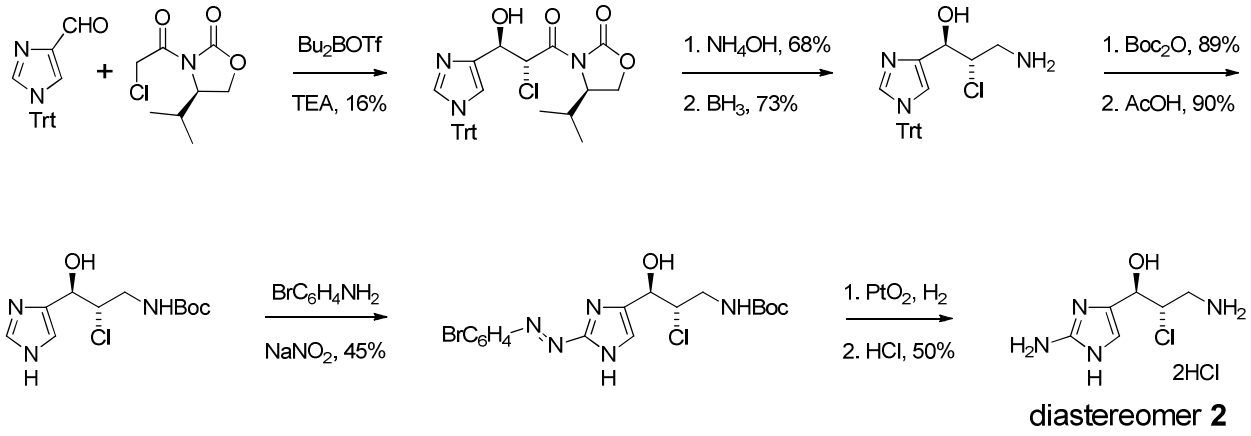
Table S3. Top 10 sensitive deletion mutants of non-essential and essential deletion mutant strains for girolline and des-chloro girolline

Girolline				Des-chloro girolline			
Non-essential	**Z-score**	**Essential**	**Z-score**	**Non-essential**	**Z-score**	**Essential**	**Z-score**
*dph1*Δ	-12.61	*rpb7*Δ	-12.43	*gga1*Δ	-14.28	*arh1*Δ	-5.94
*ygr111w*Δ	-11.03	*pup3*Δ	-11.04	*yhl005c*Δ	-12.74	*ylr132c*Δ	-5.71
*jjj3*Δ	-9.29	*ydl221w*Δ	-6.99	*ygr164w*Δ	-7.67	*crm1*Δ	-4.63
*gga1*Δ	-7.69	*hym1*Δ	-6.43	*rsf1*Δ	-6.83	*rpn3*Δ	-4.25
*dph2*Δ	-6.75	*mas2*Δ	-5.08	*dtd1*Δ	-6.75	*mec1*Δ	-4.11
*rrt2*Δ	-6.29	*rib2*Δ	-4.84	*ydr387c*Δ	-6.29	*pop4*Δ	-4.09
*yor072w*Δ	-6.20	*rsp5*Δ	-4.65	*yhr180w*Δ	-5.79	*afg2*Δ	-4.06
*sqs1*Δ	-6.03	*tub2*Δ	-4.54	*nma111*Δ	-5.72	*smc3*Δ	-3.80
*gal1*Δ	-5.97	*rps13*Δ	-4.48	*yml048wa*Δ	-4.98	*fol1*Δ	-3.65
*vhs3*Δ	-5.68	*ame1*Δ	-4.37	*aqy2*Δ	-4.70	*rpb3*Δ	-3.58

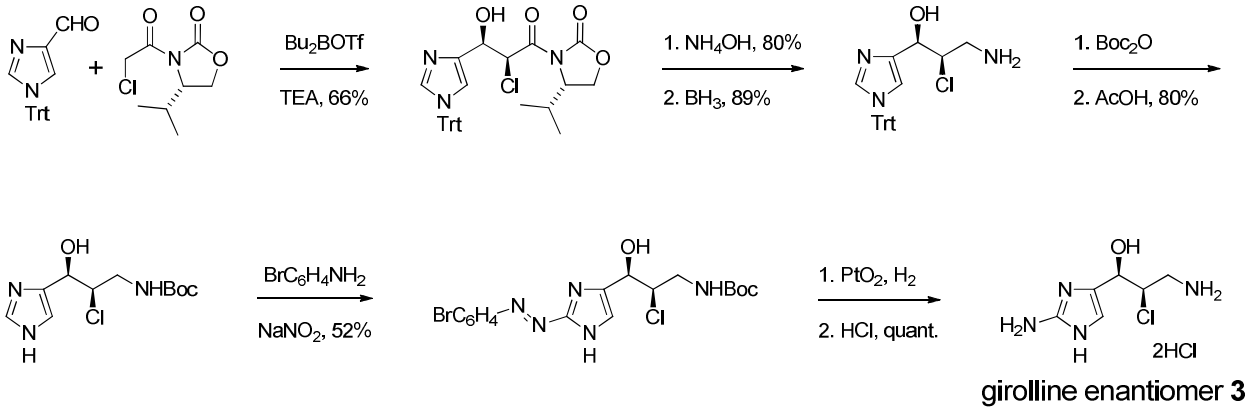
Note: The z-score is a measure of strain abundance in the presence of a drug relative to the solvent control.

Supporting Experimental

Synthetic procedures for the preparation of girolline analogs

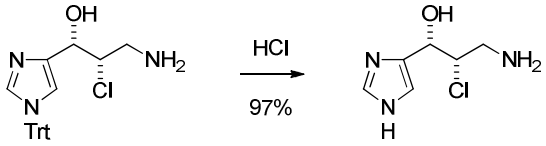


Girolline Diastereomer 2. To a solution of the corresponding azo compound (67.3 mg, 0.353 mmol) in MeOH (8.0 mL) was added PtO_2 (20 mg) and concentrated aqueous HCl (20 µL, 4.8 mmol). The reaction mixture was subjected to hydrogen atmosphere (H_2 balloon) and stirred for 4 h. The reaction mixture was then filtered through a plug of Celite and concentrated. Purification by silica gel chromatography (5:3:1:1 EtOAc:*n*BuOH:H_2O:HCO_2H) followed by Sephadex LH-20 chromatography (MeOH) afforded **2** (23 mg, 55%) as a colorless oil. Data for **2**: R_f 0.10 (5:3:1:1 EtOAc:*n*BuOH:H_2O: HCO_2H); ^{1}H NMR (D_2O, 400 MHz) δ 8.35 (s, 2H, formate), 6.85 (s, 1H), 5.11 (d, 1H, *J* = 3.3 Hz), 4.55 (dt, 1H, J_1 = 9.4 Hz, J_2 = 3.3 Hz), 3.56 (dd, 1H, J_1 = 13.8 Hz, J_2 = 3.3 Hz), 3.41 (dd, 1H, J_1 = 13.8 Hz, J_2 = 9.4 Hz); ^{13}C NMR (D_2O, 100 MHz) δ 147.2, 125.2, 111.1, 66.3, 60.1, 43.0; HRMS (ESI) calculated for $C_6H_{12}ClN_4O$ $(M+H)^+$: 191.0700, found: 191.0711, $[\alpha]_D^{23}$ = +29.1° (c = 1.2, $CHCl_3$).



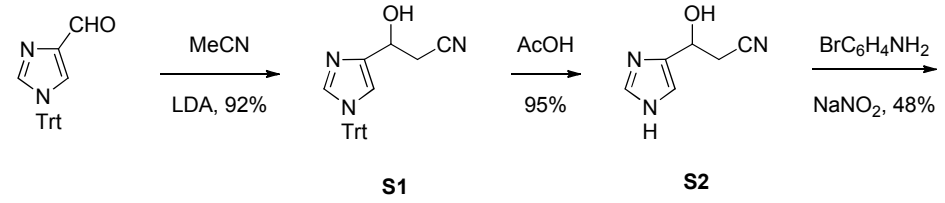
Girolline enantiomer 3. To a solution of the corresponding azo compound (77.6 mg, 0.169 mmol) in MeOH (8.0 mL) was added PtO_2 (20 mg) and concentrated aqueous HCl (20 µL, 3.0 mmol). The reaction mixture was subjected to hydrogen atmosphere (H_2 balloon) and stirred for 4 h. The reaction mixture was then filtered through a plug of Celite and concentrated. Purification by silica gel chromatography (5:3:1:1 EtOAc:*n*BuOH:H_2O:HCO_2H) followed by Sephadex LH-20 chromatography (MeOH) afforded **2** (47

mg, 100%) as a colorless oil. Data for **2**: R_f 0.08 (5:3:1:1 EtOAc:*n*BuOH:H_2O: HCO_2H); ^{1}H NMR (D_2O, 400 MHz) δ 8.30 (s, 3H, formate), 6.83 (s, 1H), 5.10 (d, 1H, *J* = 2.6 Hz), 4.54 (dt, 1H, J_1 = 9.4 Hz, J_2 = 3.3 Hz), 3.55 (dd, 1H, J_1 = 13.8 Hz, J_2 = 3.3 Hz), 3.39 (dd, 1H, J_1 = 13.8 Hz, J_2 = 9.4 Hz); ^{13}C NMR (D_2O, 100 MHz) δ 150.2, 123.8, 109.5, 64.7, 58.4, 41.3; HRMS (ESI) calculated for $C_6H_{12}ClN_4O$ $(M+H)^+$: 191.0700, found: 191.0697, $[\alpha]_D^{23}$ = -12.0° (c = 1.0, $CHCl_3$).

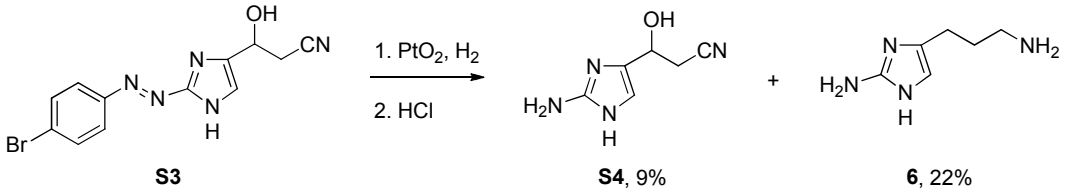


des-amino girolline **4**

Des-amino analog 4. To a solution of trityl-imidazole (46.7 mg, 0.112 mmol) in *n*PrOH (3.0 mL) was added concentrated HCl (20 µL, 4.8 mmol) and the resulting mixture was stirred at 100 °C for 1 h. After being cooled to ambient temperature, the reaction mixture was concentrated, diluted with EtOAc (30 mL), washed with water (20 mL), and concentrated affording **4** (19.0 mg, 97%) as a colorless oil. Data for **4**: R_f 0.05 (5:3:1:1 EtOAc:*n*BuOH:H_2O:HCO_2H); ^{1}H NMR (MeOD, 400 MHz) δ 8.10 (s, 1H), 7.30 (s, 1H), 5.13 (d, 1H, *J* = 3.5 Hz), 4.53 (m, 1H), 3.48 (dd, 1H, J_1 = 13.6 Hz, J_2 = 3.4 Hz), 3.34 (dd, 1H, J_1 = 13.6 Hz, J_2 = 8.8 Hz); ^{13}C NMR (MeOD, 100 MHz) δ 136.7, 134.9, 116.2, 68.4, 61.0, 42.8; HRMS (ESI) calculated for $C_6H_{11}ClN_3O$ $(M+H)^+$: 176.0591, found: 176.0588.



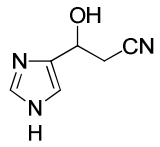
S1 **S2**



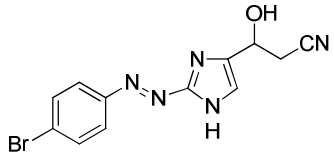
S3 **S4**, 9% **6**, 22%

Trityl-nitrile S1. To a solution of MeCN (50 µL, 0.95 mmol) in THF (2.0 mL) at –78 °C was added *n*BuLi (1.6 M in hexanes, 0.60 mL, 0.96 mmol). After the reaction mixture was stirred for 30 min, 1-triphenylmethyl-4-formyl-imidazole (295 mg, 0.872 mmol) was added dropwise as a solution in THF (3.0 mL). Following another 30 min at –78 °C, the reaction mixture was warmed up to RT, quenched with ice chunks and aqueous HCl solution (2.0 M, 0.10 mL). The mixture was then diluted with EtOAc (30 mL), washed with saturated aqueous $NaHCO_3$ (10 mL), brine (10 mL), and concentrated. Purification by silica gel chromatography (1:1 [90:10:0.6:0.6 CH_2Cl_2:MeOH:H_2O:NH_4OH]:CH_2Cl_2) afforded **S1** (304 mg, 92%) as a colorless oil. Data for **S1**: R_f 0.3 (90:10:0.6:0.6 CH_2Cl_2:MeOH:H_2O:NH_4OH); ^{1}H NMR (CDCl3, 400 MHz) δ 7.49 (s, 1H), 7.34 (m, 9H), 7.17 (m, 6H), 6.92 (s, 1H), 4.98 (t, 1H, *J* = 6.0 Hz), 2.88 (d, 2H, *J* = 6.3 Hz); ^{13}C

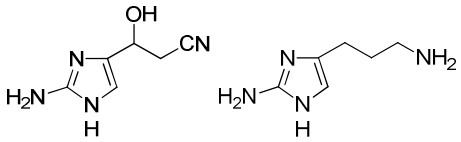
NMR ($CDCl_3$, 100 MHz) δ 142.0, 141.2, 138.9, 129.6, 128.2, 128.1, 118.8, 117.7, 75.6, 64.2, 26.2; HRMS (ESI) calculated for $C_{25}H_{22}N_3O$ $(M+H)^+$: 380.1763, found: 380.1754.



Free imidazole-nitrile S2. To a solution of **S1** (947 mg, 2.50 mmol) in *n*PrOH (16.0 mL) was added acetic acid (2.0 mL) and the resulting mixture was stirred at 100 °C for 2 h. After being cooled to ambient temperature, the reaction mixture was concentrated. Purification by silica gel chromatography (90:10:0.6:0.6 CH_2Cl_2:MeOH:H_2O:NH_4OH → 1:9 MeOH:[90:10:0.6:0.6 CH_2Cl_2:MeOH:H_2O:NH_4OH]) afforded **S2** (325 mg, 95%) as a white crystalline solid. Data for **S2**: R_f 0.07 (90:10:0.6:0.6 CH_2Cl_2:MeOH:H_2O:NH_4OH); ^{1}H NMR (MeOD, 400 MHz) δ 7.67 (s, 1H), 7.12 (s, 1H), 5.00 (t, 1H, J = 5.4 Hz), 2.96 (ddd, 1H, J_1 = 16.7 Hz, J_2 = 5.1 Hz, J_3 = 1.4 Hz), 2.89 (ddd, 1H, J_1 = 16.7 Hz, J_2 = 6.6 Hz, J_3 = 1.3 Hz); ^{13}C NMR (MeOD, 100 MHz) δ 141.6, 136.8, 119.2, 116.3, 65.4, 26.9; HRMS (ESI) calculated for $C_6H_8N_3O$ $(M+H)^+$: 138.0667, found: 138.0668.

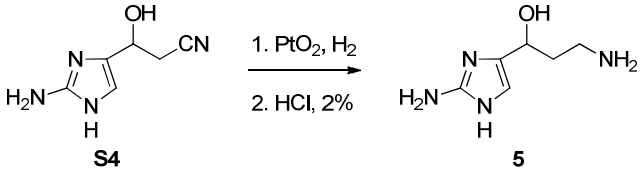


Azo-nitrile S3. To a solution of 4-bromoaniline (110 mg, 0.639 mmol) in H_2O (0.3 mL) and aqueous HCl (2.0 M, 2.0 mL) at 0 °C was added $NaNO_2$ (49.0 mg, 0.710 mmol) and the reaction mixture was stirred for 10 min. Solution of **S2** (79.4 mg, 0.579 mmol) in MeOH (18.0 mL) and Na_2CO_3 (10%, 8.0 mL) was then added all at once and the resulting mixture was stirred at 0 °C for 15 min and warmed up to RT. After 30 min at RT, the reaction mixture was diluted with EtOAc (100 mL), washed with water (20 mL), brine (10 mL), and concentrated. Purification by silica gel chromatography (1:1 [90:10:0.6:0.6 CH_2Cl_2:MeOH:H_2O:NH_4OH]: CH_2Cl_2) afforded **S3** (88 mg, 48%) as an orange solid. Data for **S3**: R_f 0.38 (90:10:0.6:0.6 CH_2Cl_2:MeOH:H_2O: NH_4OH); ^{1}H NMR (MeOD, 400 MHz) δ 7.77 (m, 2H), 7.66 (m, 2H), 7.36 (s, 1H), 5.09 (t, 1H, J = 5.8 Hz), 3.08 (dd, 1H, J_1 = 16.7 Hz, J_2 = 5.1 Hz), 3.00 (dd, 1H, J_1 = 16.7 Hz, J_2 = 6.6 Hz); ^{13}C NMR (MeOD, 100 MHz) δ 155.4, 152.6, 133.8, 133.5, 127.3, 125.6, 125.1, 119.0, 65.5, 26.9; HRMS (ESI) calculated for $C_{12}H_{11}{}^{79}BrN_5O$ $(M+H)^+$: 320.0147, found: 320.0156.



Nitrile analog S4 and **des-chlorohydroxy analog 6**. To a solution of azo-nitrile **S3** (88 mg, 0.275 mmol) in MeOH (10.0 mL) was added PtO_2 (20 mg) and concentrated aqueous HCl (20 µL, 4.8 mmol). The reaction mixture was subjected to hydrogen atmosphere (H_2 balloon) and stirred for 4 h. The reaction mixture was then filtered through a plug of Celite and concentrated. Separation by silica gel chromatography

(5:3:1:1 EtOAc:*n*BuOH:H_2O:HCO_2H) as well as purification by Sephadex LH-20 chromatography (MeOH) afforded **S4** (3.7 mg, 9%) as a colorless oil along with previously described **6** (7.6 mg, 22%). Data for **S4**: R_f 0.05 (5:3:1:1 EtOAc:*n*BuOH:H_2O:HCO_2H); ^{1}H NMR (MeOD, 400 MHz) δ 6.71 (s, 1H), 4.88 (t, 1H, obscured by the solvent peak), 2.90 (m, 2H); ^{13}C NMR (MeOD, 100 MHz) δ 132.2, 122.2, 118.6, 111.1, 63.6, 26.4; HRMS (ESI) calculated for $C_6H_9N_4O$ $(M+H)^+$: 153.0776, found: 153.0774.



S4 **5**

Des-chloro analog 5. To a solution of azo-nitrile **S4** (193 mg, 0.603 mmol) in MeOH (10.0 mL) was added PtO_2 (40 mg) and concentrated aqueous HCl (20 μL, 4.8 mmol). The reaction mixture was subjected to hydrogen atmosphere (H_2 balloon) and stirred for 4 h. The reaction mixture was then filtered through a plug of Celite and concentrated. Purification by silica gel chromatography (5:3:1:1 EtOAc:*n*BuOH:H_2O:HCO_2H) followed by Sephadex LH-20 chromatography (MeOH) afforded **5** (1.6 mg, 2%) as a colorless oil. Data for **5**: R_f 0.05 (5:3:1:1 EtOAc:*n*BuOH:H_2O: HCO_2H); ^{1}H NMR (MeOD, 400 MHz) δ 6.66 (s, 1H), 4.74 (t, 1H, *J* = 6.6 Hz), 3.10 (m, 2H), 2.07 (q, 2H, *J* = 6.8 Hz); ^{13}C NMR (MeOD, 100 MHz) δ 149.8, 129.2, 110.9, 66.4, 42.0, 37.4; HRMS (ESI) calculated for $C_6H_{13}N_4O$ $(M+H)^+$: 157.1089, found: 157.1084.

Immunoblotting

The primary antibodies for immunoblotting analysis including phospho-NF-κB p65, IκBα, β-actin, GAPDH, Oct-1 and Histone H3 were from Cell Signaling Technology (Danvers, MA), whereas the NF-κB p65 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody conjugated with the infrared dye (IRDye® 800 CW) was purchased from LI-COR Biosciences (Lincoln, NE).

Immunoblotting experiments were conducted following the procedures described previously (*1, 2*). Briefly, to generate total cell lysates, cells were harvested in RIPA lysis buffer (50 mM Tris HCl, 150 mM NaCl, 2 mM EGTA and EDTA, and 1% TrionX-100 at pH 7.5) with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Nepean, ON, Canada). After 10 min incubation at 4°C while gently shaking, cells were disrupted by sonication (Misonix S-4000 with indirect horn, Qsonica LLC, Newtown, CT) at 30 amplitude for 30 s (with 10 s off every 10 s) in a ice-cold water bath. Samples were centrifuged at 14000 rpm for 10 min at 4°C, and the supernatants were collected. For nuclear and cytoplasmic extraction, a NE-PER® extraction kit (Thermo Scientific) was used following the manufacture's instruction. The total protein concentration of the cell lysates was determined using the modified Branford assay (Coomassie

Plus Assay Kit, Thermo Scientific) and the concentration was adjusted accordingly. SDS-PAGE (10%) was performed to separate proteins, which were then transferred onto a PVDF membrane (Immobilon-FL, EMD Millipore, Billerica, MA). The membranes were blocked at room temperature for 1 h and blotted with various primary antibodies overnight at 4°C. The membranes were subsequently blotted with fluorescently-labelled secondary antibodies for 1 h at room temperature and imaged by a LI-COR Odyssey infrared imaging system (LI-COR Bioscience). The blocking and blotting steps were performed in 1x tris-buffered saline (TBS, G-Biosciences, St. Louis, MO) containing 5% bovine serum albumin (BSA) and 0.1% TWEEN 20 (EMD Millipore). The densitometry was obtained using the ImagJ freeware.

Translation inhibition assay

A luciferase-based, rabbit reticulocyte assay (Promega L4540) was conducted to test if Girolline inhibits translation as predicted. Rabbit reticulocytes were incubated in the presence of a compound and luciferase RNA for 90 minutes at 30°C. Detection of functional, translated luciferase quantified in a luminometer by adding 2.5 µl of the cell lysate reaction to 50 µL of luciferase assay reagent. The known translation inhibitor cycloheximide was used as a positive control. Rapamycin and amphotericin B, which do not target protein translation, were employed as negative controls, while DMSO as the solvent control.

References

1. Fung, S. Y., Oyaizu, T., Yang, H., Yuan, Y., Han, B., Keshavjee, S., and Liu, M. (2011) The potential of nanoscale combinations of self-assembling peptides and amino acids of the Src tyrosine kinase inhibitor in acute lung injury therapy, *Biomaterials 32*, 4000-4008.
2. Tang, A., Sharma, A., Jen, R., Hirschfeld, A. F., Chilvers, M. A., Lavoie, P. M., and Turvey, S. E. (2012) Inflammasome-Mediated IL-1beta Production in Humans with Cystic Fibrosis, *PLoS One 7*, e37689.