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Synthesis and Biological Evaluation in U87MG Glioma Cells of (Ethynylthiophene)Sulfonamido-Based Hydroxamates as Matrix Metalloproteinase Inhibitors

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Abstract

Matrix metalloproteinases (MMPs) are important factors in gliomas since these enzymes facilitate invasion into the surrounding brain and participate in neovascularization. In particular, the gelatinases (MMP-2 and MMP-9), and more recently MMP-25, have been shown to be highly expressed in gliomas and have been associated with disease progression. Thus, inhibition of these MMPs may represent a promising non-cytotoxic approach to glioma treatment. We report herein the synthesis and biological evaluation of a series of 4-butylphenyl(ethynylthiophene)sulfonamido-based hydroxamates. Among the new compounds tested, a promising derivative, **5a**, was identified, which exhibits nanomolar inhibition of MMP-2, MMP-9, and MMP-25, but weak inhibitory activity towards other members of the MMP family. This compound also exhibited anti-invasive activity of U87MG glioblastoma cells at nanomolar concentrations, without affecting cell viability.

Keywords

Matrix metalloproteinase inhibitors; hydroxamic acids; antiglioma agents; MMP-2; MMP-9; MMP-25

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1. Introduction

Glioblastoma multiforme (GBM) represents the most malignant of the primary central nervous system (CNS) tumors that aggressively infiltrate the surrounding brain tissue [1]. Two protease families, in particular, the serine proteases of the plasminogen activator/plasmin system and the matrix metalloproteinases (MMPs), seem to be implicated in tumor invasion.

MMPs are a group of structurally related zinc-dependent endopeptidases involved in the degradation of extracellular matrix (ECM) components and a diverse array of non-ECM proteins. Twenty three human MMPs have been identified so far, which have been classified into subgroups based on their structure and substrate specificity as follows: collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs (MT-MMPs). Among the members of the MMP family, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are thought to play a key role in degradation of type IV collagen and gelatin, the two major components of ECM [2]. Increased expression of MMP-2 and MMP-9 is reported in many human tumors, including ovarian, breast and prostate tumors, and melanoma [2]. Elevated levels of MMP-2 and MMP-9 have also been found in human glioblastoma tissue samples, compared with low-grade brain tumors and normal brain tissue [3]. MMP-2, in particular, is highly expressed in gliomas and its expression, at both the mRNA and protein level, increase with tumor progression [4]. It has been shown that MMP-2 is localized in both tumor and vasculature cells, thus indicating multiple roles for this enzyme in tumor progression, including angiogenesis [5]. MMP-2 expression is also known to be associated with tumor invasion and metastasis [6]. Thus, gelatinase inhibition represents a promising non-cytotoxic approach to glioma treatment.

Unfortunately, clinical trials with MMP inhibitors (MMPIs) in cancer patients conducted over the past years failed to show a significant therapeutic response, mostly because the majority of patients enrolled had advanced metastatic disease [7]. Moreover, most clinical trials were carried out using broad-spectrum MMPIs, such as marimastat **1** (BB2516) [8], prinomastat **2** (AG3340) [9] and CGS27023A [10] **3** (Figure 1), which caused undesired side effects.

In fact, the use of broad-spectrum MMPIs in clinical trials was greatly limited by musculoskeletal syndrome (MSS) side effects, which required a drug dose reduction and the consequent reduction of beneficial effects.

It is important to emphasize that not all MMPs contribute to tumour progression. Recently, it has been demonstrated that some MMPs are involved in innate immunity and host defence against cancer, others inhibit tumour growth and malignant transformation, and others exert antiangiogenic effects [11]. Therefore, some MMPs are considered to be anti-targets for cancer therapy [12]. Inhibition of MMP-1 and MMP-14, in particular, has been hypothesized to cause the MSS observed clinically with broad-spectrum inhibitors application [13], while MMP-8 [14] and MMP-3 [12] exhibited anti-tumor properties and, consequently, their inhibition may be counterproductive. Hence, both the side effects and the poor outcome due to wide inhibition of MMPs could be avoided by the use of selective MMP-2 and MMP-9 inhibitors. Therefore, the aim of our study was to identify new compounds to selectively inhibit the gelatinases, without significantly affecting the activity of other MMPs, which could potentially be used as anti-glioma agents.

Most MMPIs are based on a chelating moiety interacting with the catalytic zinc ion and a hydrophobic portion protruding into the hydrophobic S1' subsite, which is a pocket situated

in proximity to the catalytic zinc ion. Widely utilized zinc-binding groups (ZBGs) include: hydroxamic acids, carboxylic acids, pyrones [15] and pyrimidinetriones. The most used of these is the hydroxamate group [16] since it has excellent chelating properties. In this paper, we describe the synthesis and biological evaluation of a series of 4-butylphenyl(ethynylthiophene)sulfonamido-based hydroxamates, **4-7**. Previous studies on carboxylate MMPis [17] had shown that the presence of an elongated hydrophobic substituent in P1' like 4-butylphenylethynylthiophene could ensure good inhibitory activity toward the gelatinases, sparing other enzymes like MMP-1, -3 and -7. In fact, the gelatinases have a deep S1' pocket while MMP-1 and MMP-7 present a shallow pocket in the catalytic site [18]. For this reason, we chose to maintain this type of substituent in the sulfonamido moiety and to change the ZBG in an effort to improve the selectivity also over other deep-pocket MMPs, such as MMP-8 and -3. In fact, as previously shown by Cohen [19], selectivity of action against some deep-pocket MMPs can also be dependent on the nature of the ZBGs, that have different chelating geometries and different pK_a values.

With the aim of increasing selectivity and based on our previous results on MMPis [16a,20], we planned to introduce in the new inhibitors structural constraints able confer a major degree of rigidity to the system by using a combination of the appropriate chelating moiety (for instance the hydroxamate) with a rigid P1' sulfonamide chain (like a 4-substituted phenyl(ethynylthiophene) chain), and with a lengthened P1 substituent. Therefore, starting from the un-substituted secondary sulfonamide **4**, derivatives **5a-d** bearing various (*R*) aryl-alkyl chains in position α of the hydroxamate were synthesized according to the hypothesis that the selectivity profile could be also affected by the presence and structure of the α -chain (P1 group), as previously observed by Hanessian *et al.* [21]. The scaffold was further rigidified by introducing α -chains fused with the sulfonamido moiety, thus affording cyclic compounds **6** and **7** (Figure 2).

The effects of these substitutions on MMP-2 and MMP-9 activity and selectivity were examined *in vitro* using a fluorometric assay on purified enzymes. The compounds **5a-d**, **6**, **7** were then tested for their ability to inhibit *in vitro* invasion of U87MG glioma cells through matrigel and to inhibit cell growth. Finally, these compounds were evaluated as inhibitors for MMP-25, a recently discovered target in glioma progression.

2. Chemistry

Scheme 1 shows the synthesis of compound **4**. 5-bromothiophene-2-sulfonyl chloride **8** was converted into sulfonamide **9** by reaction with glycine *tert*-butyl ester hydrochloride in water and dioxane in the presence of triethylamine. Compound **9** was then protected as a Boc-sulfonamide by treatment with di-(*tert*-butyl)dicarbonate in CH_2Cl_2 using 4-(dimethylamino)pyridine (DMAP) as acylation catalyst [22]. The N-protected intermediate **10** was submitted to the Sonogashira reaction [23,24] in the presence of Pd(II), CuI, triphenylphosphine and triethylamine to afford the acetylenic derivative **11**. Acid hydrolysis with TFA allowed to deprotect the carboxylic acid and the sulfonamidic nitrogen in a single step. Finally, the condensation of carboxylate **12** with *O*-(*tert*-butyldimethylsilyl)hydroxylamine in the presence of *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) followed by acid cleavage yielded the desired hydroxamate **4** [25].

For the synthesis of the α -substituted compounds **5a-d** and **6**, **7**, an improved shorter route was followed (Scheme 2 and 3). Carboxylates **13a-d** and **18**, **19**, obtained by reaction of 5-bromothiophene-2-sulfonylchloride with the appropriate commercial non-natural amino acid, were first converted into protected hydroxamates **14a-d** and **20**, **21** by condensation with *O*-(tetrahydro-2H-pyran-2-yl)hydroxylamine (THP-hydroxylamine) [26] in the

presence of EDC and then submitted to the Sonogashira coupling. The reaction conditions were optimized and the reaction was faster with improved yields with the use of microwaves [27,28]. After mild hydrolysis with 4N HCl [26], tetrahydropyranyl intermediates **15a-d** and **22, 23** afforded hydroxamates **5a-d** and **6, 7**.

3. Results and Discussion

3.1. MMPs inhibition

The newly synthesized hydroxamates **4-7** were tested *in vitro* for their ability to inhibit human recombinant MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-14 by a fluorometric assay, which uses a fluorogenic peptide [29] as the substrate. The broad-spectrum inhibitor **3** was taken as reference compound (Table 1).

As can be seen from Table 1, the introduction of various (*R*) aryl-alkyl chains in α position of the hydroxamate did not substantially affect activity toward MMP-2 compared with the un-substituted derivative **4**. In fact, all α -substituted compounds displayed nanomolar inhibitory activity for MMP-2 (IC_{50} values between 1.3 and 17 nM) comparable to compound **4** activity (IC_{50} = 1.5 nM). As expected, these data confirmed that the inhibitory activity toward MMP-2 was mainly determined by the P1' group, a 4-butylphenylethynylthiophene substituent, which is common to all compounds. On the contrary, the selectivity profile was greatly affected by the presence of the α -chain (P1 group). The cyclic derivatives **6** and **7**, bearing a thiazolidine and a tetrahydroisoquinoline ring respectively, were poorly selective over the anti-target MMPs (MMP-3 and MMP-8)., The tetrahydroisoquinoline derivative **7**, in particular, was the least selective of the series, exhibiting nanomolar activity toward all tested enzymes, except MMP-1. Among the non-cyclic inhibitors, the benzyloxy benzyl derivative **5a** was the most active toward both MMP-2 and MMP-9 (IC_{50} = 2.3 nM and 63 nM, respectively), showing the highest selectivity over MMP-1, -3, -8 and -14. This compound exhibited a 2000-fold selectivity for MMP-2 over MMP-1, a 1000-fold selectivity over MMP-14, a 200-fold selectivity over MMP-8 and a 80-fold selectivity over MMP-3. This very good selectivity is probably due to the presence of an elongated α -chain compared to the other derivatives. The benzothienyl derivative **5d** was the weakest inhibitor of MMPs, on all tested enzymes.

3.2. Molecular Modeling

Inhibitor **5a** was docked into the MMP-2 active site using the crystal structure available in the Protein Data Bank (PDB code: 1QIB [30]). The calculation was performed with the aid of Glide 5.5 in the extra-precision mode (XP) while Glidescore was used for poses scoring. The docking experiment resulted in only one pose, in which the hydroxamic acid moiety coordinates the catalytic zinc atom in a bidentate fashion, and establishes an H-bond with the A165 carbonyl oxygen (Figure 3). The sulfonamide moiety of **5a** forms a bifurcated H-bond with L164 and A165 backbone NH and allows the 4-butylphenylethynylthiophene moiety to plunge deeply into the large S1'-specificity pocket. In here, the butylphenyl ring is engaged in multiple hydrophobic interactions with L197, A217, L218, A220, T227, F232 and L234 side chains. The ethynyl group is found sandwiched between Y223 and H201, whereas the thiophene moiety is flanked by lipophilic residues such as L164 and V198. On the other side of the molecule, the benzyloxy-benzyl branch reaches out for the S3 pocket where the terminal phenyl ring forms aromatic-aromatic interactions with F168 and Y155 side chains. Clearly, all these interactions endow **5a** with a low nanomolar inhibitory activity toward MMP-2. Looking at the SARs herein developed, it is clear that MMP-2 can tolerate extremely different P1 groups ranging from long and flexible substituents, such as the benzyloxy-benzyl (**5a**), to relatively long and rigid groups, such as the benzophenone (**5b**) or to backbone-fused substituents (**6-7**) without a substantial difference in IC_{50} . However, the

P1 group seems to be useful in increasing the inhibitors selectivity at least toward some MMPs. For example, with respect to **4**, compound **5a** is less active toward MMP-3, -8, and -14 and, at least for MMP-8, and -14, this effect could be ascribed to the fact that these MMPs have a Ser and a Thr instead of the MMP-2-Y155, respectively. Moreover, from Table 1, it is also evident that compounds **4** and **7** are the most active toward MMP-2, but generally speaking they are also the least selective. This lack of selectivity could be attributed to a better chance for the hydroxamic acid to find the most favourable chelating geometry, either when only a hydrogen is present in the alpha position (**4**), or when there is a proper backbone cyclization, respectively (**6-7**). Compound **5b** has a slightly higher IC₅₀ with respect to **5a** and this seems to be due to a greater rigidity of the P1 group. In fact, in order to preserve the interaction pattern with the S3 cavity, the benzophenone moiety adopts an energetically unfavourable conformation. On the other hand, **5c** and **5d**, both possessing a P1 group not long enough to interact with F168 and Y155 S3 pocket residues, are less active toward MMP-2 than **5a**. Of note, the indole ring of **5c** can be superimposed to the first benzene of the benzyloxy-benzyl branch of **5a**, whereas the pyrrole establishes a π -interaction with the H211. Substitution of the NH group with sulfur in **5d** induces a certain clash with the aforementioned residue side chain which results in a change in the thiophene position in the active site and further loss of activity. Moreover, according to the binding mode found for **5c**, the P1 group seems to be partially exposed to the solvent and, in this environment, the indole ring (**5c**) is certainly more suitable than the benzothiophene ring.

3.3. Effect of MMPIs on U87MG glioma cell invasion and viability

We evaluated the ability of the newly synthesized gelatinase inhibitors **4-7** and the reference compound **3** to inhibit invasion of U87MG cells through Matrigel (Table 2). The number of invading cells, quantified by counting the cells on the lower surface of the transwell membrane after fixing with p-formaldehyde and staining with crystal violet, showed a marked reduction ($p < 0.0001$) after treatment for 24 h, with 5 nM of inhibitor. This effect was not due to MMPI cytotoxicity because, as shown in Table 2, at 5 nM the inhibitors showed no effect on glioblastoma cell viability ($p > 0.05$), as ascertained by the Trypan Blue exclusion assay, over a 24 h period.

Taken together, these results identify a new series of MMPIs with anti-invasive activity towards U87MG glioblastoma cells in the nanomolar range, without causing cytotoxic effects. Furthermore, all compounds, except the cyclic derivative **6**, were more active than the broad-spectrum inhibitor **3**. As shown in Figure 4A and 5, compounds **5c** and **5a**, in particular, were the most effective invasion inhibitors (48 and 43%, respectively). As depicted in figure 4B, compound **5a** showed a dose dependent effect on cell invasion, at concentrations of 1, 5, 25, 125 nM, yielding a IC₅₀ = 12 nM. These data suggest that MMP-2 is not the only enzyme implicated in U87MG cell invasion through Matrigel, since all the compounds tested showed similar inhibitory activity towards MMP-2 (Table 1) but yet exhibited widely different invasion inhibitory activities (ranging from 16 to 48% inhibition at 5 nM).

3.4. MMP-25 inhibition

The newly synthesized compounds **4**, **5a-d**, **6**, **7** and the reference compound **3** were also tested on human recombinant MT6-MMP (known as MMP-25) by fluorometric assay. This enzyme is a membrane-type MMP that is anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI) moiety, and is highly expressed in human neutrophils [31]. MMP-25 can degrade various extracellular matrix components, including type IV collagen, gelatin, fibrin, fibronectin, and chondroitin sulfate proteoglycan [32]. The body of work on GPI-MT-MMPs today is still surprisingly limited when compared to other MT-MMPs. Recently however, elevated MMP-25 mRNA expression was found in several

human cancers including brain (anaplastic astrocytomas and glioblastomas) [33], colon [32] and prostate cancer. In gliomas, expression of MMP-25 mRNA was suggested to contribute to disease progression [34]. Thus, we decided to evaluate the inhibitory activity of the compounds toward this enzyme, to ascertain whether the anti-invasive effect on U87MG cells could be correlated with MMP-25 inhibition. As reported in Table 1, compounds **5a** and **5c**, which displayed the highest invasion inhibitory activity were potent MMP-25 inhibitors ($IC_{50} = 38$ and 76 nM, respectively). However, compound **6** which exhibited the worst anti-invasive activity displayed only a slightly lower binding affinity ($IC_{50} = 170$ nM). Moreover, compounds with very similar anti-invasive activity on cells, like **5b** and **3**, exhibited a very different inhibitory potency toward MMP-25 ($IC_{50} = 340$ and 1.5 nM, respectively). Thus, similarly to MMP-2, there is not direct correlation between MMP-25 inhibition and anti-invasive activity of U87MG glioblastoma cells through Matrigel, for the MMPis herein reported. Of note, the MMP-25 sensitivity to this class of inhibitors resembles that of the gelatinases (MMP-2 and MMP-9) as opposed to the collagenases including MT1-MMP, lending credit to the hypothesis that the GPI-anchored MT-MMPs exhibit unique inhibitor sensitivities, which may correlate with their physiological functions. Further studies are needed to better ascertain the role of MMP-25 in glioma progression.

4. Conclusions

In this study, a series of new 4-butylphenyl(ethynylthiophene)sulfonamido-based hydroxamate inhibitors selective for MMP-2, relative to MMP-1 and MMP-14, was designed, synthesized and tested for their ability to inhibit glioma cell invasion *in vitro*. Among these compounds, a very promising derivative was identified (**5a**) with nanomolar activity for MMP-2 and good selectivity over the anti-targets MMP-8 and MMP-3, by fluorometric assay on isolated enzymes. The effect of these new compounds on cell viability and invasion through Matrigel was evaluated using human U87MG glioblastoma cells. No cytotoxicity was detected in the nanomolar range (5 nM) and compound **5a** inhibited cell invasion, with a $IC_{50} = 12$ nM. This compound exhibited nanomolar inhibitory activity towards MMP-25, a GPI-anchored MMP that may be involved in glioma progression. After the disappointing clinical results of broad-spectrum MMPis in cancer therapy of the last years, we hope that our findings, and those of others [35], will contribute to accelerate the discovery of novel and more selective MMPis with potent anti-invasive activity to be used individually or in combination with cytotoxic compounds [36,26]. The use of more specific and effective inhibitors should allow a reduction of the doses necessary to have therapeutic efficacy without the side effects typically associated with broad-spectrum MMPis.

5. Experimental section

5.1. Chemistry

Melting points were determined on a Kofler hotstage apparatus and are uncorrected. 1H and ^{13}C NMR spectra were determined with a Varian Gemini 200 MHz spectrometer. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references. Coupling constants J are reported in hertz; ^{13}C NMR spectra were fully decoupled. The following abbreviations are used: singlet (s), doublet (d), triplet (t), double-doublet (dd), broad (br) and multiplet (m). Where indicated, the elemental compositions of the compounds agreed to within $\pm 0.4\%$ of the calculated value. Chromatographic separations were performed on silica gel columns by flash column chromatography (Kieselgel 40, 0.040 – 0.063 mm; Merck) or using ISOLUTE Flash Si II cartridges (Biotage). Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F254) sheets that were visualized under a UV lamp and hydroxamic acids were visualized with $FeCl_3$ aqueous solution. Evaporation was performed *in vacuo* (rotating evaporator). Sodium sulfate was always used as the drying agent.

Microwave-assisted reactions were run in a CEM Discover LabMate microwave synthesizer. Commercially available chemicals were purchased from Sigma-Aldrich. D-amino acids were purchased from Bachem. Compound **3** was synthesized as reported in literature [37].

5.1.1. *tert*-Butyl 2-(5-bromothiophene-2-sulfonamido)acetate (**9**)—5-

bromothiophene 2-sulfonyl chloride **8** (500 mg, 2.21 mmol) was added to a solution of glycine *tert*-butyl ester hydrochloride (372 mg, 2.21 mmol) in H₂O (2.20 mL) and dioxane (2.20 mL) containing Et₃N (0.61 mL, 4.42 mmol). The reaction was stirred overnight at room temperature, then dioxane was evaporated and the residue was taken up with EtOAc and washed (x3) with water and brine. Organic layers were then collected, dried over Na₂SO₄, and evaporated *in vacuo*. The crude was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 8:1) and a yellow solid was obtained (568 mg, 1.77 mmol, 80% yield). ¹H-NMR (CDCl₃) δ: 1.43 (s, 9H); 3.78 (d, *J*=5.3 Hz, 2H); 5.14 (t, *J*=5.3 Hz, 1H); 7.08-7.10 (m, 1H); 7.38-7.40 (m, 1H).

5.1.2. *tert*-Butyl 2-(5-bromo-*N*-(*tert*-butoxycarbonyl)thiophene-2-sulfonamido)acetate (**10**)—*tert*-Butyl ester **9** (560 mg, 1.75 mmol) was dissolved in dry CH₂Cl₂ (2.18 mL) and DMAP (21.4 mg, 0.175 mmol) and Et₃N (0.27 mL, 1.92 mmol) were added. A solution of di-(*tert*-butyl)dicarbonate (439 mg, 2.01 mmol) in dry CH₂Cl₂ (3.04 mL) was added dropwise to the *tert*-butyl ester solution and the mixture was stirred at room temperature overnight. The solution was diluted with CH₂Cl₂, and treated with HCl 1N, water and brine. Organic layers were dried over Na₂SO₄ and evaporated *in vacuo* to give a yellow oil (648 mg, 1.54 mmol, 88% yield). ¹H-NMR (CDCl₃) δ: 1.45 (s, 18H); 4.41 (s, 2H); 7.06-7.08 (m, 2H); 7.61-7.63 (m, 1H).

5.1.3. *tert*-Butyl 2-(*N*-(*tert*-butoxycarbonyl)-5-((4-butyphenyl)ethynyl)thiophene-2-sulfonamido)acetate (11**)**—To a solution of **10** (400 mg, 0.95 mmol) in dry THF (19 mL), 1-butyl-4-ethynyl-benzene (0.2 mL, 1.14 mmol), Et₃N (0.26 mL, 1.9 mmol), triphenylphosphine (12.6 mg, 0.048 mmol), copper(I) iodide (9.14 mg, 0.048 mmol), and dichlorobis(triphenylphosphine)palladium(II) (33.4 mg, 0.048 mmol) were added. The mixture was stirred refluxing at 70 °C under Ar atmosphere for 24h, then it was diluted with water and extracted with EtOAc. Organic phases were washed with HCl 1N, brine and with a saturated solution of NaHCO₃, dried over Na₂SO₄ and evaporated under reduced pressure. The residue was purified by flash chromatography (*n*-hexane/EtOAc 14:1) using a Isolute Si (II) cartridge to yield a brown oil (131 mg, 0.25 mmol, 26% yield). ¹H-NMR (CDCl₃) δ: 0.93 (t, *J*=6.9Hz, 3H), 1.33-1.41 (m, 2H); 1.40 (s, 18H), 1.53-1.68 (m, 2H), 2.59 (t, *J*=7.8 Hz, 2H), 3.32 (br s, 2H), 7.17-7.23 (m, 3H), 7.42-7.46 (m, 2H), 7.55 (d, *J*=4.0 Hz, 1H).

5.1.4. 2-(5-((4-Butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-hydroxyacetamide (4**)**—*tert*-Butyl ester **11** (330 mg, 0.62 mmol) was dissolved in freshly distilled CH₂Cl₂ (4.82 mL) and TFA (2.71 mL, 35.24 mmol) was added dropwise to the chilled solution. The reaction mixture was stirred at 0 °C for 5h, the solvent was evaporated and the crude product was triturated with Et₂O. The product was used in the next step without further purification. Carboxylate **12** (218 mg, 0.58 mmol) was suspended in dry CH₂Cl₂ (15 mL) and *O*-(*tert*-butyldimethylsilyl)hydroxylamine (85 mg, 0.58 mmol) and 1-[3-(Dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (EDC) (166 mg, 0.87 mmol) were added. The reaction was stirred at room temperature overnight, diluted with CH₂Cl₂, washed with water, dried over Na₂SO₄ and concentrated *in vacuo*. The crude was purified by flash chromatography on silica gel (*n*-hexane/EtOAc 5:2) to give the pure *O*-silyl-intermediate as a yellow oil (87.3 mg, 0.17 mmol, 30% yield). ¹H-NMR (CDCl₃) δ: 0.17 (s, 6H); 0.92 (t, *J*=6.9Hz, 3H); 0.94 (s, 9H); 1.29-1.40 (m, 2H); 1.52-1.67 (m, 2H); 2.62

(t, $J=7.9$ Hz, 2H); 3.71 (br s, 1H); 3.96 (br s, 1H); 6.00 (br s, 1H); 7.15-7.19 (m, 3H); 7.40-7.44 (m, 2H); 7.48-7.50 (m, 2H); 8.835 (br s, 1H).

TFA (0.75 mL, 9.79 mmol) was added dropwise to a 0 °C solution of *O*-silyl-intermediate (87 mg, 0.17 mmol) in dry CH₂Cl₂ (2 mL), and the reaction was stirred at 0 °C for 5h. Trituration with Et₂O and further purification by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 1:12) gave the pure hydroxamate **4** as a white solid (33.4 mg, 0.09 mmol, 50% yield). Mp 145-147 °C; ¹H-NMR (DMSO-*d*₆) δ: 0.89 (t, $J=7.14$ Hz, 3H); 1.22-1.33 (m, 2H); 1.48-1.62 (m, 2H); 2.62 (t, $J=7.7$ Hz, 2H); 3.45 (s, 2H); 7.26-7.30 (m, 2H); 7.42-7.44 (m, 1H); 7.48-7.52 (m, 2H); 7.54-7.56 (m, 1H); 8.94 (br s, 1H); 10.62 (br s, 1H).

¹³C-NMR (DMSO-*d*₆) δ: 14.3, 22.3, 33.4, 35.7, 41.2, 86.5, 92.7, 120.1, 122.2, 127.0, 127.8, 132.2, 138.3, 166.2. Elemental Analysis for C₁₈H₂₀N₂O₄S₂: Calculated: %C, 55.08; %H, 5.14; %N, 7.14, Found %C, 55.13; %H, 5.17; %N, 7.10.

5.1.5. General procedure for the synthesis of carboxylates **13a-d**, **18**, **19**—5-

Bromothiophene-2-sulfonylchloride (1g, 4.43 mmol) was added to a solution of the appropriate non-natural commercial amino acid (4.43 mmol) in H₂O (4.43 mL) and dioxane (13.3 mL) containing Et₃N (1.24 mL, 8.86 mmol). The mixture was stirred at room temperature overnight, the dioxane was evaporated and the residue was treated with EtOAc and washed with HCl 1N and brine. Organic layers were then collected, dried over Na₂SO₄, and evaporated *in vacuo*.

5.1.6. (*R*)-3-(4-(Benzyloxy)phenyl)-2-(5-bromothiophene-2-sulfonamido)propanoic acid (**13a**)—

The title compound was prepared from H-D-Tyr(Bzl)-OH following the general procedure. The crude product was triturated with Et₂O and *n*-hexane to yield carboxylate **13a** as a yellow solid (56% yield). ¹H-NMR (DMSO-*d*₆) δ: 2.90 (dd, $J_1=4.95$ Hz, $J_2=13.55$ Hz, 2H); 3.35-3.40 (m, 1H); 5.04 (s, 2H); 6.80-6.84 (m, 2H); 7.04-7.08 (m, 2H); 7.25-7.46 (m, 7H).

5.1.7. (*R*)-3-(4-Benzoylphenyl)-2-(5-bromothiophene-2-sulfonamido)propanoic acid (**13b**)—

The title compound was prepared from H-p-Bz-D-Phe-OH following the general procedure. The crude product was triturated with Et₂O and *n*-hexane to yield carboxylate **13b** as a yellow solid (28% yield). ¹H-NMR (Acetone-*d*₆) δ: 3.01-3.26 (m, 1H); 3.26-3.35 (m, 1H); 4.25-4.37 (m, 1H); 7.10-7.12 (m, 1H); 7.25-7.27 (m, 1H); 7.33-7.38 (m, 1H); 7.42-7.46 (m, 2H); 7.53 (t, $J=1.83$ Hz, 1H); 7.57-7.64 (m, 2H); 7.67-7.71 (m, 2H); 7.76-7.81 (m, 2H).

5.1.8. (*R*)-2-(5-Bromothiophene-2-sulfonamido)-3-(1*H*-indol-3-yl)propanoic acid (**13c**)—

The title compound was prepared from H-D-Trp-OH following the general procedure. The crude product was triturated with Et₂O and *n*-hexane and further purified by flash chromatography using a Isolute Si (II) cartridge (CHCl₃/MeOH 9:1) to give **13c** as a green oil (71% yield). ¹H-NMR (CDCl₃) δ: 3.16-3.41 (m, 2H); 4.28-4.40 (m, 1H); 5.23 (d, $J=8.23$ Hz, 1H); 5.49 (br s, 1H); 6.77 (d, $J=3.67$ Hz, 1H); 6.97 (s, 1H); 7.08-7.21 (m, 3H); 7.35 (d, $J=7.87$ Hz, 1H); 7.50 (d, $J=7.87$ Hz, 1H); 8.05 (s, 1H).

5.1.9. (*R*)-3-(Benzo[*b*]thiophen-3-yl)-2-(5-bromothiophene-2-sulfonamido)propanoic acid (**13d**)—

The title compound was prepared from H-β-(3-Benzothieryl)-D-Ala-OH following the general procedure. Clear oil (76% yield). ¹H-NMR (Acetone-*d*₆) δ: 3.18-3.30 (m, 1H); 3.39-3.51 (m, 1H); 4.29-4.40 (m, 1H); 6.85 (d, $J=4.0$ Hz, 1H); 7.05 (d, $J=4.0$ Hz, 1H); 7.37-7.43 (m, 2H); 7.46 (s, 1H); 7.78-7.83 (m, 1H); 7.90-7.95 (m, 1H).

5.1.10. (*R*)-3-(5-Bromothiophen-2-ylsulfonyl)thiazolidine-4-carboxylic acid (18**)**

—The title compound was prepared from D-thiazolidine-4-carboxylic acid **16** following the general procedure. The crude product was triturated with Et₂O and *n*-hexane to furnish **18** as a yellow solid (55% yield). ¹H-NMR (DMSO-*d*₆) δ: 3.11-3.15 (m, 2H); 4.45 (d, *J*=10.5 Hz, 1H); 4.72 (d, *J*=10.5 Hz, 1H); 4.77-4.80 (m, 1H); 7.48 (d, *J*=4 Hz, 1H); 7.74 (d, *J*=4 Hz, 1H).

5.1.11. (*R*)-2-(5-Bromothiophen-2-ylsulfonyl)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (19**)**

—The title compound was prepared from D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid **17** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 1:1) to give **19** as a yellow oil (71% yield). ¹H-NMR (CDCl₃) δ: 3.21 (d, *J*=4 Hz, 2H); 4.61 (dd, *J*₁=15.2 Hz, *J*₂=27 Hz, 2H); 4.96 (t, *J*=5 Hz, 1H); 7.00 (d, *J*=4 Hz, 1H); 7.07-7.24 (m, 4H); 7.33 (d, *J*=4 Hz, 1H).

5.1.12. General procedure for the synthesis of 2-(5-bromothiophene-2-sulfonamido)-*N*-(tetrahydro-2H-pyran-2-yloxy)acetamides **14a-d, **20**, **21**—**

Carboxylates **13a-d**, **18**, **19** (1.46 mmol) were dissolved in dry DMF (2.21 mL) and *O*-(tetrahydro-2H-pyran-2-yl)hydroxylamine (530 mg, 4.53 mmol), *N*-methylmorpholine redistilled (0.48 mL, 4.38 mmol), hydroxybenzotriazole (HOBT) (237 mg, 1.75 mmol), and 1-[3-(Dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (EDC) (392 mg, 2.04 mmol) were added under N₂ atmosphere. After stirring for 2 h, the reaction mixture was diluted with EtOAc and washed with water, saturated solution of NaHCO₃, and brine. The organic phase was dried over Na₂SO₄, filtered and concentrated *in vacuo*.

5.1.13. (2*R*)-3-(4-(Benzyloxy)phenyl)-2-(5-bromothiophene-2-sulfonamido)-*N*-(tetrahydro-2H-pyran-2-yloxy)propanamide (14a**)**

—The title compound was prepared from carboxylic acid **13a** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 3:1) to yield a yellow foaming oil (74% yield). ¹H-NMR (CDCl₃) δ: 1.58 (br s, 4H); 1.78 (br s, 2H); 2.86-3.09 (m, 2H); 3.55-3.60 (m, 1H); 3.72-3.90 (m, 2H); 4.73-4.83 (m, 1H); 5.04 (s, 2H); 5.22 (br s, 1H); 6.83-6.88 (m, 2H); 6.95-6.99 (m, 3H); 7.15-7.19 (m, 1H); 7.36-7.42 (m, 5H); 8.69 (br s, 1H).

5.1.13. (2*R*)-3-(4-Benzoylphenyl)-2-(5-bromothiophene-2-sulfonamido)-*N*-(tetrahydro-2H-pyran-2-yloxy)propanamide (14b**)**

—The title compound was prepared from carboxylic acid **13b** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 2:1) to furnish a yellow oil (67% yield). ¹H-NMR (CDCl₃) δ: 1.58 (br s, 4H); 1.78 (br s, 2H); 3.01-3.24 (m, 2H); 3.55-3.63 (m, 1H); 3.78-3.92 (m, 1H); 3.98-4.08 (m, 1H); 4.79 (br s, 1H); 5.34 (br s, 1H); 6.99 (d, *J*=4 Hz, 1H); 7.18-7.25 (m, 3H); 7.45-7.64 (m, 3H); 7.70-7.81 (m, 4H); 8.69 (br s, 1H).

5.1.14. (2*R*)-2-(5-Bromothiophene-2-sulfonamido)-3-(1*H*-indol-3-yl)-*N*-(tetrahydro-2H-pyran-2-yloxy)propanamide (14c**)**

—The title compound was prepared from carboxylic acid **13c** following the general procedure. Yellow foaming solid (95% yield). ¹H-NMR (CDCl₃) δ: 1.56 (br s, 4H); 1.77 (br s, 2H); 3.11-3.35 (m, 2H); 3.47-3.53 (m, 1H); 3.72-3.90 (m, 1H); 4.02 (br s, 1H); 4.70-4.80 (m, 1H); 5.21 (br s, 1H); 6.75-6.79 (m, 1H); 7.02-7.22 (m, 4H); 7.34-7.38 (m, 1H); 7.43-7.47 (m, 1H); 8.10 (br s, 1H); 8.69 (br s, 1H).

5.1.15. (2*R*)-3-(Benzo[*b*]thiophen-3-yl)-2-(5-bromothiophene-2-sulfonamido)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)propanamide (14d)—The title compound was prepared from carboxylic acid **13d** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 5:2) to furnish a yellow oil (83% yield). ¹H-NMR (CDCl₃) δ: 1.57 (br s, 4H); 1.79 (br s, 2H); 3.11-3.27 (m, 1H); 3.42-3.69 (m, 2H); 3.62-3.93 (m, 1H); 3.99-4.09 (m, 1H); 4.83 (br s, 1H); 5.29 (br s, 1H); 6.62-6.66 (m, 1H); 6.96-7.02 (m, 1H); 7.21 (s, 1H); 7.36-7.40 (m, 2H); 7.62-7.68 (m, 1H); 7.82-7.98 (m, 1H); 8.82 (br s, 1H).

5.1.16. (4*R*)-3-(5-Bromothiophen-2-ylsulfonyl)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)thiazolidine-4-carboxamide (20)—The title compound was prepared from carboxylic acid **18** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 3:1) to furnish a foaming clear oil (83% yield). ¹H-NMR (CDCl₃) δ: 1.62 (br s, 4H); 1.83 (br s, 2H); 2.63 (s, 1H); 2.80-2.93 (m, 1H); 3.42-3.52 (m, 1H); 3.64-3.70 (m, 1H); 3.93-4.06 (m, 1H); 4.42-4.62 (m, 1H); 4.97-5.01 (m, 1H); 7.16 (d, *J*=4 Hz, 1H); 7.42 (d, *J*=4 Hz, 1H); 9.27 (s, 1H).

5.1.17. (3*R*)-2-(5-Bromothiophen-2-ylsulfonyl)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (21)—The title compound was prepared from carboxylic acid **19** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 3:1) to furnish a clear oil (70% yield). ¹H-NMR (CDCl₃) δ: 1.58 (br s, 4H); 1.73 (br s, 2H); 2.75-2.85 (m, 1H); 3.21-3.31 (m, 1H); 3.49-3.60 (m, 1H); 3.85-3.92 (m, 1H); 4.46-4.57 (m, 2H); 6.96-7.01 (m, 1H); 7.11-7.24 (m, 4H); 7.26-7.28 (m, 1H); 9.09 (br s, 1H).

5.1.18. General Procedure for the synthesis of 2-(5-((4-butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)acetamides 15a-d, 22, 23—A mixture of the appropriate 5-bromo-derivative **14a-d**, **20**, **21** (0.37 mmol), 1-butyl-4-ethynyl-benzene (0.08 mL, 0.45 mmol), Et₃N (0.1 mL, 0.74 mmol), triphenylphosphine (4.85 mg, 0.019 mmol), copper(I) iodide (3.52 mg, 0.019 mmol), and dichlorobis(triphenylphosphine)palladium(II) (4.85 mg, 0.019 mmol) in dry THF (2.96 mL) was submitted to microwave irradiation at 60 °C for 10 min (power 300W, 250 psi). The reaction mixture was diluted with water and extracted with EtOAc. The organic phase was washed with HCl 1N, brine, NaHCO₃ saturated solution, dried over Na₂SO₄, filtered and concentrated *in vacuo*.

5.1.19. (2*R*)-3-(4-(Benzyloxy)phenyl)-2-(5-((4-butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)propanamide (15a)—The title compound was prepared from the 5-bromo-derivative **14a** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 5:2) to furnish a brown solid (25% yield). ¹H-NMR (Acetone-*d*₆) δ: 0.93 (t, *J*=7.5 Hz, 3H); 1.26-1.45 (m, 2H); 1.54-1.67 (m, 8H); 2.66 (t, *J*=7.5 Hz, 2H); 2.96-3.06 (m, 2H); 3.46-3.63 (m, 1H); 3.83-3.99 (m, 1H); 4.73 (br s, 1H); 5.09 (s, 2H); 4.74-4.77 (m, 1H); 6.85-6.91 (m, 2H); 7.09-7.47 (m, 14H); 10.39 (br s, 1H).

5.1.20. (2*R*)-3-(4-Benzoylphenyl)-2-(5-((4-butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)propanamide (15b)—The title compound was prepared from the 5-bromo-derivative **14b** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 5:2) to furnish a yellow oil (66% yield). ¹H-NMR (Acetone-*d*₆) δ: 0.93 (t, *J*=7.5 Hz, 3H); 1.26-1.40 (m, 2H); 1.54-1.68 (m, 8H); 2.66 (t, *J*=7.5 Hz, 2H); 2.94-3.06 (m,

1H); 3.12-3.24 (m, 1H); 3.45-3.58 (m, 1H); 3.80-3.98 (m, 1H); 4.17-4.24 (m, 1H); 4.74-4.77 (m, 1H); 7.19-7.27 (m, 3H); 7.37-7.71 (m, 10H); 7.75-7.78 (m, 2H); 10.43 (br s, 1H).

5.1.21. (2*R*)-2-(5-((4-Butylphenyl)ethynyl)thiophene-2-sulfonamido)-3-(1*H*-indol-3-yl)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)propanamide (15c)—The title compound was prepared from the 5-bromo-derivative **14c** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 2:1) to furnish a yellow oil (55% yield). ¹H-NMR (CDCl₃) δ: 0.93 (t, *J*=7.5 Hz, 3H); 1.29-1.46 (m, 6H); 1.54-1.76 (m, 4H); 2.63 (t, *J*=7.5 Hz, 2H); 3.21-3.25 (m, 2H); 3.42-3.56 (m, 1H); 3.72-3.82 (m, 1H); 4.74-4.77 (m, 1H); 5.34 (br s, 1H); 6.89-6.93 (m, 1H); 7.02 (s, 1H); 7.08-7.49 (m, 9H); 8.16 (br s, 1H); 8.79 (br s, 1H).

5.1.22. (2*R*)-3-(Benzo[*b*]thiophen-3-yl)-2-(5-((4-butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)propanamide (15d)—The title compound was prepared from the 5-bromo-derivative **14d** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 5:2) to furnish a yellow oil (54% yield). ¹H-NMR (Acetone-*d*₆) δ: 0.93 (t, *J*=7.5 Hz, 3H); 1.30-1.42 (m, 2H); 1.52-1.66 (m, 8H); 2.67 (t, *J*=7.5 Hz, 2H); 3.14-3.25 (m, 1H); 3.32-3.43 (m, 1H); 3.47-3.59 (m, 1H); 3.82-3.98 (m, 1H); 4.17-4.29 (m, 1H); 4.76 (br s, 1H); 7.02 (d, *J*=4 Hz, 1H); 7.29-7.52 (m, 9H); 7.74-7.83 (m, 1H); 7.88-7.96 (m, 1H); 10.44 (br s, 1H).

5.1.23. (4*R*)-3-(5-((4-Butylphenyl)ethynyl)thiophen-2-ylsulfonyl)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)thiazolidine-4-carboxamide (22)—The title compound was prepared from the 5-bromo-derivative **20** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 3:1) to furnish a yellow oil (50% yield). ¹H-NMR (Acetone-*d*₆) δ: 0.92 (t, *J*=7.5 Hz, 3H); 1.30-1.45 (m, 2H); 1.57-1.74 (m, 8H); 2.67 (t, *J*=7.7 Hz, 2H); 2.97-3.07 (m, 2H); 3.25-3.33 (m, 1H); 3.49-3.55 (m, 1H); 3.99-4.10 (m, 1H); 4.70-4.84 (m, 2H); 4.98 (br s, 1H); 7.29-7.32 (m, 2H); 7.44 (d, *J*=4 Hz, 1H); 7.48-7.53 (m, 2H); 7.76 (d, *J*=4 Hz, 1H); 10.67 (s, 1H).

5.1.24. (3*R*)-2-(5-((4-Butylphenyl)ethynyl)thiophen-2-ylsulfonyl)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (23)—The title compound was prepared from the 5-bromo-derivative **21** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 3:1) to furnish a yellow oil (62% yield). ¹H-NMR (Acetone-*d*₆) δ: 0.92 (t, *J*=7.5 Hz, 3H); 1.26-1.40 (m, 4H); 1.51-1.66 (m, 6H); 2.60 (s, 2H); 2.66 (t, *J*=7.7 Hz, 2H); 3.07-3.12 (m, 1H); 3.42-3.57 (m, 1H); 3.82-3.98 (m, 1H); 4.67-4.82 (m, 3H); 7.15-7.19 (m, 3H); 7.27-7.30 (m, 2H); 7.45-7.49 (m, 2H); 7.56-7.59 (m, 1H); 10.48 (br s, 1H).

5.1.25. General procedure for the synthesis of 2-(5-((4-butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-hydroxyacetamides 5a-d, 6, 7—Protected Hydroxamates **15a-d**, **22**, **23**. (0.33 mmol) were dissolved in dioxane (0.6 mL) and a solution of HCl 4*N* (5.94 mL) was added dropwise followed by MeOH (1.32 mL). After stirring for 30 min, the reaction was evaporated *in vacuo*.

5.1.26. (*R*)-3-(4-(Benzyloxy)phenyl)-2-(5-((4-butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-hydroxypropanamide (5a)—The title compound was prepared from the THP-protected hydroxamate **15a** following the general procedure. The crude product was triturated with Et₂O and *n*-hexane to furnish a light brown solid (99% yield). Mp

201-203 °C; ¹H-NMR (Acetone-*d*₆) δ: 0.92 (t, *J*=7.5 Hz, 3H); 1.26-1.45 (m, 2H); 1.53-1.68 (m, 2H); 2.65 (t, *J*=7.5 Hz, 2H); 3.00 (dd, *J*₁=5.9 Hz, *J*₂=7.9 Hz, 2H); 3.96-4.08 (m, 1H); 5.09 (s, 2H); 6.84-6.88 (m, 2H); 7.07-7.47 (m, 13H); 8.11 (br s, 1H); 10.39 (br s, 1H). ¹³C-NMR (Acetone-*d*₆) δ: 14.45, 23.23, 34.47, 36.41, 57.90, 70.65, 81.73, 115.64, 128.58, 128.75, 129.46, 129.90, 131.50, 132.57, 132.70, 132.90, 138.71, 145.75, 158.97. Elemental Analysis for C₃₂H₃₂N₂O₅S₂: Calculated: %C, 65.28; %H, 5.48; %N, 4.76, Found %C, 65.30; %H, 5.53; %N, 4.71.

5.1.27. (*R*)-3-(4-Benzoylphenyl)-2-(5-((4-butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-hydroxypropanamide (5b)—The title compound was prepared from the THP-protected hydroxamate **15b** following the general procedure. The crude product was triturated with *n*-hexane to give a light yellow solid (75% yield). Mp 179-181 °C; ¹H-NMR (DMSO-*d*₆) δ: 0.89 (t, *J*=7.5 Hz, 3H); 1.20-1.39 (m, 2H); 1.48-1.63 (m, 2H); 2.60 (t, *J*=7.7 Hz, 2H); 2.80-2.97 (m, 2H); 3.86-3.98 (m, 1H); 7.19-7.33 (m, 8H); 7.47-7.70 (m, 7H); 8.70 (d, *J*=8.8 Hz, 1H); 9.00 (br s, 1H); 10.77 (s, 1H). ¹³C-NMR (DMSO-*d*₆) δ: 13.75, 21.67, 32.74, 34.67, 55.47, 80.45, 95.67, 118.11, 127.44, 128.32, 128.59, 129.32, 129.40, 129.52, 131.20, 131.29, 135.02, 137.04, 142.05, 142.41, 144.14, 166.57, 195.02. Elemental Analysis for C₃₂H₃₀N₂O₅S₂: Calculated: %C, 65.51; %H, 5.15; %N, 4.77; Found %C, 65.57; %H, 5.19; %N, 4.70.

5.1.28. (*R*)-2-(5-((4-Butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-hydroxy-3-(1*H*-indol-3-yl)propanamide (5c)—The title compound was prepared from the THP-protected hydroxamate **15c** following the general procedure. The crude product was triturated with Et₂O and *n*-hexane to furnish a light gray solid (92% yield). Mp 90-91 °C; ¹H-NMR (Acetone-*d*₆) δ: 0.93 (t, *J*=7.5 Hz, 3H); 1.26-1.40 (m, 2H); 1.54-1.69 (m, 2H); 2.67 (t, *J*=7.7 Hz, 2H); 2.99-3.09 (m, 1H); 3.20-3.29 (m, 1H); 4.05-4.16 (m, 1H); 6.96-7.38 (m, 8H); 7.48-7.52 (m, 2H); 10.04 (br s, 1H). ¹³C-NMR (Acetone-*d*₆) δ: 14.45, 23.25, 34.48, 36.43, 56.74, 112.54, 119.32, 119.84, 122.38, 125.09, 129.93, 135.57. Elemental Analysis for C₂₇H₂₇N₃O₄S₂: Calculated: %C, 62.17; %H, 5.22; %N, 8.06; Found: %C, 62.21; %H, 5.24; %N, 8.00.

5.1.29. (*R*)-3-(Benzo[*b*]thiophen-3-yl)-2-(5-((4-butylphenyl)ethynyl)thiophene-2-sulfonamido)-*N*-hydroxypropanamide (5d)—The title compound was prepared from the THP-protected hydroxamate **15d** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 2:1) to furnish a yellow oil (25% yield). ¹H-NMR (Acetone-*d*₆) δ: 0.93 (t, *J*=7.5 Hz, 3H); 1.28-1.41 (m, 2H); 1.58-1.66 (m, 2H); 2.67 (t, *J*=7.5 Hz, 2H); 3.16-3.27 (m, 1H); 3.34-3.44 (m, 1H); 4.16-4.27 (m, 1H); 6.91 (d, *J*=4 Hz, 1H); 7.28-7.32 (m, 2H); 7.35-7.43 (m, 4H); 7.48-7.52 (m, 2H); 7.74-7.79 (m, 1H); 7.88-7.92 (m, 1H); 8.46 (br s, 1H); 10.42 (br s, 1H). ¹³C-NMR (Acetone-*d*₆) δ: 13.96, 22.71, 33.93, 35.90, 55.29, 80.98, 96.40, 119.69, 122.09, 123.46, 124.59, 124.81, 125.70, 129.41, 131.27, 131.89, 132.02, 139.01, 140.92, 142.09, 145.22, 167.76. Elemental Analysis for C₂₇H₂₆N₂O₄S₃: Calculated: %C, 60.20; %H, 4.86; %N, 5.20; Found: %C, 60.28; %H, 5.00; %N, 5.10.

5.1.30. (*R*)-3-(5-((4-Butylphenyl)ethynyl)thiophen-2-ylsulfonyl)-*N*-hydroxythiazolidine-4-carboxamide (6)—The title compound was prepared from the THP-protected hydroxamate **22** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 2:1) to furnish a yellow solid (25% yield). Mp 152-154 °C; ¹H-NMR (Acetone-*d*₆) δ: 0.94 (t, *J*=7.5 Hz, 3H); 1.28-1.49 (m, 2H); 1.56-1.72 (m, 2H); 2.69 (t, *J*=7.7 Hz, 2H); 3.00 (dd, *J*₁=7 Hz, *J*₂=10.5 Hz, 1H); 3.30 (dd, *J*₁=4 Hz, *J*₂=8.5 Hz, 1H); 4.65 (d, *J*₁=10.5 Hz, 1H); 4.79 (d, *J*₁=10.5 Hz, 1H); 4.69-4.76 (m, 1H); 7.29-7.32 (m, 2H); 7.44 (d, *J*=4 Hz, 1H); 7.75-7.77 (m,

2H); 7.76 (d, $J=4$ Hz, 1H); 8.22 (br s, 1H); 10.57 (br s, 1H). ^{13}C -NMR (Acetone- d_6) δ : 13.90, 14.43, 23.21, 34.43, 35.32, 36.40, 53.28, 64.95, 98.22, 119.84, 129.95, 131.83, 132.61, 133.61, 134.78, 137.78, 146.05, 160.62. Elemental Analysis for $\text{C}_{20}\text{H}_{22}\text{N}_2\text{O}_4\text{S}_3$: Calculated: %C, 53.31; %H, 4.92; %N, 6.22; Found: %C, 53.40; %H, 4.96; %N, 6.18.

5.1.31. (*R*)-2-(5-((4-Butylphenyl)ethynyl)thiophen-2-ylsulfonyl)-*N*-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (7)—The title compound was prepared from the THP-protected hydroxamate **23** following the general procedure. The crude product was purified by flash chromatography using a Isolute Si (II) cartridge (*n*-hexane/EtOAc 2:1) to furnish a yellow semi-solid (32% yield). ^1H -NMR (Acetone- d_6) δ : 0.92 (t, $J=7.5$ Hz, 3H); 1.26-1.40 (m, 2H); 1.52-1.68 (m, 2H); 2.65 (t, $J=7.7$ Hz, 2H); 2.92 (dd, $J_1=4$ Hz, $J_2=20$ Hz, 1H); 3.16 (dd, $J_1=8$ Hz, $J_2=20$ Hz, 1H); 4.52-4.72 (m, 3H); 7.14-7.30 (m, 6H); 7.45-7.49 (m, 2H); 7.58 (d, $J=4$ Hz, 1H); 8.08 (br s, 1H); 10.42 (br s, 1H). ^{13}C -NMR (Acetone- d_6) δ : 13.83, 14.38, 23.14, 34.35, 36.32, 46.92, 55.72, 81.08, 97.41, 119.86, 127.33, 127.62, 128.27, 129.07, 129.84, 130.70, 132.48, 133.17, 133.41, 133.48, 133.68, 139.58, 145.83, 167.57. Elemental Analysis for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4\text{S}_2$: Calculated: %C, 63.13; %H, 5.30; %N, 5.66; Found: %C, 63.20; %H, 5.32; %N, 5.60.

5.2. MMP inhibition assays [38]

Recombinant human MMP-14 catalytic domain was a kind gift of Prof. Gillian Murphy (Department of Oncology, University of Cambridge, UK). Pro-MMP-1, pro-MMP-2, pro-MMP-3, pro-MMP-8, and pro-MMP-9 were purchased from Calbiochem.

Proenzymes were activated immediately prior to use with *p*-aminophenylmercuric acetate (APMA 2 mM for 1 h at 37 °C for MMP-2 and MMP-8, APMA 2 mM for 2 h at 37 °C for MMP-1, 1 mM for 1 h at 37 °C for MMP-9). Pro-MMP-3 was activated with trypsin 5 $\mu\text{g}/\text{mL}$ for 30 min at 37 °C followed by soybean trypsin inhibitor 62 $\mu\text{g}/\text{mL}$. For assay measurements, the inhibitor stock solutions (10 mM in DMSO) were further diluted, at 7 different concentrations for each MMP in the fluorometric assay buffer (FAB: Tris 50 mM, pH=7.5, NaCl 150 mM, CaCl_2 10 mM, Brij 35 0.05% and DMSO 1%). Activated enzyme (final concentration 0.5 nM for MMP-2, 1.3 nM for MMP-9, 1.5 nM for MMP-8, 5.0 nM for MMP-3, 1.0 nM for MMP-14cd, 2.0 nM for MMP-1) and inhibitor solutions were incubated in the assay buffer for 2 h at 25 °C. After the addition of the fluorogenic substrate Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)- NH_2 (Sigma) for MMP-3 and Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg- NH_2 (Bachem) for all the other enzymes in DMSO (final concentration 2 μM), the hydrolysis was monitored every 15 s for 15 min, recording the increase in fluorescence ($\lambda_{\text{ex}}=325$ nm, $\lambda_{\text{em}}=395$ nm) with a Molecular Devices SpectraMax Gemini XS plate reader. The assays were performed in duplicate in a total volume of 200 μL per well in 96-well microtiter plates (Corning black, NBS). The MMP inhibition activity was expressed in relative fluorescent units (RFU). Percent of inhibition was calculated from control reactions without the inhibitor. IC_{50} was determined using the formula: $v_i/v_o = 1/(1 + [I]/\text{IC}_{50})$, where V_i is the initial velocity of substrate cleavage in the presence of the inhibitor at concentration $[I]$ and V_o is the initial velocity in the absence of the inhibitor. Results were analyzed using SoftMax Pro software [39] and GraFit software [40].

5.3. Molecular Modeling. Docking simulations

Molecular docking of **5a** into the X-ray structure of MMP-2 (PDB code: 1QIB) was carried out using the Glide 5.5 program [41]. Maestro 9.0.211 [42] was employed as the graphical user interface and Figure 4 was rendered by the Chimera software package [43].

5.3.1. Ligand and Protein Setup—The inhibitor structure was first generated through the Dundee PRODRG2 Server [44]. Then, CM1 atomic charges [45,46] were computed via

single-point AM1 calculations using OPLS-AA [47] optimized geometries and the BOSS program [48,49]. The target protein was prepared through the Protein Preparation Wizard of the graphical user interface Maestro and the OPLS-2001 force field. Water molecules were removed, hydrogen atoms were added, a +2 charge was assigned to the zinc ion in the active site and minimization was performed until the RMSD of all heavy atoms was within 0.3 Å of the crystallographically determined positions. The binding pocket was identified by placing a 20 Å cube around the catalytic ion.

5.3.2. Docking Setting—Molecular docking calculations were performed with the aid of Glide 5.5 in extra-precision (XP) [50,51] mode, using Glidescore for ligand ranking. A constraint that forced the interaction with the metal ion was included. For multiple ligand docking experiments, an output maximum of 5000 ligand poses per docking run with a limit of 100 poses for each ligand was adopted.

5.4. Cells and Reagents

Cell culture media and growth supplements were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Non-essential amino acids (1%) were from GIBCO (Milan, Italy). Trypan blue was from Sigma/RBI (Natick, MA). All other reagents were from standard commercial sources. The human GBM cell line U87MG was obtained from the National Institute for Cancer Research of Genoa. It was maintained in RPMI medium supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1% non-essential amino acids, at 37 °C in humidified atmosphere composed of 5% CO₂ and 95% O₂.

5.4.1. Cell treatment—U87MG cells were seeded at 30,000 cells/cm² in serum-free medium and incubated in the absence (control) or presence of the MMPi for 24 h. The MMPi were tested at a single concentration (5 nM). Compound **5a** was also assayed at concentrations of 1, 5, 25, 125 nM to ascertain its effect on cell invasion through Matrigel. The vehicle (DMSO) in which the compounds were dissolved never exceeded 1% of the final volume of culture medium.

5.4.2. Trypan Blue exclusion assay—Cell viability was ascertained in serum free medium containing 5 nM of the various compounds, using the Trypan Blue exclusion assay, as previously reported [36,52]. Briefly, after 24 h incubation, lightly trypsinized cells were collected by centrifugation at 800 x g for 5 min, stained with Trypan Blue dye (0.4% solution in 0.9% NaCl), and counted using a haemocytometer chamber (Burker counting chamber).

5.4.3. Invasion assay of U87MG glioblastoma cells through Matrigel—The effect of MMPi on cell invasion through Matrigel was evaluated using a human glioblastoma cell line (U87MG) as follows: the surface of the transwell (24 Transwell 6.5 mm diameter 8.0 µm pore size polycarbonate filters, Costar, Corning, NY) was coated with 25 µL of Matrigel basement membrane matrix (BD Biosciences) (0.32 mg/mL) at room temperature overnight to form a genuine reconstituted basement membrane. The U87MG cells were suspended in serum free medium (60,000/400 µL), treated with 5 nM of each MMP inhibitor, and added to the upper compartment of the transwell, while 200 µL of RPMI containing 10% foetal bovine serum was added to the lower compartment. The cells were allowed to invade through the matrices at 37 °C for 24 h. The number of invading cells was quantified by counting the cells on the lower surface of the transwell membrane after fixing with *p*-formaldehyde and staining with crystal violet. Non-migrating cells on the upper surface were removed with a cotton bud. Pictures of randomly picked light microscope fields were taken (5 fields for each filter), and cells were counted using ImageJ Software[53]. Each

compound concentration was tested in duplicate, and the experiments were repeated at least three times.

Statistical analyses were performed by one-way ANOVA (with Bonferroni post-test) by using the GraphPad Prism computer program [54]. P value <0.05 was considered statistically significant. All data are presented as the mean $\pm$ S.E.M., derived from at least three independent experiments done in duplicate.

5.5. MMP-25 inhibition assay

The catalytic domain of MT6-MMP (MMP-25) was expressed in house, in *E. coli* followed by purification and refolding. Fluorescence was measured with a Photon Technology International (PTI) spectrofluorometer, equipped with the RadioMaster™ and FeliX™ hardware and software, respectively. The assays were carried out at 25 °C and the cuvette holder was thermostated at the same temperature. Excitation and emission band passes of 3 and 3 nm, respectively, were used. Fluorescence measurements were taken every 4 s. The enzymatic activity of MMP-25 was monitored with the fluorescence-quenched substrate MOCacPLGLA₂pr(Dnp)AR-NH₂ at excitation and emission wavelengths of 328 and 393 nm, respectively. Fluorogenic substrate was obtained from Peptides International, Inc. (Louisville, KY). For competitive inhibition, initial rates were obtained by adding enzyme to a mixture of fluorogenic substrate and varying concentrations of inhibitor in buffer R (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.01% Brij-35, and 5% DMSO) final volume 1 mL, and monitoring the increase in fluorescence with time for 10 minutes. The initial velocities were determined by linear regression analysis of the fluorescence versus time traces using FeliX™. The initial rates were fitted to Eq. 1, where v_i and v_0 represent the initial velocity in the presence and absence of inhibitor, respectively, using the program SCIENTIST [55].

$$v_i/v_0 = (K_m + [S]) / (K_m(1 + [I]/K_i) + [S]) \quad (\text{Eq. 1})$$

Finally, MMP-25 inhibition data expressed as K_i have been converted in IC₅₀ values, according to the equation:

$$\text{IC}_{50} = K_i(1 + [S]/K_m)$$

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Research highlights

- MMP-2, MMP-9 and MMP-25 have been recognized to be highly expressed in gliomas.
- A series of sulfonamido-based hydroxamates was designed and synthesized.
- Derivative **5a** was found to have nanomolar activity toward MMP-2, MMP-9 and MMP25.
- This compound also proved to have anti-invasive activity on U87MG glioma cell line.

Figure 1.
Broad spectrum MMPi used in clinical trials for cancer.

Figure 2.

a) Evolution of the new 4-butyphenylethynyl-thiophene derivatives scaffold. **b)** Schematic representation of the new compounds substituents (**P1'**, **P1**), interacting with the principal binding pockets of MMPs (**S1'**, **S1**).

Figure 3. Docked conformation of **5a** (cyan) in the MMP-2 active site shown as an orange ribbon. The Zn(II) ion has been represented as violet sphere. Interacting residues are displayed as orange sticks. H-Bonds are displayed as blue lines.

Figure 4. Effect of MMPIs on U87MG cell invasion. **(A)** Treatment of cells with compounds **4-7**, and the reference compound **3** (5 nM), for 24 h reduced glioma cell invasion. **(B)** Dose-dependent cell invasion inhibition following treatment of compound **5a** for 24 h. The IC_{50} value represents the compound concentration that caused a 50% reduction of tumor cell invasion. The results are expressed as the mean value of the percentage of cell invasion inhibition after treatment with the MMPIs compared with untreated control cells. *** = statistically significant result ($p < 0.0001$).

Figure 5. Effect of **5a**, **5c** and **3** on U87MG cell invasion. Cell invasion was ascertained using the Matrigel basement membrane transwell assay, as described in the Experimental Section. Photographs of the invasive cells treated with vehicle (DMSO), compounds **5a**, **5c** and the reference compound **3**, at 200 $\times$ magnification.

Scheme 1.

Reagents and conditions: (a) glycine *tert*-butyl ester HCl, Et₃N, H₂O, dioxane; (b) (Boc)₂O, Et₃N, DMAP, CH₂Cl₂; (c) 1-butyl-4-ethynyl-benzene, CuI, Et₃N, Pd(Ph₃)₂Cl, PPh₃, THF, 70 °C; (d) TFA, CH₂Cl₂, 0 °C; (e) TBDMSiONH₂, EDC, CH₂Cl₂; (f) TFA, CH₂Cl₂, 0 °C.

Scheme 2.

Reagents and conditions: (i) appropriate D-amino acid, Et₃N, H₂O, dioxane; (ii) THPONH₂, HOBT, NMM, EDC, DMF; (iii) 1-butyl-4-ethynylbenzene, CuI, Et₃N, Pd(Ph₃)₂Cl, PPh₃, THF, microwaves, 300 W, 60 °C, 10 min; (iv) 4N HCl, dioxane, MeOH.

Scheme 3.

Reagents and conditions: (a) 5-bromothiophene-2-sulfonyl chloride, Et₃N, H₂O, dioxane; (b) THPONH₂, HOBT, NMM, EDC, DMF; (c) 1-butyl-4-ethynyl-benzene, CuI, Et₃N, Pd(Ph₃)₂Cl, PPh₃, THF, microwaves, 300 W, 60 °C, 10 min; (d) 4N HCl, dioxane, MeOH.

Table 1

In vitro^a activity (IC₅₀ nM values) of 4-butylethynylthiophene derivatives **4**, **5a-d**, **6**, **7** and the reference compound **3**.

Compd	R	MMP-1	MMP-2	MMP-3	MMP-8	MMP-9	MMP-14	MMP-25
4	H	13000	1.5	46	200	79	700	34
5a		4800	2.3	180	490	63	2100	38
5b		37000	4.0	84	440	50	6600	340
5c		2300	6.0	93	280	77	1700	76

4, 5a-d

6

7

Compd	R	MMP-1	MMP-2	MMP-3	MMP-8	MMP-9	MMP-14	MMP-25
5d		56000	17	320	1700	650	23000	220
6		21000	2.5	38	500	21	1600	170
7		6700	1.3	11	30	4.3	112	3.4
3		56	25	16	7.7	4.8	23	1.5

^a Enzymatic activity data are mean values for three independent experiments performed in duplicate. SD were within $\pm 10\%$.

Table 2

Effect of compounds **4-7** and reference compound **3** on U87MG glioblastoma cell viability and invasion through Matrigel.

Compd	Dead Cells (%)	Inhibition of Cell Invasion (%) ^a
DMSO	10.0±1.04	0.00
4	11.5±0.20	39.5±1.55
5a	12.3±0.44	42.6±0.97
5b	9.38±2.77	27.5±1.66
5c	6.80±1.24	48.4±2.80
5d	14.4±0.07	34.0±1.45
6	17.8±1.28	16.0±0.60
7	13.6±4.15	35.9±1.45
3	10.8±1.81	28.3±0.40

^aPercent inhibition of cell invasion observed at 5 nM of compound, corrected for cell death by subtracting the percentage of dead cells which, on average, did not exceed ~15%, as shown in column 2.