Development of a Selective, Exosite-Binding Matrix Metalloproteinase-13 Inhibitor

Joshua Roth,[a] Dmitriy Minond,[b,c] Etzer Darout,[a] Qin Liu,[a] Janelle Lauer,[d,e] Peter Hodder,[b] Gregg B. Fields,[c,d] and William R. Roush[a]*

[a]*Department of Chemistry, Scripps Florida, 130 Scripps Way, Jupiter, FL 33458*
[b]*Lead Identification Department, The Scripps Research Institute Molecular Screening Center, Scripps Florida, 130 Scripps Way, Jupiter, FL 33458*
[c]*Torrey Pines Institute for Molecular Studies, Port St. Lucie, FL 34987*
[d]*Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX*
[e]*Department of Molecular Therapeutics, Scripps Florida, 130 Scripps Way, Jupiter, FL 33458*

roush@scripps.edu

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Supporting Information

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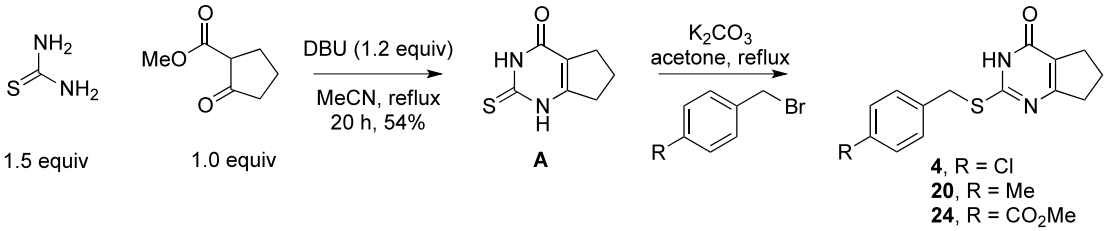
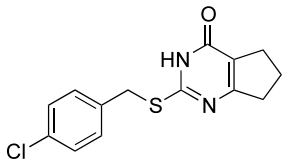
(1) General Procedure for Synthesis of MMP-13 Inhibitors 4, 20, and 24

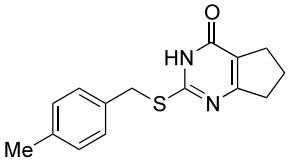


5,6-Trimethylene-2-thiouracil (**A**) was synthesized following a literature procedure.[1] A mixture of 0.5 mmol **A**, 0.5 mmol *p*-substituted benzyl bromide, and 1.5 mmol potassium carbonate in 3 mL of acetone was heated at reflux for 12 h. The reaction was allowed to cool to ambient temperature, and the resulted slurry was diluted with water and extracted with methylene chloride. The organic layer was diluted with methanol (to dissolve residual solids), dried over magnesium sulfate and then filtered. Silica gel was added to the filtrate, which was then concentrated *in vacuo*. The resulted solid was loaded onto the top of a silica gel column and eluted with 1% to 5% methanol in methylene chloride to afford the targeted MMP-13 inhibitors.



inhibitor **4**

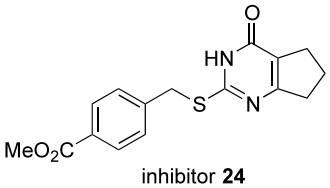
Yield: 90%; **Rf** = 0.42 (CH_2Cl_2-MeOH = 9:1); **^{1}H NMR** (400 MHz, DMSO-d6) δ ppm: 12.54 (s, 1 H), 7.42-7.46 (m, 2 H), 7.35-7.39 (m, 2 H), 4.38 (s, 2 H), 2.76 (dd, *J* = 7.3, 7.5 Hz, 2 H), 2.58 (dd, *J* = 7.3, 7.4 Hz, 2 H), 1.96 (dddd, *J* = 7.8, 7.6, 7.5, 7.5 Hz, 2 H); **^{13}C NMR** (100 MHz, DMSO-d6) δ ppm: 136.6, 131.8, 130.9, 128.3, 34.2, 32.7, 26.7, 20.5; **IR**: 3450, 1651, 1049, 826, 764; **ESIMS**: $C_{14}H_{13}ClN_2OS$ 292.04 (calculated), 292.01 (found).



inhibitor **20**

Yield: 92%; **Rf** = 0.54 (CH_2Cl_2-MeOH = 9:1); **^{1}H NMR** (400 MHz, DMSO-d6) δ ppm: 12.48 (s, 1 H), 7.28 (d, *J* = 8.0, 2 H), 7.12 (d, *J* = 7.8, 2 H), 4.34 (s, 2 H), 2.76 (dd, *J* = 7.3, 7.6 Hz, 2 H), 2.59 (dd, *J* = 7.3, 7.5 Hz, 2 H), 2.27 (s, 3H), 1.95 (dddd, *J* = 7.8, 7.6, 7.6, 7.5 Hz, 2 H); **^{13}C NMR** (100 MHz, DMSO-d6) δ ppm: 136.5, 133.9, 130.9, 129.0, 128.9, 34.2, 33.4, 26.7, 20.6, 20.5; **IR**: 3400, 1648, 992, 499, 482; **ESIMS**: $C_{15}H_{16}N_2OS$ 272.10 (calculated), 271.96 (found).

[1] WO 03/016287 A2 (PCT/EP02/09067)



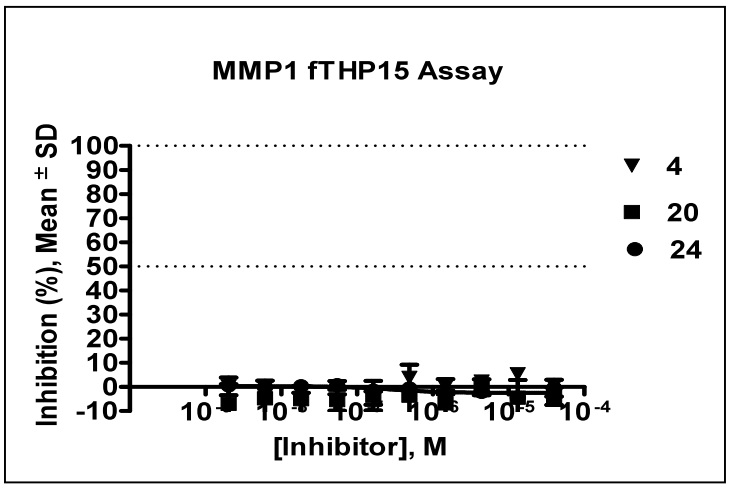
inhibitor **24**

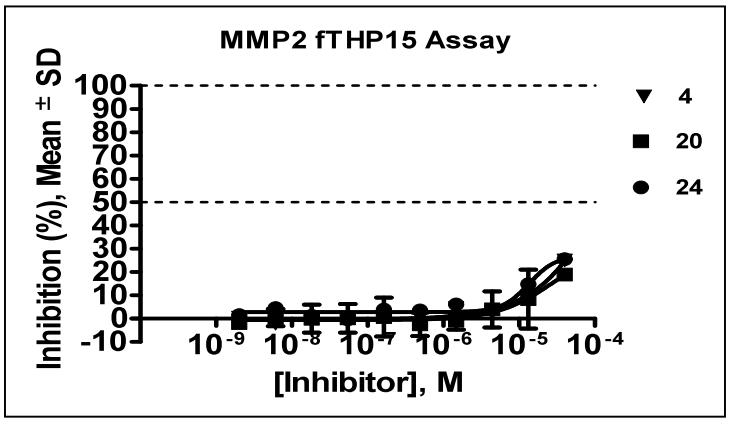
Yield: 85%; **Rf** = 0.52 (CH_2Cl_2-MeOH = 9:1); **^{1}H NMR** (400 MHz, DMSO-d6) δ ppm: 12.54 (s, 1 H), 7.90 (d, *J* = 8.4, 2 H), 7.55(d, *J* = 8.4, 2 H), 4.45 (s, 2 H), 3.84 (s, 3H), 2.76 (dd, *J* = 7.3, 8.0 Hz, 2 H), 2.59 (dd, *J* = 7.2, 7.4 Hz, 2 H), 1.95 (dddd, *J* = 7.7, 7.7, 7.5, 7.4 Hz, 2 H); **^{13}C NMR** (100 MHz, DMSO-d6) δ ppm: 165.9, 143.2, 129.4, 129.2, 128.4, 52.1, 34.2, 33.0, 26.6, 20.6, 20.5; **IR**: 3417, 1660, 1530, 1434, 1283; **ESIMS**: $C_{16}H_{16}N_2O_3S$ 316.09 (calculated), 315.98 (found).

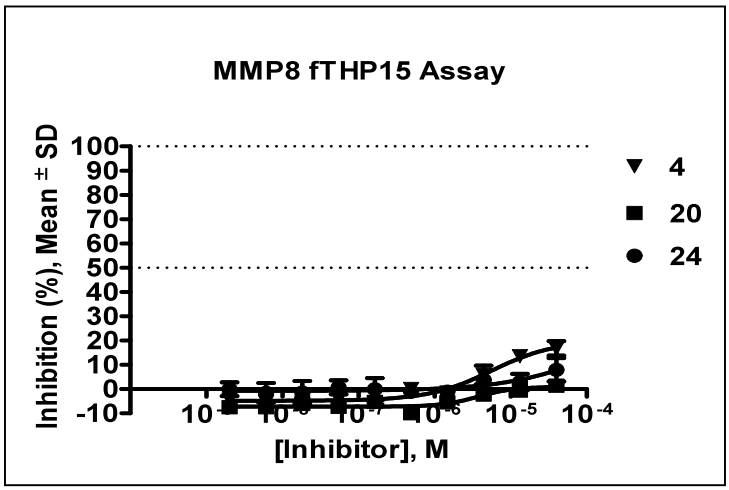
(2) MMP Inhibition Assays

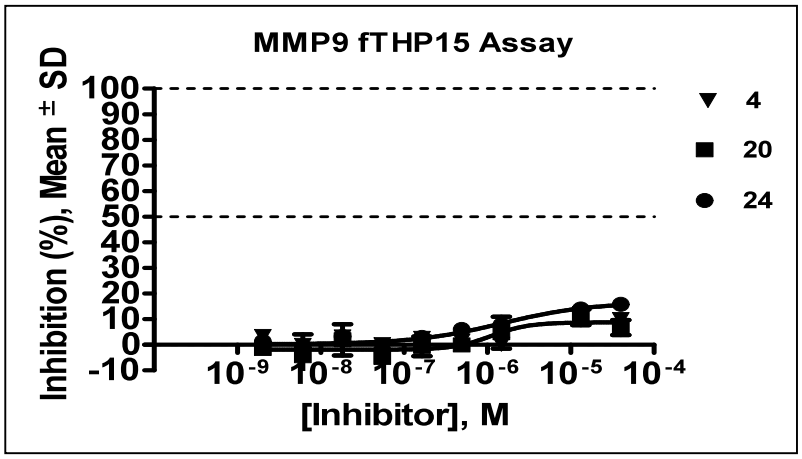
MMP inhibition assays were performed to determine whether compounds **4**, **20**, and **24** were nonselective. Assays were performed primarily using the triple-helical substrate fTHP-15 [(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2], as this substrate can be used for screening of exosite binding compounds. Experiments with fTHP-15 were performed in 1536-well white microtiter plates (Greiner Bio-One, Monroe, NC). To begin the assay 2.5 μL of 8 μM fTHP-15 in enzyme assay buffer (EAB; 50 mM Tris•HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35) was added to the wells using a FRD™ IB Workstation (Aurora Discovery, Carlsbad, CA). 20 nL of DMSO–water (3:1) containing the control compounds, test compounds, or no compounds was dispensed using a 1536-head Pintool system (GNF Systems, San Diego, CA). The DMSO concentration in the assay was 0.3%. Reactions were initiated by addition of 2.5 μL of MMP in EAB. After 4 h of incubation at 25 °C the reaction was stopped by addition of 5 μL of 50 mM EDTA (product # E7889; Invitrogen, Carlsbad, CA). All reagents were dispensed at ambient temperature. Plates were incubated for 10 min at 25 °C and emission fluorescence was read on the Viewlux (Perkin-Elmer, Turku, Finland) microplate reader ($\lambda_{excitation}$ = 325 nm, $\lambda_{emission}$ = 450 nm).

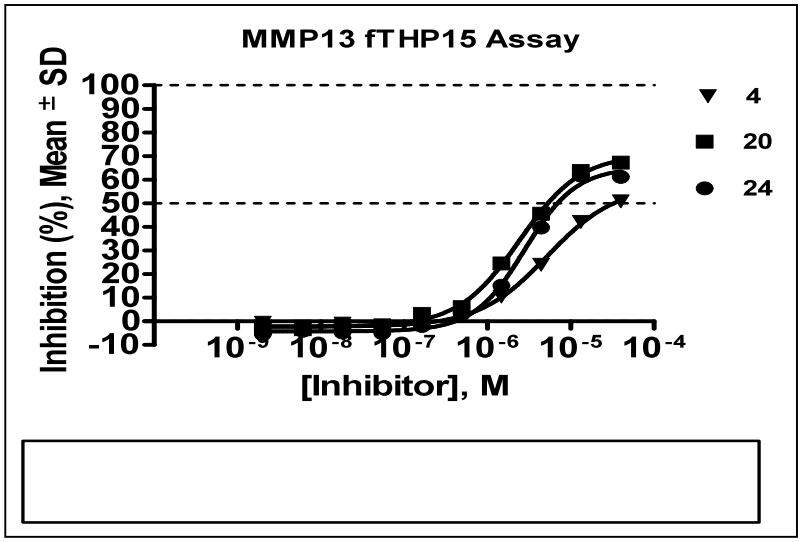
Figure S-1. Dose-dependent inhibition of MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, or MMP-14 hydrolysis of fTHP-15 by compound **4**, **20**, or **24**.








MMP9 fTHP15 Assay
Inhibition (%), Mean ± SD
100
90
80
70
60
50
40
30
20
10
0
-10
10-9 10-8 10-7 10-6 10-5 10-4
[Inhibitor], M
4
20
24


MMP13 fTHP15 Assay
Inhibition (%), Mean ± SD
100
90
80
70
60
50
40
30
20
10
0
-10
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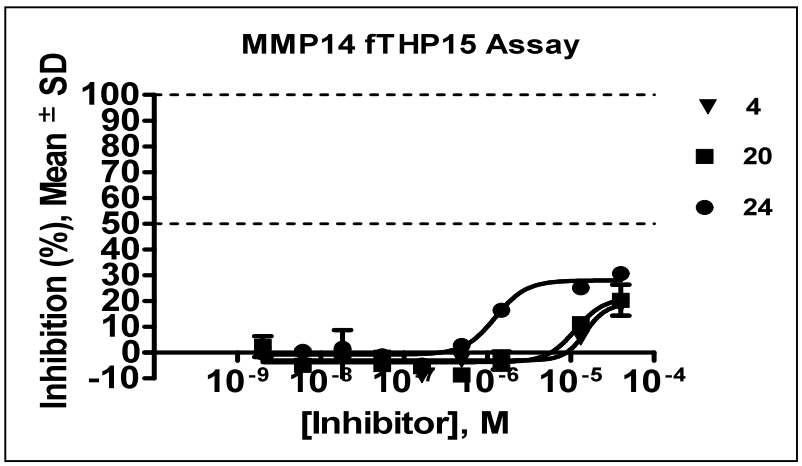
[Inhibitor], M
4
20
24

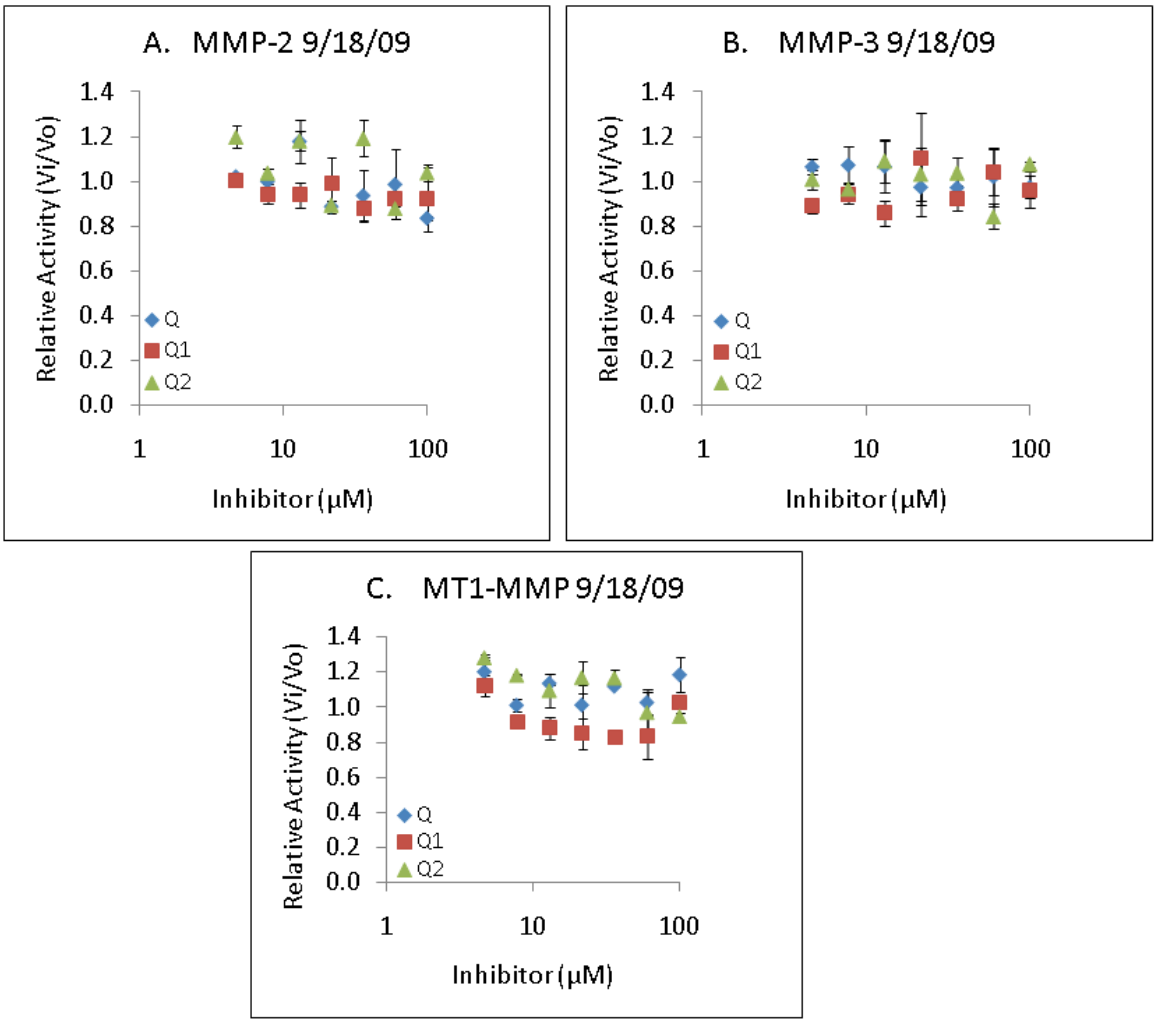

MMP14 fTHP15 Assay
Inhibition (%), Mean ± SD
100
90
80
70
60
50
40
30
20
10
0
-10
10-9 10-8 10-7 10-6 10-5 10-4
[Inhibitor], M
4
20
24

To further examine the selectivity of inhibitors, MMP assays were performed using Knight SSP [Mca-Lys-Pro-Leu-Gly-Leu-Lys(Dnp)-Ala-Arg-NH_2]. This shorter substrate can be used to identify exosite inhibition based on differential activities between it and a triple-helical substrate (fTHP-15). Inhibitors were prepared as 10 mM solutions in DMSO and then further diluted with EAB. Assays were conducted in EAB by pretreating 1-10 nM enzyme with up to 100 μM inhibitor for 1 h at 37 °C, then incubating 10 μM Knight fSSP for 1.5-4 h. Fluorescence readings ($\lambda_{excitation}$ = 324 nm and $\lambda_{emission}$ = 393 nm) were obtained over time on a Molecular Devices SPECTRAmax Gemini EM Dual-Scanning Microplate Spectrofluorimeter, and the reaction was quenched by addition of 25 mM EDTA. Rates of hydrolysis (ΔRFU) were obtained from plots of fluorescence versus time, using data points from only the linear portion of the hydrolysis curve.

Table S-1: Inhibition of MMP hydrolysis of Knight fSSP by compounds 4, 20, and 24

Target	Activity (%) at [inhibitor] = 100 μM		
	4	**20**	**24**
MMP-1	92.7 ± 1.5	105.5 ± 7.2	101.9 ± 9.1
MMP-2	83.0 ± 3.1	97.6 ± 2.8	84.7 ± 4.3
MMP-3	~96	~100	~100
MMP-8	81.7 ± 3.6	106.2 ± 12.3	93.8 ± 1.2
MMP-12	91.6 ± 9.2	77.3 ± 10.1	80.6 ± 8.0
MMP-13	44.0 ± 3.3	38.6 ± 4.8	35.1 ± 1.8
MMP-14	106.6 ± 0.6	100.1 ± 6.4	104.0 ± 2.8
MMP-20	105.0 ± 5.0	101.4 ± 0.9	96.4 ± 2.5

Figure S-2. Dose-dependent inhibition of (A) MMP-2, (B) MMP-3, or (C) MMP-14/MT1-MMP hydrolysis of Knight fSSP by compound **4** (Q), **20** (Q1), or **24** (Q2). Compounds **20** and **24** did not inhibit MMP-1, MMP-2, MMP-3, and MMP-8 (see also Table S-1). This is distinct from compound **4**, which exhibited 20% inhibition of MMP-8 at 100 μM. Compound **20** did not inhibit MMP-14, while compound **24** showed 15% inhibition of MMP-14 (see also Table S-1).



(3) Mode and Potency of Inhibition

MMP-13 assays were performed to determine the inhibition constant (K_i) and modality of these compounds. Kinetic assays were conducted by incubating a range of substrate concentrations (2 to 25 μM) versus varying inhibitor concentrations with 4 nM enzyme at room temperature in EAB (50 mM Tris•HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35). Substrate hydrolysis was continuously monitored. Raw fluorescence was measured by a Tecan Safire2 using $\lambda_{excitation}$ = 324 nm and $\lambda_{emission}$ = 393 nm. Initial velocities were obtained from plots of fluorescence at λ = 394 nm versus time, using data points from only the linear portion of the hydrolysis curve. All kinetic parameters were calculated using GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA). All K_i values were determined by non-linear regression (hyperbolic equation) analysis using the mixed inhibition model which allows for simultaneous determination of the mechanism of inhibition. The mechanism of inhibition was determined using the "alpha" parameter derived from a mixed-model inhibition by GraphPad Prism. The mechanism of inhibition was additionally evaluated by Hanes-Woolf and Lineweaver-Burk plots.