Towards Aspirin-Inspired Self-Immolating Molecules which Target the Cyclooxygenases
Supporting Information

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1. Synthetic Chemistry: Experimental Details

1.1. General Considerations

All commercial reagents were purchased from Sigma-Aldrich, VWR, Fisher Scientific or TCI America with purities of over 95% and, unless otherwise stated, were used as received. Anhydrous solvents were purchased from Sigma-Aldrich or VWR and used as received. Purified COX-1 (ovine) and COX-2 (human recombinant) were purchased from Cayman Chemicals. RAW264.7 murine macrophages were purchased from ATCC and cultured in DMEM + 10% heat-inactivated FBS. LPS was a kind gift from Dr. Stefanie Sowinski. Interferon-γ was purchased from Calbiochem. Media, media supplements and PBS were purchased from the University of California San Francisco’s cell culture facility.

All reactions were performed in dry solvents under an inert nitrogen atmosphere and with vigorous stirring unless otherwise stated. Temperatures stated refer to the external medium. Oil baths, in combination with IKA heating mantles and thermocouples, were used to achieve elevated temperatures. Ice baths were used to cool to 0 °C. Solid CO₂ in acetone was used to cool to -78 °C. TLC was performed on EMD 60 F254 aluminum backed plates and visualized under UV light or stained with KMnO₄ solution. Purification over silica was achieved using EMD 60 (230-400 mesh). ¹H and ¹³C NMR were recorded on a Varian 400 MHz spectrometer and processed using ACD/NMR Processor Academic Edition using residual isotopic solvent as an internal reference for organic deuterated solvents. The following abbreviations were used during assignment: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad. Mass spectra were measured by the University of Notre Dame Mass Spectrometry and Proteomics Facility. HPLC spectra were recorded on a Hitachi LaChrom Elite system using UV detection at 254 nm. UV-vis spectra were measured using a Thermo Scientific NanoDrop 2000c. Emission spectra and fluorescence data were measured using a Gemini EM fluorescence microplate reader and black-sided 96-well plates.
2. HPLC Traces:

A.

Figure S1. Measurement of Probe Purity by RP-HPLC. A. **CP-1**: The purity of **CP-1** was measured via HPLC using the following gradient of MeCN (+0.1% TFA) in H₂O (+0.1% TFA): 2-20%, 0-10 mins; 20-90%, 10-25 mins. Under these conditions 6-aminoquinoline eluted at 10.2 mins. B. **CP-2**: The purity of **CP-2** was measured via HPLC using the following gradient of MeCN (+0.1% TFA) in H₂O (+0.1% TFA): 40-60%, 0-20 mins; 60-100%, 20-25 mins. Under these conditions 6-aminoquinoline eluted at 8.8 mins.
3. UV-Vis Spectra

Figure S2. UV-Vis Spectra of A) CP-1 and B) CP-2. Compounds were dissolved at 0.1 mgmL$^{-1}$ in either water or deprotection buffer (2:1:1, v:v:v, methanol:water:saturated NaHCO$_3$) for 30 minutes prior to measurement of spectra.
4. NMR Spectra

1,3-Di(tert-butyldimethylsilyl)propan-2-yl 2-hydroxy-5-(hydroxymethyl)benzoate (4):
1,3-Di(tert-butyldimethylsilyl)propan-2-yl 2-(acetyloxy)-5-(hydroxymethyl)benzoate (5):
3-(Chlorosulfonyl)-4-hydroxybenzoic acid (7):
4-(Hydroxymethyl)-2-(oct-2-yn-1-ylsulfanyl)phenol (9):
4-(Hydroxymethyl)-2-(oct-2-yn-1-yloxy)sulfanyl)phenyl acetate (10):
5. Probe Stability at Various pHs:

Either CP-1 or CP-2 were dissolved in one of the following buffers at a concentration of 100 μM:
100 mM citrate-PBS (pH 6.0); phosphate-buffered saline (PBS, pH 7.4, 0.2 g/L KH₂PO₄, 2.16
g/L Na₂HPO₄·7H₂O, 0.2 g/L KCl, 8.0 g/L NaCl, 0.1 g/L CaCl₂, 0.1 MgCl₂·6H₂O); 100 mM Tris (pH
8.0), 100 mM glycine (pH 9.0). The fluorescent signal (λₑₓ = 355 nm, λₑₓₘ = 535 nm) was
measured and the solutions were then incubated at 37 °C for 30 mins. The fluorescent signal
was measured again and the increase in fluorescence was calculated to give the data in figure
S3.

![Graph](image-url)

**Figure S3.** Stability of CP-1 and CP-2 against hydrolysis at various pHs.
6. Analysis of CP-1 Treated COX-2 by LC/MS/MS

All data were acquired and analyzed by MS BioWorks LLC (Ann Arbor, MI).

**Data Processing:** Data was searched using a local copy of Mascot with the following parameters:

- Enzyme: Trypsin or none
- Database: SwissProt Human (forward and reverse appended with common contaminants)
- Fixed modification: Carbamidomethyl (C)
- Variable modifications: Oxidation (M), Acetyl (protein N-term, KS), Deamidation (NQ)
- Mass values: Monoisotopic
- Peptide mass tolerance: 10 ppm
- Fragment mass tolerance: 0.5 Da
- Mixed mass cleavages: 3

Mascot DAT files were parsed into the Scaffold software for validation, filtering and to create a nonredundant list per sample. Data were filtered using a minimum protein value of 99.9%, a minimum peptide value of 50% (Prophet scores) and requiring at least two unique peptides per protein.

**Results:** The target protein, PGH2_HUMAN, was observed with 96% sequence coverage after combining the data from all three enzymes:

sp|P35354|PGH2_HUMAN (100%), 68,997.8 Da; Prostaglandin G/H synthase 2 OS=Homo sapiens GN=PTGS2 PE=1 SV=2

661 unique peptides, 1048 unique spectra, 1439 total spectra, 580/604 amino acids (96% coverage)
A. Summary of acetylation sites on CP-1 treated COX-2, as measured via LC/MS/MS analysis following enzymatic degradation. Lysine acetylation sites highlighted in green, serine acetylation sites in light blue. Non-highlighted residues not covered by analysis. B. Typical mass spectrum for lysine acetylation site (R)IKLFLKPTPN(T).

**Figure S4.**