Bioorganic & Medicinal Chemistry Letters 23 (2013) 5566–5570

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Design and synthesis of MMP inhibitors with appended fluorescent tags for imaging and visualization of matrix metalloproteinase enzymes

John N. Freskos [a,*], Bethel Asmelash [a], Kimberly R. Gaston [a,b,†], Amol Karwa [b], Tim A. Marzan [a], Maureen A. Nickols [b], Thomas E. Rogers [a,b,‡], Tasha Schoenstein [a], Carolyn J. Sympson [b], Bich Vu [a]

[a] Department of Chemistry, Mallinckrodt Pharmaceuticals, 675 McDonnell Blvd., Hazelwood, MO 63042, United States
[b] Department of Biology, Mallinckrodt Pharmaceuticals, 675 McDonnell Blvd., Hazelwood, MO 63042, United States

ARTICLE INFO

Article history:
Received 8 July 2013
Revised 6 August 2013
Accepted 9 August 2013
Available online 16 August 2013

Keywords:
Imaging agents
MMP inhibitors
Tumor visualazation

ABSTRACT

We describe the synthesis, MMP-2 and 9 potency, and in vitro evaluation of a series of α-sulfone hydroxamate MMP inhibitors conjugated to a series of dyes with different absorption/emission lamina maxima's that can be used to visualize tumors.

Matrix metalloproteinases (MMPs) are a class of zinc-dependent endopeptidases having functionality for degradation and remodeling of the extracellular matrix and subendothelial basement membrane.[1] This family of more than twenty enzymes is further classified into several main groups, including gelatinases, interstitial collagenases, stromelysins, and membrane-type matrix metalloproteinases. In particular, there is considerable evidence demonstrating a close association between upregulation of certain MMPs and the proliferation, invasive behavior and metastatic potential of certain types of tumors.[2–4] The discovery of the α-sulfone hydroxamates by researchers at Searle/Pharmacia illustrates the focus on selectivity, potency, and achieving suitable ADME properties. The optimization of these ADME properties led to inhibitors with good oral bioavailability and an approriate $T_{1/2}$ to achieve efficacy in various models of osteoarthritis, cancer, and myocardial remodeling after an ischemic event.[5a,b] While MMPs are a well recognized target for the development of therapeutic and diagnostic agents, their potential for biomedical imaging and visualization remains much less explored. Optical agents for imaging the expression of MMPs in certain tumors and lesions has potential application for diagnosis, staging, and monitoring of cancer. This approach may also be useful for evaluation of tissue degradation associated with pathological conditions such as osteoarthritis, atherosclerosis, and wound repair. In addition, optical agents capable of selective delivery to cells and to tissue expressing MMPs also have potential for visually differentiating the tissue of a tumor or lesion from normal tissue during a surgical procedure, such as a biopsy or therapeutic tissue removal. This approach was used to conjugate a derivative of the broad spectrum MMP inhibitor CGS25966 to a NIR dye and in vitro results indicated that this approach was viable.[6]

Recently we reported the discovery of a novel set of pyrazine dyes whose properties as pure GFR agents are in the process of being evaluated in the clinic for noninvasive, realtime monitoring of kidney function.[7a,b] It was of interest to see if these pyrazines, as well as other dyes, could be tethered to the α-sulfone hydroxamate MMP inhibitor class and still retain MMP activity as well as maintaining good optical fluorescence properties. Indeed from the data presented in Table 1 we now demonstrate that this is possible.[8] As shown earlier, through numerous X-ray co-crystal structures of the α-sulfone hydroxamate series of MMP inhibitors, substituents attached to the piperidine nitrogen are solvent exposed and thus ammenable to putting large groups off the nitrogen while still retaining high potency for MMP's of interest and sparing MMP-1.[5a–c] This is demonstrated quite profoundly by the fact that it is possible to put a 5000 mer Peg-DSPE (Distearylphosphitylester) off of the piperidine nitrogen and still maintain low nanomolar

* Corresponding author. Tel.: +1 1 314 654 3148; fax: +1 1 314 654 8343.
E-mail addresses: john.freskos@mallinckrodt.com, freskos@covidien.com (J.N. Freskos).
† Current address: Monsanto Co., 800 N. Lindberg Blvd., St. Louis, MO 63167, United States.
‡ Current address: Center for Emerging Technologies, 4041 Forest Park Ave., St. Louis, MO 63108, United States.

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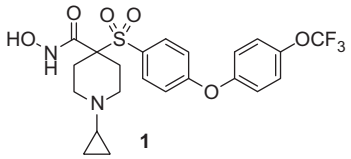
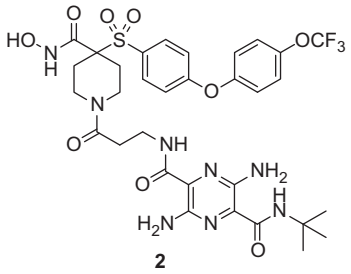
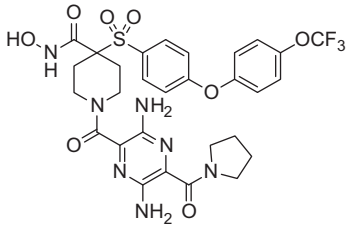
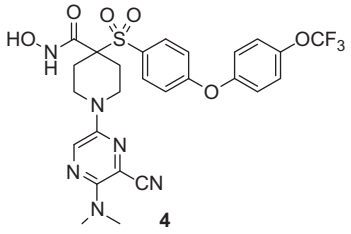
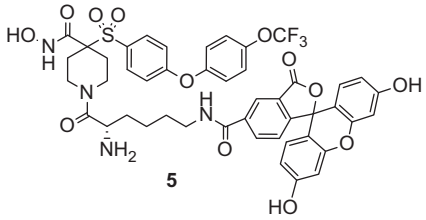
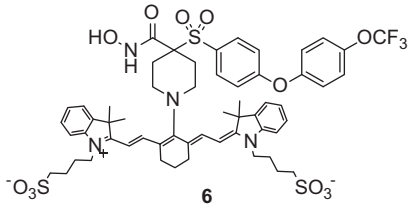
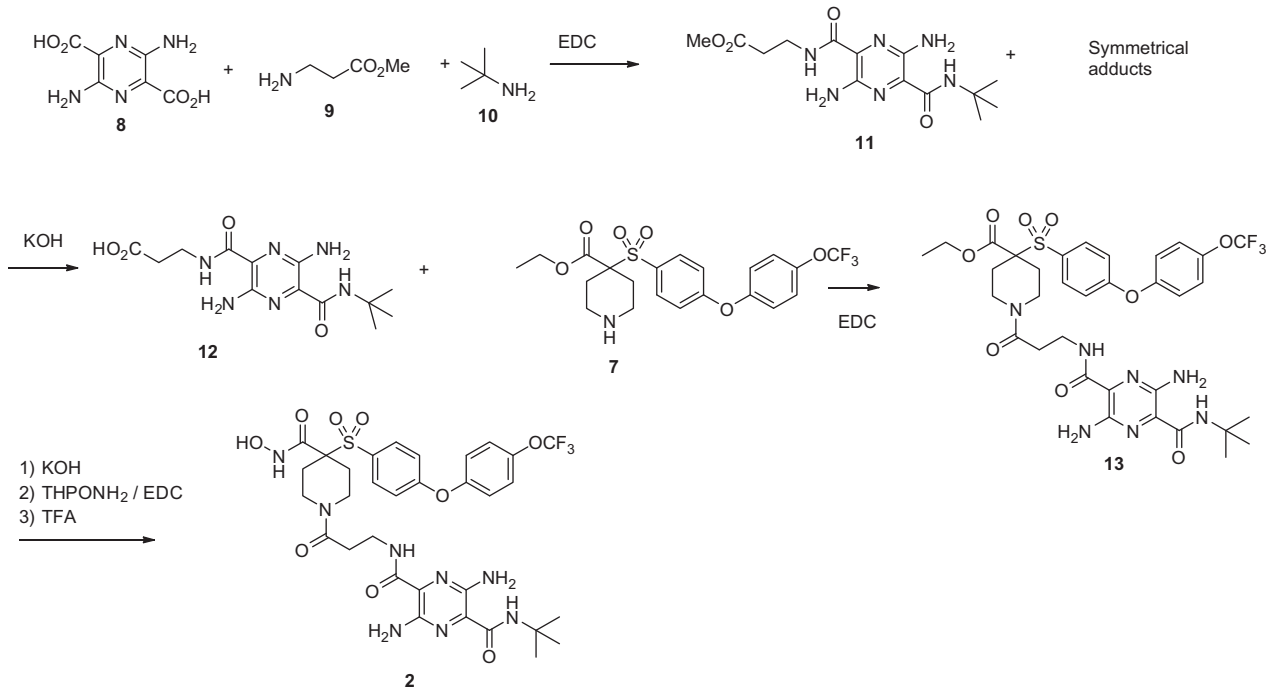
http://dx.doi.org/10.1016/j.bmcl.2013.08.050

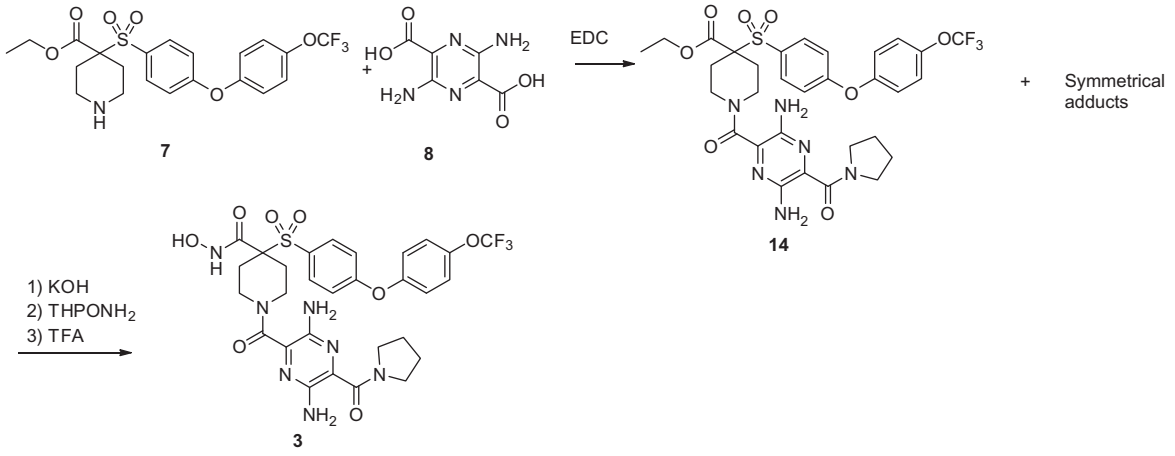
Table 1
IC_{50} and UV absorption and emission properties (λ_{max}) of MMP–dye conjugates

Compound	hMMP-2 (nM)	hMMP-9 (nM)	λ (abs) nM	λ (emis) nM
 **1**	<1	<1	ND	ND
 **2**	5.1	4.1	450	550
 **3**	<1	<1	420	520
 **4**	1.6	<1	400	500
 **5**	<1	<1	488	525
 **6**	2.8	1.6	>700	>700

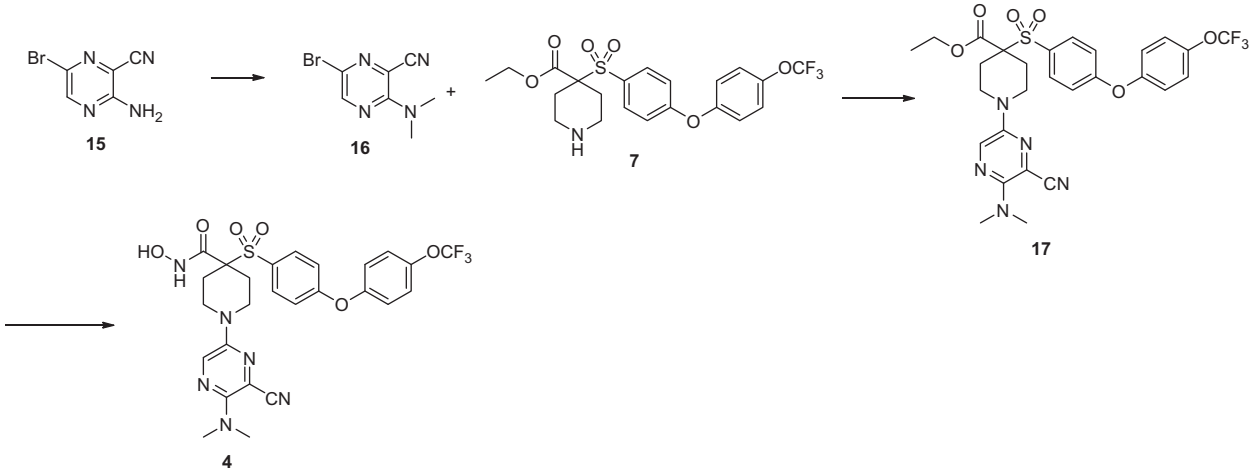
potency.[10] Interestingly, unlike the approach with CGS25966 where a fairly long (3-mer Peg-amide-5-carbon alkyl) linker was needed to separate the MMP-inhibitor from the dye,[6] we have found that short linkers are acceptable and that the linker may even be omitted. Compound **1** was our internal standard and its synthesis has been described elsewhere.[5a,b] Like compound 1 all of our MMP–dye's will spare MMP-1 and be active against not only MMP-2 and -9 but also against MMP-3, -8, and -13.[5a,b] This is due to the phenyl-*O*-phenyl-OCF_3 moiety that lies in the $S_{1'}$ binding pocket of the various MMP enzymes, unlike the smaller phenyl-OMe which is present in CGS25966 and enables it to fit in the $S_{1'}$ binding site of the MMP-1 enzyme, which is much smaller than MMP's-2, 3, 9, 13, and 14. The MMP-imaging agents **2** and **5** have very short linkers while **3**, **4** and **6** have no linker and all yet still retain excellent potency for MMP-2 and -9. The synthesis of compounds **2**–**6** are shown below. The synthesis of MMP-pyrazine (Scheme 1) **2** starts with commercially available diacid **8**, and the EDC/HOBt mediated coupling affords the desired product plus the two symmetrical isomers which were separated by Flash chromatography. Basic hydrolysis (KOH/EtOH 80 °C) to acid **12** followed by standard (EDC–HOBt) coupling to the key amine intermediate **7** yielded the amide **13**. The preparation of **7** has been



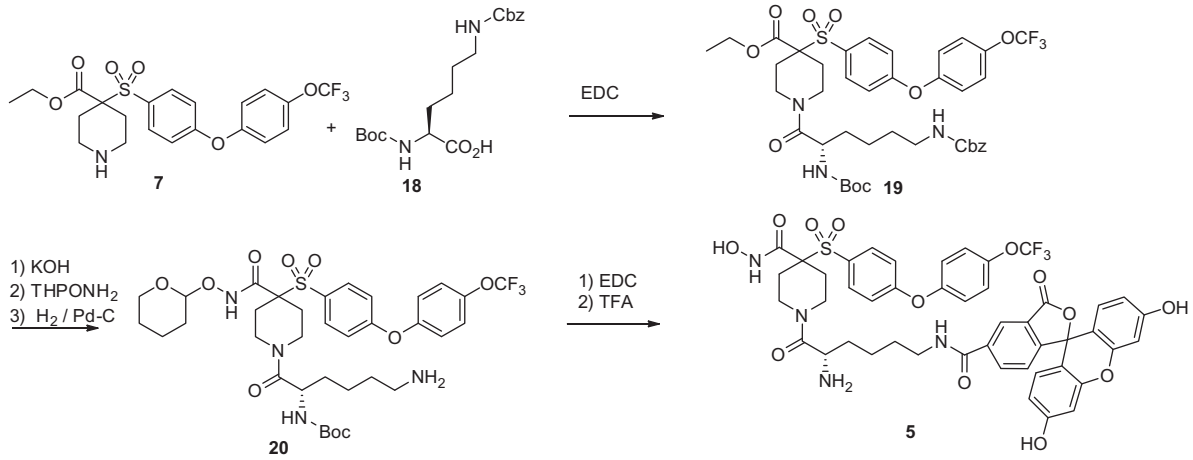
Scheme 1. Synthesis of imaging agent **2**.



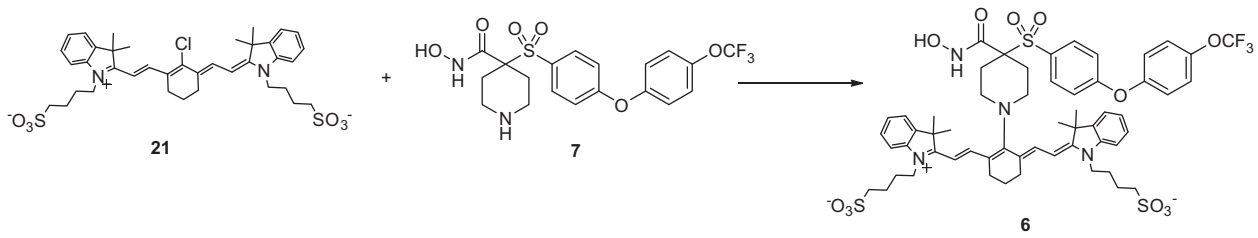
Scheme 2. Synthesis of imaging agent **3**.



Scheme 3. Synthesis of imaging agent **4**.



Scheme 4. Synthesis of imaging agent **5**.



Scheme 5. Synthesis of imaging agent **6**.

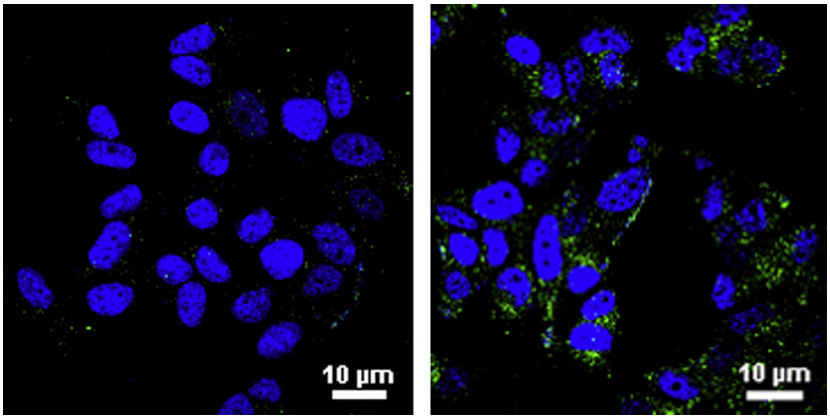


Figure 1. Compound **5** uptake in MCF7 cells at 37 °C.

described elsewhere.[5a] The conversion from **13** to the desired hydroxamate **2** was accomplished by the three-step sequence shown below involving hydrolysis, EDC-mediated coupling with THP-protected hydroxylamine, and removal of the THP protecting group with trifluroacetic acid.

The synthesis of compound **3** is shown in Scheme 2 and involves the direct coupling of **7** and **8** in the presence of 1 equivalents of pyyrolidine to afford, after chromatography, the unsymmetrical amide **14**, which was converted to **3** as described above.

The synthesis of imaging agent **4** is shown in Scheme 3. The commercially available pyrazine **15** was alkylated with methyl iodide and Cs_2CO_3 in DMF at room temperature to afford **16**. Palladium catalyzed coupling afforded, after RPHPLC purification, 60% of the desired adduct **17**. The conversion to hydroxamate **4** was accomplished as described above.

The synthesis of imaging agent **5** is shown in Scheme 4. The amine **7** was coupled with Boc-Lys(Z)-OH under standard conditions to afford amide **19**. This was then hydrolyzed and coupled to afford the protected hydroxamate that was then hydrogenated with Pd–C to afford the amine **20**. EDC mediated coupling with 5-FAM followed by de-protection afforded imaginng agent **5**.

The synthesis of the Near Infared Red (NIR) dye-MMP inhibitor **6** is shown in Scheme 5. Reaction of the amine **7** with the cy5.5 derivative **21** afforded the desired imaging agent in 10–20% yield and the main product obtained was from displacement of chloride with hydroxide. No attempts were made to optimize these conditions.

In Figure 1 we show the specificity of compound **5** when incubated with parental MCF7 and MMP-14 (MT-1 MMP) overexpressing MCF7 cells as visualized by confocal microscopy. This compound showed preferential accumulation in the MMP-14

overexpressing compared to parental cells when incubated at 37 °C.[9,10] Compound **5** was tested against MMP-14 and found to have an IC_{50} of ~100 nM.[11]

MCF7 parental cells are shown at left and the MMP-14 overexpressing MCF7 cells at right.

*Summary:*We have described a novel series of MMP–dye conjugates that possess the ability to visualize MMP-bearing cells in vitro. Compounds having increased potency toward MMP-14 to elaborate this technology will be described in a susquent disclosure.

Acknowledgements

The authors would like to thank Professor Alex Strongin from the Burnham Research Institute for kindly providing the MMP-14 overexpressing MCF7 cells that were employed in the confocal microscopy experiments. The authors would like to thank Ms. Laura Lorenz and Ms. Michelle Rieken for help in the preparation of this manuscript.

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11. MMP-2 and MMP-14 enzyme activities were measured using recombinant human proteins with substrate and protocols provided by R&D Systems, Minneapolis, MN. For the assays, 25 μL of 5× test samples and 50 μL of activated recombinant human protein were combined in 96 black well plates at ambient temperature, and 50 μL of substrate (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, R&D ES010) was then added to start the reaction. (All components were prepared at concentrations and in appropriate buffers as listed below.) The plates were then read at 320 nm excitation/405 nm emission using the kinetic mode of a fluorescence plate reader. A substrate blank was subtracted from duplicate determinations and IC_{50} values from six-point dose response curves were calculated using GraphPad Prism software, La Jolla, CA. For the MMP-14 enzyme assay, 40 μg/mL of rhMMP-14 (R&D 918-MP) was activated with 0.86 μg/mL rhFurin (R&D 1503-SE) in activation buffer (50 mM Tris, 1 mM $CaCl_2$, 0.05% (v/v) Brij-35, pH 9) and incubated at 37 °C for 1.5 °h. The activated protein was then diluted to 1.24 μg/mL in assay buffer (50 mM Tris, 3 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 8.5), for use in the assay. The substrate was used at 20 μM in assay buffer. For the MMP-2 enzyme assay, 100 μg/mL of rhMMP2 (R&D 902-MP) was activated with 1 mM APMA (*p*-aminophenylmercuric acetate) in assay buffer (50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (v/v) Brij-35, pH 7.5) and incubated at 37 °C for 1 h. The activated protein was then diluted to 248 ng/mL in assay buffer for use in the assay. The substrate was used at 25 μM in assay buffer.