Inhibition of Hypoxia Inducible Factor-2 Transcription: Isolation of Active Modulators from Marine Sponges.

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S1. Sponge collection details.

The sponge *Aaptos aaptos* (0M9I5552) was collected by SCUBA in March 2003 by Steve Oakley (Coral Reef Research Foundation) at Luconia (05° 39.60′ N, 112° 27.00′ E), NW of Miri, Sarawak, Malaysia and identified by John N. A. Hooper (Queensland Museum, Brisbane, Australia).

The sponge *Amphimedon* sp. (0CDN481) was collected by SCUBA in August 1992 by Patrick Colin (Coral Reef Research Foundation) from reefs at the mouth of the east mangrove lagoon at Chuuk Lagoon (07° 22.93′ N, 151° 53.90′ E), Dublon Island, Chuuk State, Caroline Islands. Michelle Kelly (National Institute of Water and Atmospheric Research (NIWA), Auckland, New Zealand) determined the taxonomy of the sponge.

The sponge *Xestospongia* sp. (0CDN7718) was collected by SCUBA in November 2000, by Patrick Colin (Coral Reef Research Foundation) on the northern slope of Aore Island on the south side of the segond channel (15° 32.55′ S, 167° 12.94′ E), Espiritu Santo, Vanuatu. Michelle Kelly (NIWA, Auckland, New Zealand) determined the taxonomy of the sponge.

The sponge *Haliclona velinea* (0M9I5016) was collected by SCUBA in January 2003, by Steve Oakley (Coral Reef Research Foundation) from the reef 10 km offshore of Miri Island (04° 20.67′ N, 113° 53.76′ E), Sarawak, Malaysia. Lori J.B. Colin (Coral Reef Research Foundation, Koror, Palau) determined the taxonomy of the sponge.

The sponge *Hyrtios reticulatus* (0M9H2286) was collected by SCUBA in August 2002 by Belinda Alvarez de Glasby (Museum and Art Gallery of the Northern Territory) from 500 m SE of the east arm of the wharf, 5.5 km SE of Darwin City (12° 29.86′ S,

130° 53.14′ E), Darwin Harbor, Australia. Patricia Berquist (University of Auckland, deceased) determined the taxonomy of the sponge.

The sponge *Kirkpatrickia variolosa* (Q66C3083) was collected by SCUBA in October 1989 by Peter Murphy (SeaPharm, Inc) from the north jetty at McMurdo Base (77° 51.0′ S, 166° 39.0′ E), Cape Armitage, Ross Island, Antarctica. Paul K. Dayton (Scripps Institution of Oceanography, San Diego, CA) determined the taxonomy of the sponge.

The sponge *Haliclona* sp. (Q66C334) was collected by SCUBA in February 1987 by Peter Murphy (SeaPharm, Inc) from the southern tip of Bowden Reef (19° 3.15′ S, 147° 55.6′ E), Great Barrier Reef, Australia. Jane Fromont (Western Australian Museum, Perth, Australia) determined the taxonomy of the sponge.

The sponge *Petrosia* sp. (0M9H2607) was collected by SCUBA in March 2004 by Belinda Alvarez de Glasby (Museum and Art Gallery of the Northern Territory) from 200 m off the northern headland at the east end of Wigram Island (11° 45.82′ S, 136° 31.9′ E) Northern Territory, Australia. Belinda Alvarez de Glasby (Museum and Art Gallery of the Northern Territories, Darwin, Australia) determined the taxonomy of the sponge.

The sponge *Lendenfeldia chondrodes* (0M9H2352) was collected by SCUBA in May 2003 by Belinda Alvarez de Glasby (Museum and Art Gallery of the Northern Territory) 3 km SSE of Rankin Point (12° 42.19′ S, 130° 35.43′ E), SE Cox Peninsula, Bynoe Bay, The Northern Territory, Australia. Patricia Berquist (University of Auckland, deceased) determined the taxonomy of the sponge.

The sponge *Fascaplysinopsis* sp. (0M9I5678) was collected by SCUBA in April 2003 by Steve Oakley (Coral Reef Research Foundation) offshore of the Sibuti River (04° 3.60′ N, 113° 41.70′ E), Sarawak, Malaysia. John N. A. Hooper (Queensland Museum, Brisbane, Australia) determined the taxonomy of the sponge.

S2. Isolation methods for compounds **1-12.**

Aaptamine (1):[11] A portion of the organic extract (1.02 g) was dissolved in 9:1 MeOH/H_2O (200 mL) and taken through a solvent-solvent partition scheme that concentrated the activity in the H_2O soluble fraction. These materials (600 mg) were then permeated through a Sephadex LH-20 column (2.5 × 90 cm) eluting with MeOH/H_2O (9:1) which afforded 65 eight mL fractions that were combined into eight fractions (A-G) based on the resulting UV trace monitored at 254 nm. Fraction C (121.3 mg) was further processed through a vacuum C_{18} reverse phase column (5 cm × 10 cm). Fractions were eluted with a step-wise gradient from H_2O (100%) to MeOH (100%). Aaptamine (**1**, 7.3 mg, 0.71 % crude extract) eluted as a pure compound in fraction B (9:1 H_2O/MeOH).

Mimosamycin (2):[12-13] A portion of the organic extract (500 mg) was subjected to a solvent-solvent partitioning scheme, concentrating the HIF activity into the EtOAc fraction (104.6 mg). Size exclusion chromatography of the EtOAc fraction on Sephadex LH-20 (2.5 × 90 cm) using hexane/CH_2Cl_2/MeOH (2:5:1) yielded 10 fractions (A-J). Fraction E was purified by HPLC using a Rainin Dynamax C_{18} column (8 μ, 1 × 25 cm) employing a gradient of 50% CH_3CN/50% H_2O (+0.1% AcOH) to 80% CH_3CN at 4.5 mL/min over 17 min to yield mimosamycin (**2**, 0.7 mg, 0.14% crude extract).

Xestoquinone (3):[14] A portion of the organic extract (500 mg) of *Xestospongia* sp. was separated into five fractions on a vacuum Diol column (5 g) eluted with the following solvent combinations: hexane/EtOAc (9:1, A), CH_2Cl_2/EtOAc (20:1, B), EtOAc (100%, C), EtOAc/MeOH (1:1, D) and MeOH (100%, E). Upon evaporation of the solvents, the B fraction (72 mg) was further separated on a Sephadex LH-20 column (2.5 × 90 cm) using hexane/CH_2Cl_2/MeOH (2:5:1). The major peak observed in the chromatogram was

separated further by HPLC on Diol (5 μ, 1 × 25 cm, hexane/EtOAc, 80:20, 2 mL/min). Finally, HPLC purification on Diol (5 μ, 1 × 25 cm, EtOAc 100%; 2 mL/min) provided compound **3** (8 mg, 12.5% crude extract).

Compounds 4[15] **and 5:**[16] A portion of the organic extract (311 mg) was separated using a Diol SPE cartridge (2 g), eluting with hexane/CH_2Cl_2 (9:1, A), CH_2Cl_2/EtOAc (20:1, B), EtOAC (100%, C), EtOAc/MeOH (5:1, D) and MeOH (100%, E) in a step-wise fashion. The materials in fractions B-D were very similar by NMR and were therefore combined and further processed by a reverse phase C_4 flash column (1 × 20 cm) eluted with MeOH/H_2O (8:2), MeOH (100%) and CH_2Cl_2 (100%). The MeOH/H_2O and MeOH fractions were further processed through a Sephadex LH-20 column (2.5 × 90 cm) eluting with MeOH/CH_2Cl_2 (1:1) which separated the materials into 2 fractions based on color and UV monitoring of the eluent at 254 nm. The early eluting, red fraction was further fractionated by Sephadex LH-20 column (2.5 × 80 cm) eluting with MeOH (100%) to yield compound **5**, a 2:1 mixture of isomers of *N*-formyl-1,2-dihydrorenierone (2.1 mg, 0.68% crude extract) and a fraction that yielded compound **4**, *O*-demethylrenierone (1.4 mg, 0.45% crude extract) after reverse phase C_{18} flash chromatography using MeOH/H_2O (6:4).

Puupehenone (6):[17-19] A portion of the organic extract (181 mg) was separated by two Diol SPE cartridges (2 g each), and the equivalent fractions were combined to give five total fractions: hexane/CH_2Cl_2 (9:1, A), CH_2Cl_2/EtOAc (20:1, B), EtOAc (100%, C), EtOAc/MeOH (5:1, D), and MeOH (100%, E). Size exclusion chromatography of the active fraction B on Sephadex LH-20 (2.5 × 90 cm) using hexanes/CH_2Cl_2/MeOH (2:5:1) yielded 10 fractions (A-J). The active fraction J was chromatographed on phenyl resin (5

× 1.5 cm) using a step gradient of 100% H_2O (+ 0.1% TFA) to 100% MeOH in 25% steps to yield puupehenone (**6**, 21.0 mg, 11.6% crude extract). $[\alpha]_D^{25}$ +282 (*c* 0.33, CH_2Cl_2), Lit. $[\alpha]_D^{25}$ +315 (*c* 1.64, CCl_4).[18]

Variolin B (7):[20] A portion of the organic extract (300 mg) was fractionated on pre-packed Diol SPE cartridges (100 mg of extract per 500 mg cartridge) by the elution of solvents as follows : hexane/EtOAc (9:1, A), CH_2Cl_2/EtOAc (20:1, B), EtOAc (100%, C), EtOAc/MeOH (1:1, D), MeOH (100%, E). Fractions B, C and D were combined (36 mg) and further separated on a Sephadex LH-20 column (2.5 × 90 cm) using hexane/CH_2Cl_2/MeOH (2:5:1) which afforded compound **7** (1.6 mg, 0.53% crude extract).

Haliclonadiamine (8):[21-22] A portion of the organic extract (208 mg) was subjected to a solvent-solvent partitioning scheme that concentrated the activity in the water-soluble fraction (185.1 mg). Size exclusion chromatography of the active material on Sephadex LH-20 (2 x 95 cm) with MeOH/H_2O (9:1) provided 4 fractions (A-D). Fraction B was further purified by C_{18} VLC (1 × 10 cm) eluting with a gradient from 100% H_2O to 100% MeOH. Final purification was achieved by reverse phase HPLC (Rainin Dynamax, C_{18}, 8 μ, 2 × 25 cm) eluting with a 65% - 50% CH_3CN/H_2O-0.05% TFA gradient (8 mL/min) to give 4.1 mg of haliclonadiamine (**8**, 1.97% crude extract). $[\alpha]_D^{25}$ -40.7 (*c* 0.68, MeOH), Lit. $[\alpha]_D^{25}$ -18.2 (*c* 1.3, MeOH).[21]

Neopetroformyne A (9):[23] A portion of the organic extract (1 g) was subjected to a solvent-solvent partitioning scheme that concentrated the activity in the MeOtBu-soluble fraction (185.1 mg). Size exclusion chromatography of the active MeOtBu material on Sephadex LH-20 (2 x 85 cm) with CH_2Cl_2/MeOH (1:1) provided 5 fractions (A-E).

Fraction B was further purified by HPLC on a Rainin Dynamax CN (10 μ, 2 x 25 cm) column eluting with hexane/EtOAc (6:4) over 30 min (8 mL/min) to provide neopetroformyne A (**9**, 20.1 mg, 2.0% crude extract). $[\alpha]_D^{25}$ +11 (*c* 0.092, MeOH), Lit. $[\alpha]_D^{25}$ +19 (*c* 0.45, MeOH).[23]

Compounds 10 and 11:[24] A portion of the organic extract (222 mg) was subjected to a solvent-solvent partitioning scheme that concentrated the activity in the MeOtBu-soluble fraction (52.3 mg). Size exclusion chromatography of the active MeOtBu material on Sephadex LH-20 (2 x 90 cm) with hexane/CH_2Cl_2/MeOH (2:5:1) provided 5 fractions (A-E). Fraction C was further purified by HPLC on a Rainin Dynamax CN (2 x 25 cm) column eluting with hexane/EtOAc 65:35 over 30 min (12 mL/min) to provide 12α-acetoxy-24-methyl-24-oxoscalar-16-en-22, 25-dial (**10**) (2.4 mg, 1.08% crude extract) $[\alpha]_D^{25}$ +5.4 (*c* 0.16, $CHCl_3$), (no lit. value) and (-)-22-acetoxy-16β-hydroxy-24-methyl-24-oxoscalarano-25, 12β-lactone A (**11**) (6.1 mg, 2.75% crude extract) $[\alpha]_D^{25}$-10.1 (*c* 0.08, $CHCl_3$), Lit. $[\alpha]_D$+27.7 (*c* 1.0, $CHCl_3$) for (+)-22-acetoxy-16β-hydroxy-24-methyl-24-oxoscalarano-25,12β-lactone A. (Reference: Kazlauskas, R; Murphy, PT; Wells, RJ *Aust. J. Chem.* **1982**, *35*, 51-59.)

Heteronemin (12):[25-27] A portion of the organic extract (220 mg) was subjected to a solvent-solvent partitioning scheme that concentrated the activity in the hexane and MeOtBu soluble fractions (158 mg). Size exclusion chromatography of the active material on Sephadex LH-20 (2.5 x 90 cm) with hexane/CH_2Cl_2/MeOH (2:5:1) provided four major fractions (A-D). Fraction B (58 mg) was further purified on Sephadex LH-20 (2.5 x 90 cm) with CH_2Cl_2/MeOH (1:1) yielding four additional fractions (A-D). Final purification of fraction B (19.4 mg) was achieved by HPLC (Rainin; Dynamax Si, 8μ

size 2 x 25 cm), eluting with hexane/EtOAc (7:3) in 12 min (8 mL/min) to provide heteronemin (**12**, 12.2 mg, 5.54% crude extract). $[\alpha]_D^{25}$ -51.1 (*c* 0.13, $CHCl_3$), Lit. $[\alpha]_D^{25}$ -59.7 (*c* 0.01, $CHCl_3$).[25]

S3. qPCR results for compounds **7**, **8** and **9**.

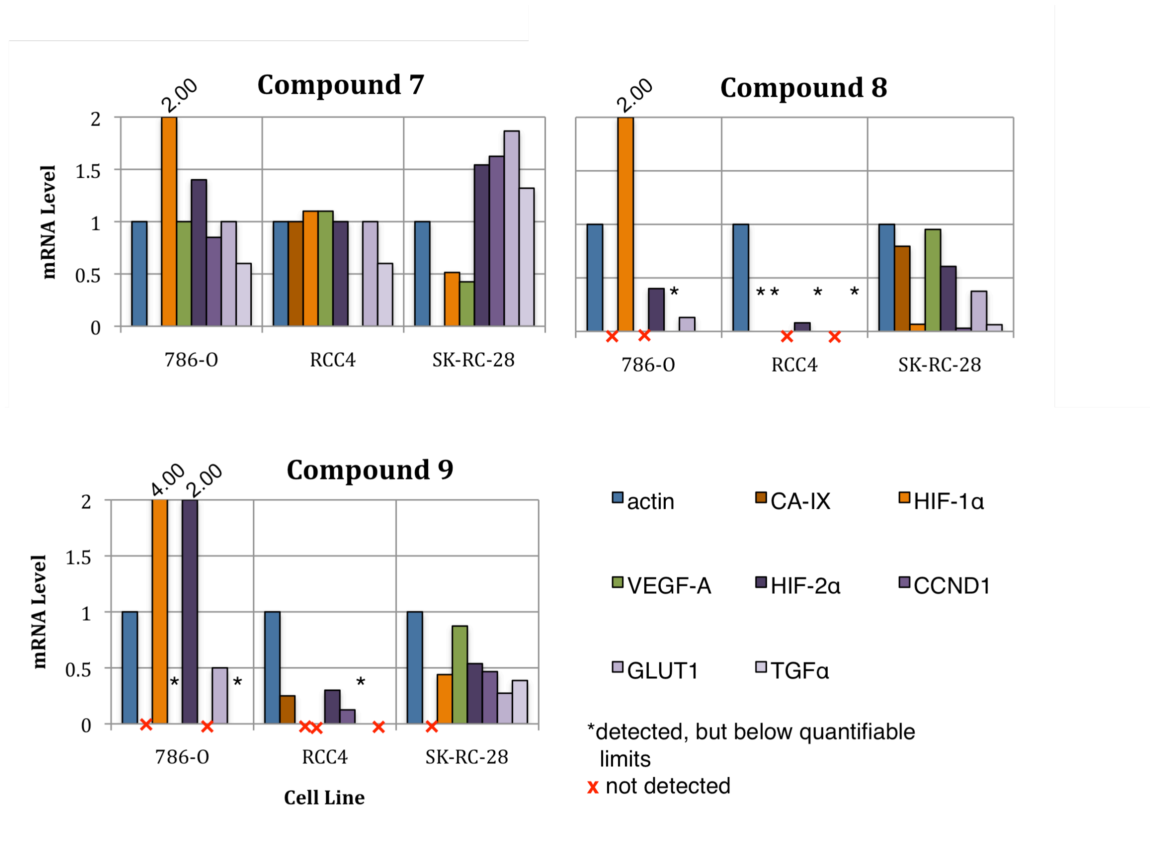


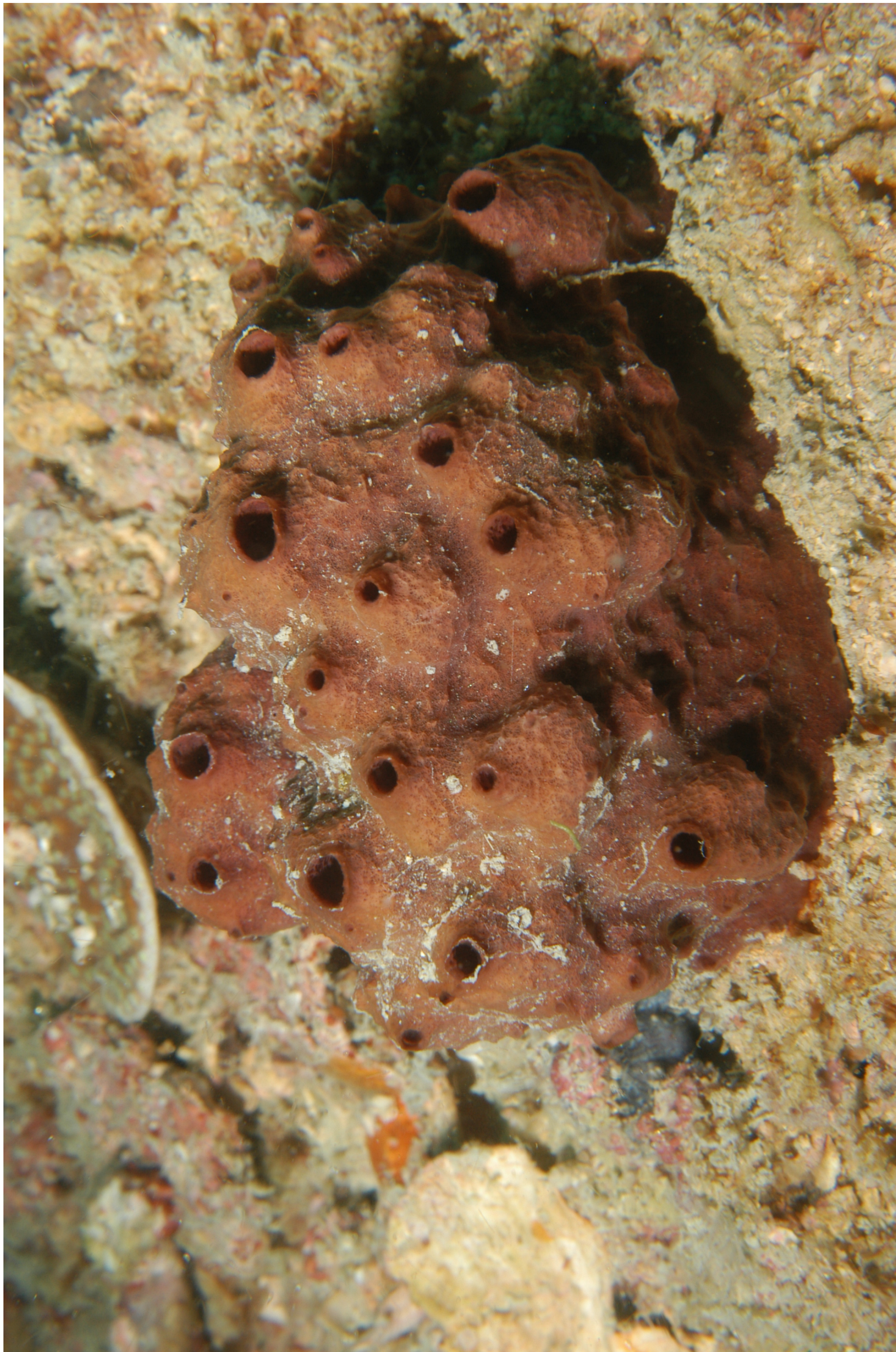
Figure 2. qPCR results for compounds **7**, **8** and **9** in three different cell lines: 786-0 (HIF-2α only), RCC4 (both HIF-1α and HIF-2α) and engineered SK-RC-28 (HIF-1α only). The effect of treatment on the transcription of mRNA of five HIF-inducible genes was quantified relative to actin. Of the five mRNA transcripts measured, carbonic anhydrase 9 (CA-IX) is mediated by HIF-1α, transforming growth factor alpha (TGFα), glucose transporter 1 (GLUT1) and cyclin D1 (CCND1) are mediated by HIF-2α, and vascular endothelial growth factor A (VEGF-A) is mediated by both HIF-1α and HIF-2α. * Indicates that mRNA was detected, but below quantifiable levels. **X** Indicates that mRNA was not detected. Results that exceeded 2-fold are indicated by values above the respective bars.

S4. Photograph of *Amphimedon* sp. (0CDN481) by Dr. Pat Colin, Coral Reef

S5. Photograph of *Xestospongia* sp. (0CDN7718) by Dr Pat Colin, Coral Reef

S6. Photograph of *Haliclona* sp. (Q66C334) by Dr Peter Murphy, AIMS

S7. Photograph of *Petrosia* sp. (0M9H2607) by Dr Belinda Glasby, Museum of the Northern Territories, Darwin.



S8. Photograph of *Fascaplysinopsis* sp. (0M9I5678) by Dr Pat Colin, Coral Reef