Sugar-based arylsulfonamide carboxylates as selective and water-soluble Matrix Metalloproteinase-12 inhibitors

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Abstract: Matrix Metalloproteinase-12 (MMP-12) can be considered an attractive target to study selective inhibitors useful in the development of new therapies for lung and cardiovascular diseases. In this study, a new series of arylsulfonamide carboxylates, with increased hydrophilicity due to the conjugation with a □-N-acetyl-D-glucosamine moiety, were designed and synthesized as MMP-12 selective inhibitors. Their inhibitory activity was evaluated on human MMPs by fluorimetric assay and a crystallographic analysis was carried out to characterize their binding mode. Among these glycoconjugates, a nanomolar MMP-12 inhibitor with improved water solubility (compound 3) was identified.

Introduction

MMP-12, or macrophage metalloelastase, belongs to the matrix metalloproteinases (MMPs) family of enzymes, a well known class of endopeptidases able to degrade all the components of the extracellular matrix (ECM). MMP-12 is a zinc-dependent enzyme mainly produced by macrophages and its principal substrates are elastin, the major constituent of alveolar walls, and other proteins such as type IV collagen, fibronectin, laminin, gelatin, vitronectin, and chondroitin sulfates.[1] MMP-12 has been involved in acute and chronic pulmonary inflammatory diseases. such as chronic obstructive pulmonary disease (COPD) and emphysema.[2] Moreover, overexpression of MMP-12 has been associated with lung cancer. In particular, the clinical importance of MMP-12 has been demonstrated in non-small cell lung cancer (NSCLC) development[3] with a critical role played by MMP-12 up-regulation in the transition from emphysema to lung cancer.[4] Finally, MMP-12 has been shown to be critical in the initiation and progression of atherosclerosis by stimulating the transformation of fatty streaks into fibrous plaques.[5] These observations suggest that MMP-12 selective inhibitors could be potentially useful for the treatment of lung and cardiovascular diseases. Selective MMP-12 inhibitors were recently reported by Dive et al. for atherosclerosis (RXP470.1),[6] by Li et al. for the potential treatment of asthma (MMP145)[7] and by Astra Zeneca for COPD (AZD1236),[8] among others. The principal obstacles that hinder clinical development of MMP inhibitors (MMPI) are related to the inadequate selectivity for the target enzyme, the poor water solubility with consequent poor oral bioavailability, and long-term toxicity. A good water solubility of MMPI is highly desirable above all for inhibitors used in the topical treatment of lung pathologies, such as emphysema and COPD, that require good solubility in aqueous biological fluids. Compounds with an improved hydrophilicity are likely to have a better bioavailability and less side effects due to cross-inhibition of anti-target zinc-proteases

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.2016xxxxx. It contains synthesis and characterization of all intermediate compounds, and a table with detailed crystallographic data.

(caused by a high dosage).[9] Therefore, in order to obtain potent and selective MMP-12 inhibitors endowed with an increased water solubility, we undertook the design and synthesis of a series of arylsulfonamide carboxylates conjugated with a □-*N*-acetyl-D-glucosamine moiety, compounds **2-11** (Figure 1). Our starting hypothesis was that the insertion of a sugar moiety in a proper position of the arylsulfonamido scaffold could increase water solubility without interfering with the inhibitor potency.[10] To prove that, the inhibitory activity of the new glycoconjugates was tested *in vitro* using recombinant MMPs and X-ray crystallographic studies were carried out to rationalize the results. Furthermore, the physicochemical properties of the most promising derivatives were evaluated.

Results and Discussion

Design of glycoconjugate MMP-12 inhibitors.

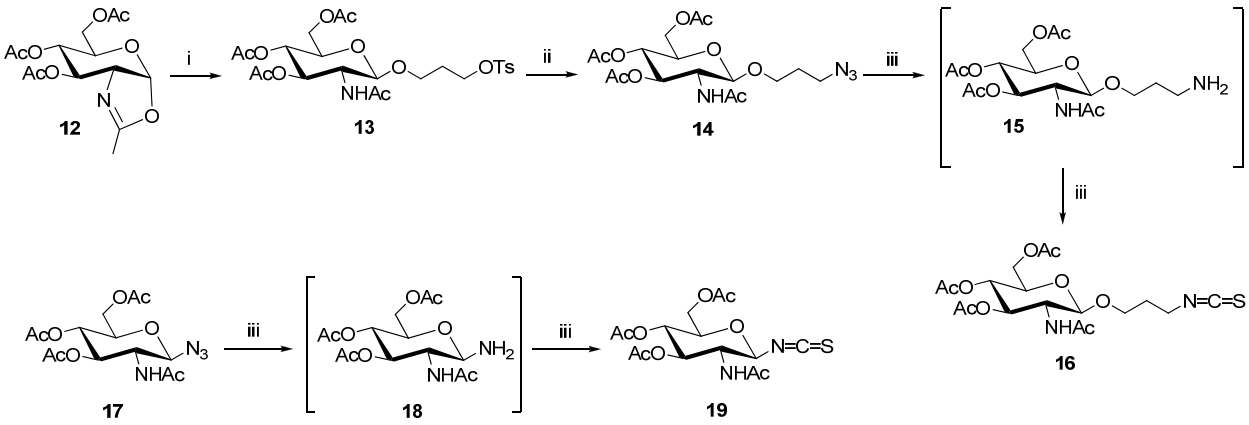
Previous studies by Attolino et al.[11] have proved that the introduction of hydrophilic chains on the nitrogen atom of a hydroxamate-based sulfonamide scaffold does not substantially change the affinity of the inhibitors for MMPs. Based on these findings, in the present study we modified the structure of the recently reported carboxylate MMP-12 inhibitor **1**[12] (Figure 1) by introducing a glycosidic residue on its sulfonamide nitrogen (P2' substituent) through insertion of a suitable spacer. This choice was also supported by the X-ray structure of compound **1**-MMP-12 complex[12] which has shown that the benzamidoethyl group does not directly participate in binding. □-*N*-acetyl-D-glucosamine (GlcNAc) was chosen for the preparation of the novel inhibitors **2-11** (Figure 1) because it is a very common monosaccharide in nature. In fact, GlcNAc is present in many glycostructures (e.g. chitin and chitooligomers), is crucial for protein and lipid glycosylation, and has a relevant role in the synthesis of the aberrant glycosylated molecules on cancer cell surfaces (multiantennary structures).[13] The conjugation between GlcNAc and the MMP inhibitor scaffold was achieved through the introduction of a thioureido group (compounds **2-5** and **10-11**, Table 1 and 3) or a 1,2,3-triazole group (compounds **6**-**9**, Table 2). These groups were chosen as linkers because 1,2,3-triazoles show topological and electronic similarities with amide bonds and for their inertness *in vivo* with respect to oxidation and reduction processes,[14] while the thioureido group is biocompatible and stable in most bio-systems. A *n*-propyloxy chain was inserted between the inhibitor and the sugar moiety in both series of derivatives, compounds **4**, **5** (Scheme 3) and **8**, **9** (Scheme 4), in order to evaluate the influence of a longer spacer on the affinity for the enzyme. Finally, also the sugar-protected analogues, acetylated on hydroxyl groups (compounds **2**, **4** and **6**, **8**), were evaluated.

Figure 1

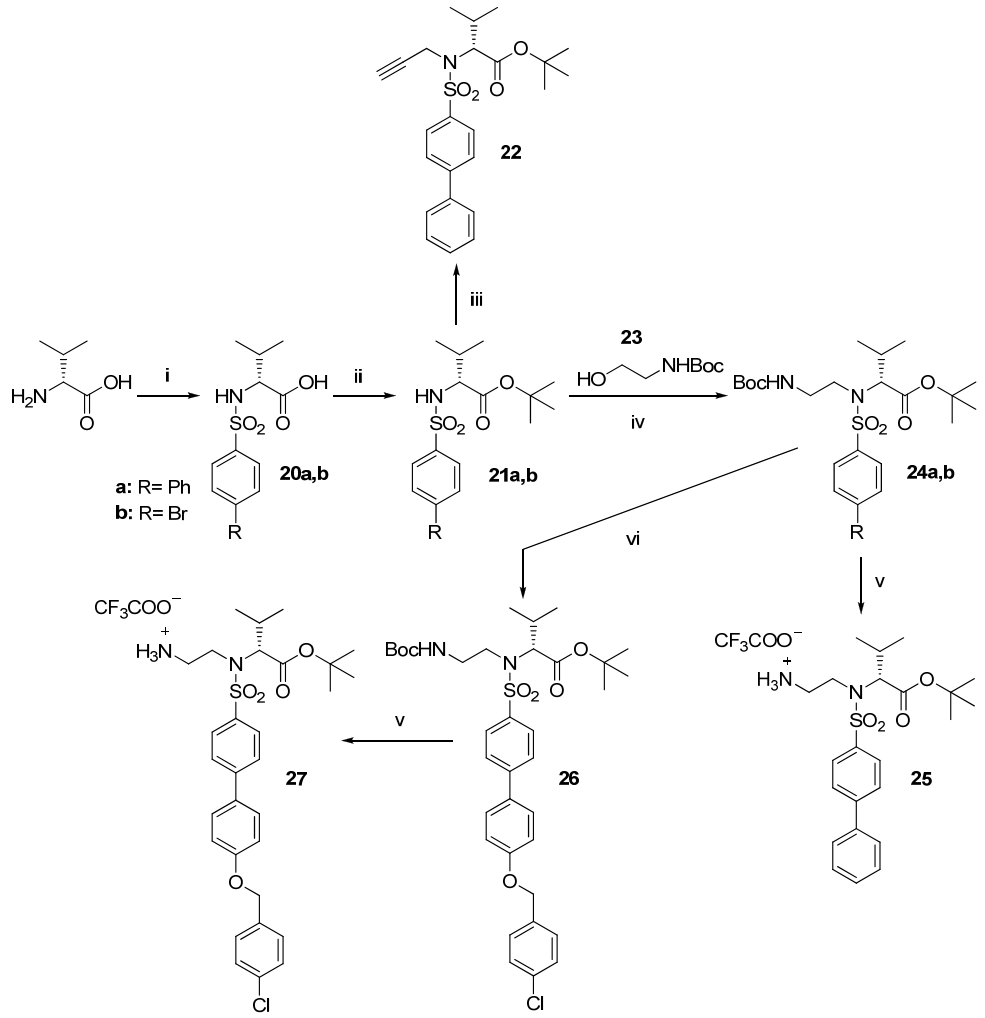
Chemistry.

The preparation of all sugar moieties used for the coupling with the MMP inhibitor is shown in Scheme 1. The known oxazoline **12**[15] and azide **17**[16] were obtained in two steps from commercial D-glucosamine hydrochloride according to published procedures. The glycosylation of **12** with 3-*O*-tosyl-1,3-propandiol[17] was conducted in 1,2-dichloroethane (DCE) with camphorsulfonic acid (CSA) as catalyst, affording stereoselectively the β-glycoside **13** ($J_{1,2}$ = 8.5 Hz) in good yield (69 %). The known azidopropyl *N*-acetyl-D-glucosamine **14**[18] was prepared by conversion of **13** into the azide through an S_N2 reaction (NaN_3, TBAI, DMF) in a nearly quantitative yield (98 %). The azido functionality in **14** and **17**[16] was reduced to the corresponding amine **15**[19] and **18**[16] (Scheme 1) in quantitative yields by Staudinger conditions (resin-PPh_3, THF/H_2O). Without further purification, the amines **15** and **18** were transformed into the corresponding isothiocyanate derivatives **16** and **19** through treatment with di-2-pyridyl thionocarbonate (DPT). DPT[20] is a commercially available, solid, non-toxic reagent that can be used in the preparation of isothiocyanates as a safe alternative to thiophosgene. After 5-24 h the intermediate β-glycosyl isothiocyanates **16** (64 % yield from **14**) and **19** (46 % yield from **17**) were obtained after removal of the solvents and purification by flash chromatography. The MMP inhibitor precursors **22**, **25** and **27** were prepared as described in Scheme 2.

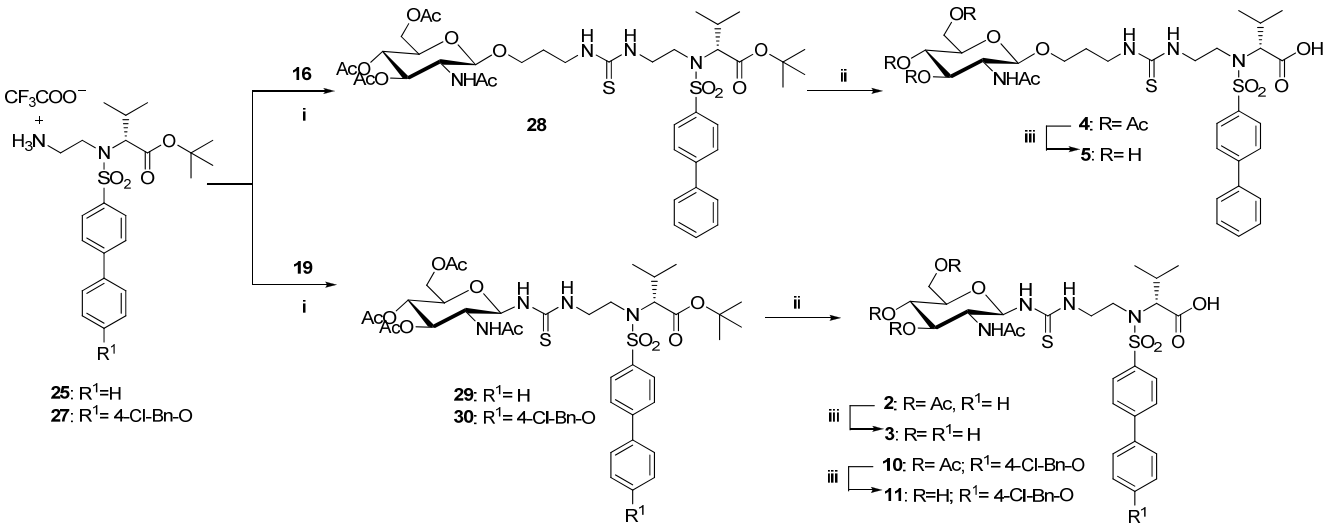
Commercially available biphenyl-4-sulfonyl chloride or *p*-bromobenzenesulfonyl chloride were respectively converted into sulfonamides **20a,b** by reaction with D-valine in H_2O and dioxane in the presence of triethylamine as base (44-45 % yield), according to a previously reported procedure.[21] Sulfonamides **20a,b** were then protected on the carboxylic moiety as *tert*-butyl esters (*R*)-**21a,b** by using *N*,*N*-dimethylformamide-di-*tert*-butyl acetal at 95 °C (46-60 % yield). Sulfonamide **21a** was *N*-alkylated by treatment with propargyl bromide in DMF using potassium carbonate as base to afford the alkyne **22** in 82 % yield. A Mitsunobu condensation of sulfonamides **21a,b** with commercial Boc-protected ethanolamine **23** in the presence of diisopropyl azodicarboxylate (DIAD) and triphenylphosphine gave the *tert*-butyl esters (*R*)-**24a,b** (62-85 % yield). A Pd-catalyzed Suzuki coupling of commercially available 4-(4'-chlorobenzyloxy)-phenylboronic acid with *p*-bromo ester **24b** in the presence of K_3PO_4 afforded (*R*)-*tert*-butyl ester **26** in 80.6 % yield. Finally, amines **25** and **27** were obtained as trifluoroacetate salts by selective hydrolysis of Boc group in the presence of *tert*-butyl group upon treatment of compounds **24a** and **26** with TFA in controlled conditions (2 h at 0 °C under argon atmosphere, 74-78 % yield). Thioureido derivatives **2-5** and **10-11** were obtained through the coupling of the MMP inhibitor amino-precursors **25** and **27** with the appropriate isothiocyanate β-*N*-acetyl-D-glucosamine derivatives **16** or **19** (Scheme 3), while the copper-catalyzed azide-alkyne cycloaddition (CuAAC)[22] was used to connect the MMP inhibitor alkynyl-precursor **22** to the azide β-*N*-acetyl-D-glucosamine derivatives **14** or **17** (Scheme 4) to give 1,2,3-triazole derivatives **6-9**. The condensation reactions between the isothiocyanates **16** and **19** and the MMP inhibitor precursors **25** and **27** (Scheme 3) were performed in a 4:1 CH_2Cl_2/DMF mixture, in presence of Et_3N at 60 °C for 12-24 h. The purification by flash chromatography of crude products yielded **29** pure (96 %) while the thiureido derivatives **28** and **30** were obtained with an impurity of the corresponding isothiocyanates (about 5 %, NMR analysis). Their structures have been confirmed by the subsequent reaction. The β-glycosyl azide **14** and **17** were conjugated to the alkyne **22** (Scheme 4) by CuAAC click chemistry according to reported conditions.[22] The reactions were performed in a mixture DMF/H_2O (4:1) with copper(II) sulfate, sodium ascorbate catalytic system and heated under microwave irradiation at 80 °C for 30 min. The desired 1,2,3-triazole derivatives **31-32** were isolated after flash chromatographic purification with complete regiospecificity and good yield (81 % and 94 % respectively).



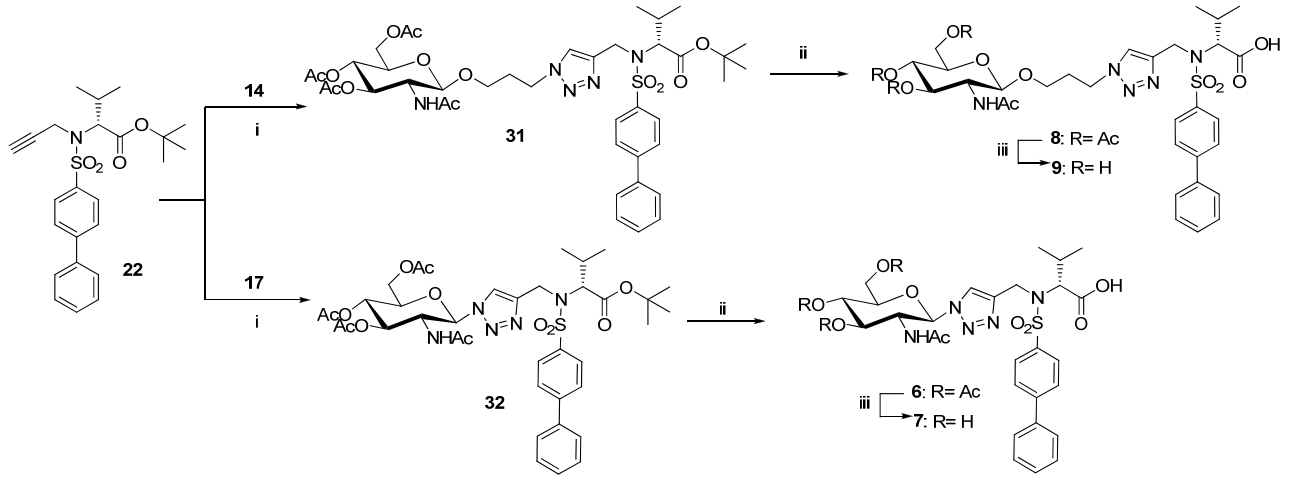
Scheme 1. Synthesis of sugar intermediates **14**, **16**, **17** and **19**. i) CSA, $HO(CH_2)_3OTs$, 4Å MS, DCE, 80 °C, 12 h, 69%; ii) NaN_3, Bu_4NI, DMF, 50 °C, 1 h, 98%; iii) (1) PPh_3-resin, THF/H_2O 20:1, 24 h; (2) DPT, CH_2Cl_2, 1.5-3 h, 46-64% (from azide).



Scheme 2. Synthesis of the MMP inhibitor intermediates **22**, **25** and **27**. i) biphenyl-4-sulfonyl chloride or *p*-bromobenzenesulfonyl chloride, Et_3N, H_2O, dioxane, RT, 18 h, 44-45%; ii) $(CH_3)_2NCH[OC(CH_3)_3]_2$, toluene, 95 °C, 4 h, 46-60%; iii) propargyl bromide, K_2CO_3, DMF, RT, 48 h, 82.6%; iv) PPh_3, DIAD, THF, RT, 18 h, 62-85%; (v) TFA, CH_2Cl_2, 0°C, 2 h, 74-78%; vi) 4-(4'-chlorobenzyloxy)-phenylboronic acid, K_3PO_4, $Pd(PPh_3)_4$, dioxane/H_2O 1:4.4, 70 °C, 2 h, 80.6%.



Scheme 3. Synthesis of thioureido derivatives **2-5** and **10-11**. i) Et_3N, CH_2Cl_2-DMF 4:1, 60 °C, 20-24 h, 96%; ii) CF_3COOH, CH_2Cl_2, RT, 24 h, 92%; iii) NH_3/MeOH 7N, MeOH, RT, 20-24 h, 79-94%.



Scheme 4. Synthesis of 1,2,3-triazole carboxylates **6-9**. i) $CuSO_4.5H_2O$, Sodium Ascorbate, DMF/H_2O 4:1, microwave, 80 °C, 30 min, 81-94%; ii) CF_3COOH, CH_2Cl_2, RT, 24 h, 55-81%; iii) NH_3/MeOH 7N, MeOH, RT, 20-24 h, 67-70%.

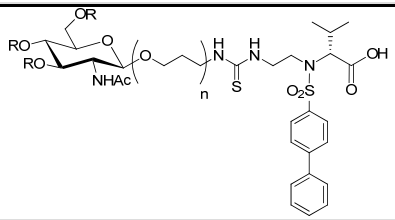
NMR analysis (^1H, ^{13}C and 2D NMR experiments) of **31-32** confirmed the structure. The exclusive formation of the 1,4-disubstituted 1,2,3-triazole **31-32**, were identified by the large Δ (ΔC_4-ΔC_5) values (about 20 ppm) observed by ^{13}C NMR spectroscopy for the cycloadducts. The removal of the *tert*-butyl group in **28-32** (Scheme 3 and 4) by treatment with TFA gave the desired carboxylic acids **4**, **2**, **10**, **8** and **6** respectively, after trituration of crude product with Et_2O. Finally, the de-*O*-acetylation of **4**, **2**, **10**, **8** and **6** by treatment with NH_3/MeOH 3.5N afforded the deprotected derivatives **5**, **3**, **11**, **9** and **7** in good yields (67-94 %), after trituration of crude products with Et_2O. The NMR (^1H, ^{13}C and 2D NMR experiments) and elemental analyses of all compounds confirmed the structures shown.

MMP Inhibition and crystallographic studies.

The first series of carboxylic acids synthesized, the thioureido **2-5** (Table 1) and 1,2,3-triazole derivatives **6-9** (Table 2), were assayed *in vitro* on human recombinant MMP-12 using a fluorogenic peptide[23] as the substrate. Inhibitor **1**, devoid of any sugar moiety, was used as the reference compound. Inhibitory activity against MMP-9 (gelatinase B) was chosen on a first instance to evaluate selectivity for MMP-12. MMP-9, together with MMP-2 and MMP-8, differs from MMP-12 by having an “intermediate S1' pocket” while the latter is classified as a “deep S1' pocket MMP”.[24] Improved selectivity for MMP-12 over MMP-9 could be

indicative of the ability of the new derivatives to discriminate among the various MMP family members. In general: 1) all new derivatives showed a stronger affinity for MMP-12 than for MMP-9 and 2) thioureido derivatives **2-5** resulted more active than their triazole analogues, compounds **6-9**, on both enzymes. In particular, among the thioureido derivatives the best results were obtained for compound **2**, an *O*-acetylated derivative with a short spacer between the sugar moiety and the biphenyl sulfonamide. The activity of compound **2** on MMP-12 (IC_{50} = 18 nM) was similar to that of the reference compound **1** with a 5-fold better selectivity over MMP-9. Noteworthy, in both series of compounds the introduction of an *n*-propyloxy chain between the linker group and GlcNAc did not substantially affect activity towards MMP-12. Only in the thioureido series of compounds, the acetylation of the hydroxyl groups on the carbohydrate moiety led to a small increase of activity in the compound with a short spacer (**2** was twice-fold more active than **3**).

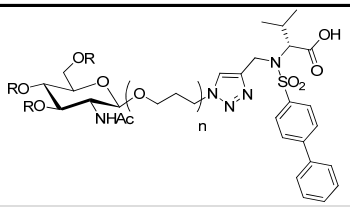
Table 1. MMP-12 and MMP-9 inhibitory activity (IC_{50} nM values)[a] of thioureido derivatives **2-5** and the reference compound **1**.



Compd	R	n	MMP-12	MMP-9
2	Ac	0	18	1,200
3	H	0	40	5,400
4	Ac	1	36	1,220
5	H	1	28	1,200
1			35	510

[a] The IC_{50} values are the average of three determinations with a standard deviation of <10%.

Table 2. MMP-12 and MMP-9 inhibitory activity (IC_{50} nM values)[a] of 1,2,3-triazole derivatives **6-9** and the reference compound **1**.



Compd	R	n	MMP-12	MMP-9
6	Ac	0	72	4,400
7	H	0	53	3,100
8	Ac	1	63	6,800
9	H	1	73	9,400
1			35	510

[a] The IC_{50} values are the average of three determinations with a standard deviation of <10%.

To understand the selectivity of compound **2** for MMP-12 compared to MMP-9, this inhibitor was co-crystallized with the catalytic domain of both enzymes and the crystal structure determined to discern the details of ligand binding (Figure 2).

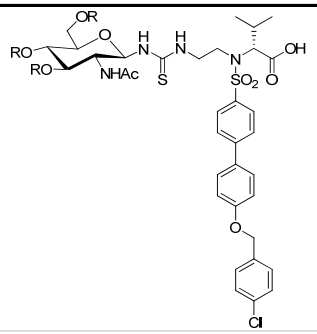
Figure 2

In MMP-12 the acetylated sugar moiety that in compound **2** replaces the phenyl of compound **1**, improves its positioning to better reflect the change in character from a hydrophobic phenyl to a hydrophilic sugar (Figure 2d). Differently, in MMP-9 the sugar maintains the same position of the benzene-dicarboxyamide present in compound **1-1** (a bi-functional analogue of compound **1** whose X-ray structure in complex with MMP-9 catalytic domain was previously reported[12]) (Figure 2e). This results in improved MMP-12 inhibition and deterioration of that for MMP-9. The sum of the various effects, translates into a substantial increase in selectivity of **2** for MMP-12 compared to MMP-9. Comparison of the crystal structures of compounds **2** and **3** with a thioureido linker, with those for compounds **6**, **7** and **8** with a triazole linker in MMP-12, shows that the sugar moieties of the two classes of compounds do not overlap (Figure 3). As noted for compound **2**, the sugar moieties for all sugar derivatives select alternative positions different from that preferred by compound **1** to optimize their binding (Figure 3a).

Figure 3

The absence of the acetyl groups on compound **3**, makes the site used by **2** less favorable, forcing the ligand to dock closer to the MMP-12 surface. The improved activity of the inhibitors with the more flexible thioureido linker, is likely to be associated to their ability to select more appropriate positions to improve their binding. On the basis of these findings, in order to further increase activity and selectivity for MMP-12 of this class of inhibitors, we decided to replace the biphenyl group present in the sulfonamide moiety (P1' group) of **2** with a 4-(4'-chlorobenzyloxy)-biphenyl group. In fact, the introduction of this substituent in P1' has been previously reported to confer a good selectivity for MMP-12 and -13 to similar sulfonamido-based derivatives.[25] 4-(4'-Chlorobenzyloxy)-biphenyl thioureido derivatives **10** and **11** (Table 3) were subsequently synthesized and tested on MMP-12 and -9. Unsurprisingly, **10** resulted the most promising compound, with a nanomolar activity against MMP-12 (IC_{50} = 12 nM) and a 70-fold selectivity over MMP-9.

Table 3. MMP-12 and MMP-9 inhibitory activity (IC_{50} nM values)[a] of 4-(4'-chlorobenzyloxy)-biphenyl thioureido derivatives **10-11**.



Compd	R	MMP-12	MMP-9
10	Ac	12	860
11	H	42	1,470

[a] The IC_{50} values are the average of three determinations with a standard deviation of <10%.

Also for these last compounds, like for the other thioureido derivatives endowed with a short spacer (compounds **2** and **3**), the *O*-acetylation led to a small increase of activity and the sugar-deprotected derivative **11** resulted less potent (IC_{50}= 42 nM) than its acetylated analogue **10**. At this point the selectivity profile of the most promising MMP-12 sugar-based inhibitors, **2**, **3** and **10**, was evaluated by testing them on MMP-1, MMP-2 and MMP-14 in comparison with **1** (Table 4). Compounds **2** and **10** showed a similar

inhibitory potency on MMP-12 (IC_{50}= 18 and 12 nM, respectively), slightly higher than reference compound **1** (IC_{50}= 35 nM). But, as expected, the introduction of the 4-(4'-chlorobenzyloxy)-biphenyl group in P1' led to a great improvement of selectivity over MMP-1 and MMP-14, two MMPs widely believed to be responsible for some side effects that have been clinically observed with the use of broad spectrum MMP inhibitors, such as musculoskeletal syndrome (MSS).[26] In fact, the 4-(4'-chlorobenzyloxy)-biphenyl thioureido derivative **10** displayed a 6-fold increase in selectivity for MMP-12 over MMP-14 and a 41-fold increase of selectivity over MMP-1 with respect to the biphenyl analogue **2**. Compound **3** showed the same potency of **1** on MMP-12 and a selectivity profile similar to that of its sugar-acetylated analogue, **2**. In particular, it had a 200-fold selectivity for MMP-12 over MMP-14 and a 1000-fold selectivity over MMP-1.

Table 4. Selectivity profile (IC_{50} nM values)[a] of compounds **2**, **3** and **10** in comparison with **1**.

Compd	MMP-1	MMP-2	MMP-9	MMP-12	MMP-14
2	19,000	330	1,200	18	3,900
3	40,000	320	5,400	40	8,900
10	50,000	100	860	12	15,500
1	16,000	170	510	35	1,830

[a] The IC_{50} values are the average of three determinations with a standard deviation of <10%.

The improvement in hydrophilicity caused by the presence of the glycosidic residue, can be evaluated by the octanol/water partition coefficient (logP). The logP value for the most active thioureido derivatives **2, 3** and **10**, **11** have been calculated as clogP values, and compared to the value calculated for the starting compound **1**, devoid of the glycosidic residue (Table 5). The deprotected glycosidic derivative **3** had a cLogP value of 3.15 which was lower than its acetylated analogue **2** and reference compound **1**, with cLogPs of 4.75 and 5.68, respectively. Also the water solubility of the new compounds was determined at pH 7.4 (see Table 5) and both glycoconjugates **2** and **3** showed increased solubility (> 5 mM) with respect to the reference compound **1**. Regarding the 4-(4'-chlorobenzyloxy)-biphenyl thioureido derivatives **10** and **11**, they showed high cLogPs (> 5) and low solubility (~ 200 µM). In this case the introduction of the 4-(4'-chlorobenzyloxy)-biphenyl portion strongly contributed to improving the lipophilicity of the molecules, countering the positive effect due to the presence of the glycosidic moiety.

Table 5. Physicochemical properties of compounds **2**, **3** and **10**, **11** in comparison with **1**.

Compd	clogP[a]	Solubility (µM)[b]
1	5.68	790
2	4.75	> 5000
3	3.15	> 5000
10	6.81	180
11	5.21	230

[a] Hydrophobicity calculated as the partition coefficient between octanol and water, log *P* (o/w), using ACD laboratory software version 14.0 (Advanced Chemistry Development, Inc. Toronto, Canada). [b] Solubility was determined in 50 mM phosphate buffer pH 7.4, at room temperature after 24 h of equilibration. Concentrations were assessed for supernatants of centrifuged samples by UV-Vis spectroscopy at 270 nm.

Overall these biological and physicochemical data show that the introduction of a glycosidic portion in P2', linked through a flexible linker (as in compound **3**), represents a good compromise to maintain nanomolar activity against MMP-12 while boosting bioavailability.

Conclusions

In the present study, a new series of carboxylate-based MMP-12 inhibitors was designed and synthesized starting from the previously reported compound **1**. In order to improve the hydrophilicity and bioavailability of these arylsulfonamides without reducing their affinity for the target, we linked a β-*N*-acetyl-D-glucosamine (GlcNAc) moiety in P2'. The conjugation between GlcNAc and the MMP inhibitor scaffold was achieved through the introduction of a thioureido group or a 1,2,3-triazole group as linker. The new glycoconjugates were tested on human MMPs by fluorimetric assay. All new derivatives showed a stronger affinity for MMP-12 than for MMP-9 and thioureido derivatives resulted more active than their triazole analogues. In particular compound **2**, an *O*-acetylated thioureido biphenyl sulfonamide, was selected for further studies, given its high affinity for MMP-12 (IC_{50}= 18 nM). Crystallographic analysis provided insights into the binding mode of **2** to MMP-12, its selectivity over MMP-9 and the higher affinity for MMP-12 with respect to triazole analogues. To further optimize its activity and selectivity, the structure of **2** was modified by introducing a 4-(4'-chlorobenzyloxy)-biphenyl group in P1'. As expected, 4-(4'-Chlorobenzyloxy)-biphenyl thioureido derivative **10** showed a nanomolar activity against MMP-12 (IC_{50} = 12 nM) and a 70-fold selectivity over MMP-9. At this point the physicochemical properties of the most promising derivatives, **2**, **10** and their respective sugar-deprotected derivatives **3** and **11**, were evaluated in comparison with the starting compound **1**. Compound **3** was the one able to conjugate a nanomolar activity for MMP-12 and good selectivity over MMPs with an improved hydrophilicity (water solubility > 5 mM and cLogP = 3.15). These preliminary results suggest that the introduction of a sugar moiety in P2', linked through a flexible linker as in compound **3**, not only can improve the hydrophilicity of MMP-12 inhibitors but also can influence their biological activity on isolated enzymes (higher selectivity over other MMPs). Of course, to fully appreciate the effect of the improved bioavailability of **3** with respect to **1**, an in vivo assay will be necessary.

Experimental Section

Chemistry. Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20±2°C. 1H NMR spectra were recorded in appropriate solvents with a Bruker Avance II operating at 250.12 MHz or a Bruker Avance III HD 400 spectrometer operating at 400 MHz. 13C NMR spectra were recorded with the spectrometers operating at 62.9 MHz or 100.57 MHz. The assignments were made, when possible, with the aid of DEPT, COSY, HSQC experiments. The first order proton chemical shifts □□ are referenced to residual solvents and J-values are given in Hz. Where indicated, the elemental compositions of the compounds agreed to within 0.4% of the calculated value. All reactions were followed by TLC on Kieselgel 60 F254 with detection by UV light and/or with ethanolic 10% phosphomolybdic or sulfuric acid, and heating. Kieselgel 60 (Merck, 70-230 and 230-400 mesh, respectively) was used for column and flash chromatography. Some of flash chromatography were conducted by the automated system Isolera Four SVTM (Biotage®), equipped with UV detector with variable wavelength (200-400 nm) or using prepacked ISOLUTE Flash Si II cartridges (Biotage). Microwave-assisted reactions were run in a CEM Discover LabMate microwave synthesizer. Unless otherwise noted, solvents and reagents were obtained from commercial suppliers and used without further purification. All reactions involving air- or moisture-sensitive reagents were performed under an argon atmosphere using anhydrous solvents. Anhydrous dimethylformamide (DMF), dichloromethane (CH2Cl2), 1,2-dichloethane (DCE) and THF were purchased from Sigma-Aldrich. Other dried solvents were obtained by distillation according to standard procedure,[27] and stored over 4Å molecular sieves activated for at least 24 h at 200 °C. MgSO4 or Na2SO4 were used as the drying agents for solutions. Compound 2-methyl-(3,4,6-tri-O-acetyl-1,2-dideoxy-□-D-glucopyrano)-[2,1-d]-2oxazoline (12),[15] 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl azide (17),[16] and 3-O-tosyl-1,3-propandiol[17] were prepared according to the reported procedures.

Synthesis of thioureido tert-butyl ester 29. The isothiocyanate 19 (0.49 mmol, 1.1 equiv) was solubilized in dry 4:1 CH2Cl2/DMF mixture (26 mL) and the solution of amine salt 25 (0.45 mmol, 1 equiv) in dry CH2Cl2/DMF 4:1 (26 mL) containing Et3N (62 □L, 0.45 mmol) was added dropwise. The reaction mixture was stirred at 60 °C until TLC analysis (EtOAc) revealed the complete disappearance of the amine salt 25 (7 h) and

concentrated under diminished pressure. Purification of crude product by flash chromatography (petroleum ether/EtOAc 2:1) using a Isolute Flash Si II cartridge afforded pure 29 (353 mg, 96 % yield calculated from 25) as a clear syrup; Rf = 0.65 (EtOAc); [□]D23 = +14.0 (c=1.07 in CHCl3); 1H NMR (CD3CN, 250.12 MHz): □ = 7.93-7.78 (m, 4H; Ar-H), 7.65 (m, 2H; Ar-H), 7.53-7.40 (m, 3H; Ar-H), 7.30, 7.16 (2bs, each 1H; 2×NHCS), 6.70 (d, J2,NH = 8.7 Hz, 1H; NHAc), 5.25-5.15 (m, 2H; H-1, H-3), 4.97 (dd, J3,4 = 9.6 Hz, J4,5 = 9.7 Hz, 1H; H-4), 4.23 (dd, J5,6b = 4.4 Hz, J6a,6b = 12.1 Hz, 1H; H-6b), 4.05 (m, 2H; H-6a, H-2), 3.88-3.20 (m, 5H; H-5, CHNSO2, CH2NSO2, CH2NHCS), 3.35 (m, 1H; CH2NSO2), 2.25 (m, 1H; Me2CH), 1.98, 1.97, 1.96 (3s, each 3H; 3×MeCOO), 1.87 (s, 3H; MeCON), 1.18 (s, 9H; Me3C), 1.00 (d, Jvic = 6.3 Hz, 3H; Me2CH), 0.91 (d, Jvic = 6.5 Hz, 3H; Me2CH); 13C NMR (CD3CN, 62.9 MHz): □ = 184.1 (C=S), 172.7, 171.3, 171.2, 170.6, 170.4 (5×C=O), 146.4 (Ar-C-SO2), 139.9, 138.9 (2×Ar-C), 130-128.2 (Ar-CH), 83.8 (C-1), 82.8 (Me3CO), 73.4 (C-3), 73.2 (C-5), 69.5 (C-4), 67.7 (CHN), 62.8 (C-6), 53.6 (C-2), 45.9 (CH2NH), 44.3 (CH2NSO2), 29.8 (CHMe2), 27.9 (Me3C), 23.0 (MeCON), 21.1, 20.9, 20.8 (3×MeCOO), 20.5, 19.5 (Me2CH); elemental analysis calcd (%) for C38H52N4O12S2: C 55.59, H 6.38, N 6.82; found: C 55.56, H 6.35, N 6.79.

General Procedure for the synthesis of triazole-linked tert-butyl esters 31-32. The alkyne 22 (0.1 mmol) and the opportune sugar azide derivative (14 or 17, 0.11 mmol, 1.1 equiv), CuSO4·5H2O (0.1 mmol, 1.5 equiv), sodium ascorbate (0.3 mmol, 3 equiv) were dissolved in a mixture of DMF/H2O 4:1 (3.2 mL). The solution was heated under microwave irradiation to 80 °C for 30 min, then diluted with Et2O (12 mL), washed by saturated aq NaHCO3 (12 mL), the organic phase was separated and the aq layer extracted with Et2O (2×12 mL). The collected organic extracts were dried (MgSO4·H2O), filtered and concentrated under diminished pressure. Flash chromatographic purification over silica gel of the crude product gave triazole-linked derivatives pure 31-32.

Triazole-linked tert-butyl ester (31). Flash chromatography on silica gel (n-hexane/EtOAc 3:7 + 0.01% Et3N) afforded pure 31 (81% yield calculated from 22) as a clear syrup; Rf = 0.64 (EtOAc); [□]D23 = +46.2 (c=1.06 in CHCl3); 1H NMR (CD3CN, 250,12 MHz): □ = 7.92-7.76 (m, 5H; 4×Ar-H, CH-triazole), 7.69-7.64 (m, 2H; 2×Ar-H), 7.54-7.40 (m, 3H; 3×Ar-H), 6.63 (d, J2,NH = 9.2 Hz, 1H; NHAc), 5.16 (dd, J2,3 = 10.6 Hz, J3,4 = 9.5 Hz, 1H; H-3), 4.95 (dd, J4,5 = 9.9 Hz, 1H; H-4), 4.87, 4.69 (AB system, JA,B = 16.6 Hz, 2H; CH2NSO2), 4.61 (d, J1,2 = 8.5 Hz, 1H; H-1), 4.37 (t, Jvic = 7.2 Hz, 2H; CH2N), 4.22 (dd, J5,6b = 4.9 Hz, J6a,6b = 12.3 Hz, 1H; H-6b), 4.06 (dd, J5,6a = 2.2 Hz, 1H; H-6a), 3.93 (d, Jvic = 10.6 Hz, 1H; CHNSO2), 3.85 (ddd, 1H; H-2), 3.72, 3.40 (2dt, Jvic = 6.0 Hz, Jgem = 10.6 Hz, each 1H; CH2O), 3.70 (m, 1H; H-5), 2.22 (m, 1H; Me2CH), 2.05 (m, 2H; CH2), 2.00, 1.96, 1.94 (3s, each 3H; 3×MeCOO), 1.86 (s, 3H; MeCON), 1.23 (s, 9H; CMe3), 0.90 (d, Jvic = 6.6 Hz, 3H; Me2CH), 0.72 (d, Jvic = 6.5 Hz, 3H; Me2CH); 13C NMR (CD3CN, 62.9 MHz): δ = 171.3, 171.1, 171.0, 170.5, 170.5 (5×C=O), 146.3, 146.0 (Ar-C-SO2, C-triazole), 139.9, 139.8, (2×Ar-C), 130.1-128.2 (Ar-CH), 125.4 (CH-triazole), 101.7 (C-1), 82.9 (Me3CO), 73.5 (C-3), 72.4 (C-5), 69.7 (C-4), 67.7 (CHN), 66.9 (CH2O), 62.9 (C-6), 54.8 (C-2), 47.5 (CH2N), 41.0 (CH2NSO2), 30.9 (CH2), 29.7 (CHMe2), 27.9 (Me3C), 23.2 (MeCON), 20.9 (3×MeCOO), 20.0, 19.4 (Me2CH); elemental analysis calcd (%) for C41H55N5O13S: C 57.40, H 6.46, N 8.16; found: C 57.37, H 6.41, N 8.25.

Triazole-linked tert-butyl ester (32). Flash chromatography on silica gel (n-hexane/EtOAc 3:7 + 0.01% Et3N) afforded pure 32 (94% yield calculated from 22) as a white foam; Rf = 0.53 (EtOAc); [□]D23 = +22.0 c=0.9 in CHCl3); 1H NMR (CD3CN, 250.12 MHz): δ = 8.05 (s, 1H; CH-triazole), 7.90-7.77 (m, 4H; Ar-H), 7.69-7.64 (m, 2H; Ar-H), 7.54-7.41 (m, 3H; Ar-H), 6.56 (d, J2,NH = 9.2 Hz, 1H; NHAc), 5.19 (d, J1,2 = 10.0 Hz, 1H; H-1), 5.43 (dd, J2,3 = 10.4 Hz, J3,4 = 9.5 Hz, 1H; H-3), 5.17 (dd, J4,5 = 9.7 Hz, 1H; H-4), 4.56 (m, 1H; H-2), 4.89, 4.68 (AB system, JAB = 16.7 Hz, 2H; CH2N), 4.21 (dd, J5,6b = 5.4 Hz, J6a,6b = 12.7 Hz, 1H; H-6b), 4.13-4.04 (m, 2H; H-6a, H-5), 3.88 (d, Jvic = 10.5 Hz, 1H; CHN), 2,21 (m, 1H; CHMe2), 2.01 (s, 3H; MeCOO), 1.98 (s, 6H; 2×MeCOO), 1.63 (s, 3H; MeCOON), 1.20 (s, 9H; Me3C), 0.89 (d, Jvic = 6.6 Hz, 3H; Me2CH), 0,67 (d, Jvic =6.5 Hz, 3H; Me2CH); 13C NMR (CD3CN, 62.9 MHz): δ = 171.2, 170.9, 170.8, 170.5, 170.4 (5×C=O), 146.3 (Ar-C-SO2, C-triazole), 140.0, 139.7 (2×Ar-C), 130.1-128.2 (Ar-CH), 124.8 (CH-triazole), 86.1 (C-1), 82.9 (Me3CO), 75.4 (C-5), 73.2 (C-3), 69.1 (C-4), 67.6 (CHN), 62.7 (C-6), 53.9 (C-2), 40.7 (CH2NSO2), 29.7 (CHMe2), 27.8 (Me3C), 22.8

(MeCON), 20.8 (3×MeCOO), 20.0, 19.4 (Me2CH); elemental analysis calcd (%) for C38H49N5O12S: C 57.06, H 6.17, N 8.76; found: C 57.02, H 6.14, N 8.74.

General procedure for the transformation of tert-butyl esters 29 and 31-32 into carboxylic acids 2 and 8, 6. The appropriate ester (29 or 31, 32, 0.1 mmol) was dissolved in CH2Cl2 (1.7 mL), treated with CF3COOH (0.6 mL) and stirred at 0 °C for 1 h and then at RT. After 24 h, TLC analysis (EtOAc) revealed the complete disappearance of the starting material and the formation of a more retained product. The solution was coevaporated with toluene (4×10 mL) under diminished pressure and the trituration of crude product with Et2O afforded pure carboxylic acids 2 and 8, 6.

Carboxylic acid (2). 92% yield as white solid; Rf = 0.41 (EtOAc); m.p. 134-135°C; [□]D23 = +9.46 (c=1.3 in CHCl3); 1H NMR (CD3OD-D2O, 250.12 MHz): δ = 7.93-7.90 (m, 2H; Ar-H), 7.76-7.73 (m, 2H; Ar-H), 7.64 (m, 2H; Ar-H), 7.50-7.35 (m, 3H; Ar-H), 5.52 (bs, 1H; H-1), 5.19 (dd, J3,4 =10.3 Hz, J2,3 = 9.6 Hz, 1H; H-3), 5.02 (dd, J4,5 = 9.9 Hz, 1H; H-4), 4.28 (dd, J5,6b = 3.8 Hz, J6a,6b = 12.5 Hz, 1H; H-6b), 4.20-3.75 [m, 7H; H-6a, H-2, H-5, CHNSO2, CH2NSO2 (1H), CH2NHCS], 3.41 (m, 1H; CH2NSO2), 2.25 (m, 1H; Me2CH), 1.95 (s, 9H; 3×MeCOO), 1.91 (s, 3H; MeCON), 0.98 (m, 2H; Me2CH); 13C NMR (CD3OD-D2O, 62.9 MHz): δ = 182.1 (C=S), 171.7-169.2 (5×C=O), 145.8 (Ar-C-SO2), 138.9 (2×Ar-C), 128.9-127.2 (Ar-CH), 83.4 (C-1), 72.8, 72.7 (C-3, C-5), 67.7, 67.6 (C-4, CHN), 61.8 (C-6), 53.8 (C-2), 43.9, 43.8 (CH2NH, CH2NSO2), 29.6 (CHMe2), 23.1 (MeCON), 20.7-20.6 (3×MeCOO), 20.5, 19.6 (Me2CH); elemental analysis cald (%) for C34H44N4O12S2: C 53.39, H 5.80, N 7.33; found: C 53.38, H 5.79, N 7.30.

Carboxylic acid (8). 55% yield as white solid; Rf = 0.24 (CHCl3/MeOH 95:5); m.p. 114-115°C; [□]D23 = +6.93 (c=1.0 in CHCl3); 1H NMR (CD3CN/CDCl3, 250.12 MHz): δ = 7.79-7.42 (m, 11H, CH-triazole, 9×Ar-H, COOH), 6.82 (d, J2,NH = 7.8 Hz, 1H; NHAc), 5.19 (dd, J2,3 = 10.1 Hz, J3,4 = 9.6 Hz, 1H; H-3), 4.94 (t, J4,5 = 9.6 Hz, 1H; H-4), 4.80-4.45 (m, 2H; CH2NSO2), 4.60 (d, J1,2 = 8.5 Hz, 1H; H-1), 4.30-4.12 (m, 3H; H-6b, CH2N), 4.04 (dd, J5,6a = 1.9 Hz, J6a,6b = 12.1 Hz, 1H; H-6a), 3.87-3.72 (m, 3H; H-5, H-2, CHNSO2), 3.70, 3.28 (2bs, each 1H; CH2O), 2.28-2.08 (m, 3H; CH2, Me2CH), 1.98, 1.96, 1.93 (3s, each 3H; 3×MeCOO), 1.85 (s, 3H; MeCON), 0.92, 0.83 (2bs, each 3H; Me2CH); 13C NMR (CD3CN/CDCl3, 62.9 MHz): δ = 171.9, 171.3, 171.2, 171.0, 170.5 (5×C=O), 145.7, 147.6 (Ar-C-SO2, C-triazole), 139.8, 139.7 (2×Ar-C), 129.9-128.0 (Ar-CH), 128.4 (CH-triazole), 101.4 (C-1), 73.2 (C-3), 72.3 (C-5), 69.6 (C-4), 66.5 (CH2O), 66.2 (CHN), 62.8 (C-6), 54.9 (C-2), 47.5 (CH2N), 41.2 (CH2NSO2), 30.8 (CH2), 30.7 (CHMe2), 23.1 (MeCON), 20.9 (3×MeCOO), 20.8, 19.8 (Me2CH); elemental analysis calcd (%) for C37H47N5O13S: C 55.42, H 5.91, N 8.73. Found: C 55.40, H 5.89, N 8.70.

Carboxylic acid (6). 81% yield as white solid; Rf = 0.57 (EtOAc); m.p. 144-146°C; [□]D23 = +18.7 (c=0.98 in MeOH); 1H NMR (CDCl3, 250.12 MHz): δ = 8.06-7.80 (m, 3H; 2 × Ar-H, CH-triazole), 7.74-7.35 (m, 8H; 7×Ar-H, NH), 5.93 (d, J1,2 = 8.9 Hz, 1H; H-1), 5.31 (dd, J2,3 = 9.3 Hz, J3,4 = 10.1 Hz, 1H; H-3), 5.06 (bt, J4,5 = 10.1 Hz, 1H; H-4),4.81, 4.45 (AB system, JA,B = 17.1 Hz, 2H; CH2N), 4.65 (m, 1H; H-2), 4.24 (d, Jvic = 9.2 Hz, 1H; NCH), 4.10-3.90 (m, 3H; H-5, H-6a, H-6b), 2.25 (m, 1H; Me2CH), 2.04, 2.00, 1.99 (3s, each 3H; 3×MeCOO), 1.84 (s, 3H; MeCOON), 0.88 (d, Jvic = 4.9 Hz, 3H; Me2CH), 0.68 (d, Jvic = 5.5 Hz, 3H; Me2CH); 13C NMR (CDCl3, 62.9 MHz): δ = 174.6 (COOH), 171.8, 171.4, 171.3, 169.8 (4×C=O), 146.6 (Ar-C-SO2, C-triazole), 139.7, 139.3 (2×Ar-C), 129.9-126.0 (Ar-CH), 122.0 (CH-triazole), 86.8 (C-1), 75.5 (C-5), 73.2 (C-3), 68.8 (C-4), 64.6 (CHN), 62.4 (C-6), 53.7 (C-2), 40.4 (CH2NSO2), 26.4 (CHMe2), 23.6 (MeCON), 21.3-21.2 (3×MeCOO), 22.1, 19.4 (Me2CH); elemental analysis calcd (%) for C34H41N5O12S: C 54.90, H 5.56, N 9.42; found: C 54.88, H 5.54, N 9.39.

Synthesis of carboxylic acid (4). The isothiocyanate 16 (76 mg, 0.17 mmol) was solubilized in dry CH2Cl2/DMF 4:1 (17.5 mL) and a solution of amine 25 (93 mg, 0.17 mmol) in dry CH2Cl2/DMF 4:1 (17.5 mL) containing Et3N (50 □L, 2 equiv) was added dropwise. The reaction mixture was stirred at 60°C for 24 h, until TLC analysis (EtOAc) the formation of a major product (Rf = 0.42). The reaction was concentrated at reduced pressure and the crude residue taken up in Et2O (10 mL), washed with H2O (10 mL) and the aqueous layer was extracted with Et2O (2×10 mL). The collected organic extracts were dried (MgSO4·H2O), filtered and concentrated

under diminished pressure. The crude residue (136 mg) was submitted to flash chromatography (n-hexane-EtOAc 2:8) on silica gel to give a foam solid (125 mg) constituted (NMR) by ester 28 impure of isothiocyanate 16 (5%). Spectroscopy data of 28: 1H NMR (CD3CN, 250.12 MHz): δ = 7.95-7.89 (m, 3H; 2×Ar-H, NHCS), 7.83-7.77 (m, 2H; 2×Ar-H), 7.68-7.63 (m, 2H; 2×Ar H), 7.53-7.40 (m, 3H; 3×Ar-H), 6.89 (d, J2,NH = 9.4 Hz, 1H; NHAc), 6.68 (bt, J = 5.1Hz, 1H; NHCS), 5.14 (dd, J2,3 = 10.7 Hz, J3,4 = 9.4 Hz, 1H; H-3), 4.95 (dd, J4,5 = 9.9 Hz, 1H; H-4), 4.53 (d, J1,2 = 8.4 Hz, 1H; H-1), 4.22 (dd, J5,6b = 4.8 Hz, J6a,6b = 12.3 Hz, 1H; H-6b), 4.05 (dd, J5,6a = 2.6 Hz, 1H; H-6a), 3.87 (d, Jvic = 10.4 Hz, 1H; CHNSO2), 4.10-3.45 [m, 6H; H-2, H-5, CH2NH (1H), CH2NH (1H), CH2O (1H), CH2NSO2 (1H)], 3.65-3.45 [m, 3H; CH2NH (1H), CH2O (1H), CH2NSO2 (1H)], 3.38-3.25 [m, 1H, CH2NH (1H)], 2.21 (m, 1H; Me2CH), 1.75 (m, 2H; CH2), 2.00, 1.96, 1.95 (3s, each 3H; 3×MeCOO), 1.90 (s, 3H; MeCON), 1.19 (s, 9H; CMe3), 1.03 (d, Jvic = 6.5 Hz, 3H; Me2CH), 0.92 (d, Jvic = 6.6 Hz, 3H; Me2CH); 13C NMR (CD3CN, 62.9 MHz): δ = 183.4 (C=S), 172.4, 171.3, 171.1, 170.5, 170.3 (5×C=O), 146.2 (Ar-C-SO2), 139.9, 139.1, (2×Ar-C), 130.1-128.1 (Ar-CH), 101.8 (C-1), 82.7 (Me3CO), 73.4 (C-3), 72.3 (C-5), 69.7 (C-4), 69.7 (CH2O), 67.6 (CHNSO2), 62.9 (C-6), 55.3 (C-2), 45.3, 44.9, 44.8 (2×CH2N, CH2NSO2), 29.4 (CH2), 29.8 (CHMe2), 27.9 (Me3C), 23.3 (MeCON), 20.9 (3×MeCOO), 20.5, 19.5 (Me2CH). The crude 28 (125 mg) was dissolved in CH2Cl2 (2.3 mL), treated with CF3COOH (0.7 mL) and stirred at 0 °C for 1 h and then at room temperature. After 24 h, TLC analysis (EtOAc) revealed the complete disappearance of the starting material and the formation of a more retained product (Rf = 0.26). The solution was coevaporated with toluene (4×10 mL) under diminished pressure and the trituration of crude product with Et2O afforded pure carboxylic acid 4 (101 mg, 72 % yield calculated from 16) as a white solid; Rf = 0.26 (EtOAc); m.p. 103-105°C; [□]D23 = +17.1 (c=0.9 in CHCl3); 1H NMR (CD3CN, 250.12 MHz): δ = 7.93-7.90 (m, 3H; 2×Ar-H, OH), 7.81-7.77 (m, 2H; 2×Ar-H), 7.73-7.60 (m, 2H; 2×Ar-H), 7.58-7.18 (m, 3H; 3×Ar-H, NHCS), 6.893-6.78 (m, 2H; NHAc, NHCS), 5.104 (dd, J2,3 = 10.6 Hz, J3,4 = 9.4 Hz, 1H; H-3), 4.95 (dd, J4,5 = 9.8 Hz, 1H; H-4), 4.56 (d, J1,2 = 8.6 Hz, 1H; H-1), 4.22 (dd, J5,6b = 5.0 Hz, J6a,6b = 12.3 Hz, 1H; H-6b), 4.03 (dd, J5,6a = 2.5 Hz, 1H; H-6a), 4.01 (d, Jvic = 10.42 Hz, 1H; CHNSO2), 3.97-3.43 [m, 10H; H-2, H-5, 2×CH2NH, CH2O, CH2NSO2), 2.18 (m, 1H; Me2CH), 1.74 (m, 2H; CH2), 1.99, 1.96, 1.94 (3s, each 3H; 3×MeCOO), 1.88 (s, 3H; MeCON), 0.95 (d, Jvic = 6.6 Hz, 3H; Me2CH), 0.92 (d, Jvic = 6.5 Hz, 3H; Me2CH); 13C NMR (CD3CN, 62.9 MHz): δ = 182.4 (C=S), 172.8, 171.3, 171.2, 171.1, 170.5 (5×C=O), 146.1 (Ar-C-SO2), 139.9, 139.7, (2×Ar-C), 130.0-128.2 (Ar-CH), 101.7 (C-1), 73.1 (C-3), 72.3 (C-5), 69.7 (C-4), 69.5 (CH2O), 66.4 (CHNSO2), 62.8 (C-6), 54.8 (C-2), 44.9, 44.8, 44.7 (2×CH2N, CH2NSO2), 29.4 (CH2), 29.2 (CHMe2), 23.2 (MeCON), 20.9 (3×MeCOO), 20.4, 19.7 (Me2CH); elemental analysis calcd (%) for C37H50N4O13S2: C 54.00, H 6.12, N 6.81; found: C 54.02, H 6.10, N 6.79.

Synthesis of carboxylic acid (10). The isothiocyanate 19 (49.8 mg, 0.133 mmol) was solubilized in dry CH2Cl2/DMF 4:1 (13.5 mL) and a solution of amine 27 (89.4 mg, 0.133 mmol) in dry CH2Cl2/DMF 4:1 (13.5 mL) containing Et3N (37 □L, 2 equiv) was added dropwise. The reaction mixture was stirred at 60 °C for 20 h, until TLC analysis (EtOAc) the formation of a major product (Rf = 0.40). The reaction mixture was concentrated at reduced pressure and the crude residue taken up in Et2O (10 mL), washed with H2O (10 mL) and the aqueous layer was extracted with Et2O (2×10 mL). The collected organic extracts were dried (MgSO4·H2O), filtered and concentrated under diminished pressure. The crude product (114 mg) was purified by flash chromatography on silica gel (n-hexane-EtOAc 2:8) to afford a white solid (80 mg) constituted (NMR) by ester 30 impure of isothiocyanates 19 (5%). Spectroscopy data of 30: 1H NMR (CD3CN, 250.12 MHz): δ = 7.88-7.84 (m, 2H, Ar-H), 7.77-7.64 (m, 2H, Ar H), 7.61-7.57 (m, 2H, Ar H), 7.45-7.37 (m, 4H, Ar-H), 7.29 (bs, 1H, NHCS), 7.18 (bt, 1H, J = 5.3 Hz, NHCS),7.10-7.04 (m, 2H, Ar-H), 6.67 (d, 1H, J2,NH = 8.8 Hz, NHAc), 5.24 (bs, 1H, H-1), 5.19 (dd, 1H, J2,3 = 10.4 Hz, J3,4 = 9.3 Hz, H-3), 5.12 (s, 2H, ArCH2O), 4.98 (dd, 1H, J4,5 = 9.8 Hz, H-4), 4.21 (dd, 1H, J5,6b = 4.7 Hz, J6a,6b = 12.3 Hz, H-6b), 4.10-4.05 (m, 2H, H-2, H-6a), 3.84 (d, 1H, Jvic = 10.5 Hz, CHNSO2), 3.98-3.71 [m, 4H, H-5, CH2NH (1H), CH2NSO2], 3.35[m, 1H, CH2NH (1H)], 2.22 (m, 1H, Me2CH), 1.99, 1.97, 1.96 (3s, each 3H, 3×MeCOO), 1.86 (s, 3H, MeCON), 1.18 (s, 9H, CMe3), 1.00 (d, 3H, Jvic = 6.4 Hz, Me2CH), 0.91 (d, 3H, Jvic = 6.6 Hz, Me2CH). 13C NMR (CD3CN, 62.9 MHz): δ = 184.3 (C=S), 172.8, 171.3, 171.2, 170.5, 170.4 (5×C=O), 160.0 (Ar-C-O), 145.8 (Ar-C-SO2), 138.2, 137.0, 134.1, 132.5 (4 ×Ar-C), 130.2-128.0 (Ar-CH), 116.3 (2×Ar-CH), 83.9 (C-1), 82.8 (Me3CO), 73.4 (C-5), 73.2 (C-3), 69.5 (C-4), 69.8

(ArCH2O), 67.6 (CHNSO2), 62.8 (C-6), 55.6 (C-2), 45.9 CH2NSO2), 44.2 (CH2NH), 29.8 (CHMe2), 27.9 (Me3C), 23.0 (MeCON), 20.9 (3×MeCOO), 20.5, 19.5 (Me2CH). The crude ester 30 (80 mg) was dissolved in CH2Cl2 (1.4 mL), treated with CF3COOH (0.54 mL) and stirred at 0 °C for 1 h and then at RT. After 24 h, TLC analysis (EtOAc) revealed the complete disappearance of the starting material and the formation of a more retained product (Rf = 0.33). The solution was co-evaporated with toluene (4×10 mL) under diminished pressure and the trituration of crude product with Et2O afforded pure carboxylic acid 10 (52 mg, 43 % yield calculated from 19) as a white solid; Rf = 0.33 (EtOAc); m.p. 119-120°C; [⍰]D23 = +12.6 (c=0.9 in CH3OH); 1H NMR (CD3CN, 250.12 MHz): δ = 7.88-7.71 (m, 2H; 2×Ar-H), 7.67-7.60 (m, 2H; 2×Ar-H), 7.58-7.52 (m, 2H; 2×Ar-H), 7.47-7.38 (m, 5H; 4×Ar-H, OH), 7.34-7.17 (m, 2H; 2×NHCS), 7.14-7.08 (m, 2H; Ar-H), 6.79 (d, J2,NH = 9.0 Hz, 1H; NHAc), 5.41 (bs, 1H; H-1), 5.19 (dd, J2,3 = 10.1 Hz, J3,4 = 9.4 Hz, 1H; H-3), 5.11 (s, 2H; ArCH2O), 4.97 (dd, J4,5 = 10.0 Hz, 1H; H-4), 4.28 (dd, J5,6b = 4.4 Hz, J6a,6b = 12.4 Hz, 1H; H-6b), 3.98 (d, Jvic = 10.2 Hz, 1H; CHNSO2), 4.10-3.60 (m, 5H; H-2, H-6a, H-5, CH2NSO2), 3.35 [m, 2H; CH2NH), 2.17 (m, 1H; Me2CH), 1.97, 1.96, 1.94 (3s, each 3H; 3×MeCOO), 1.84 (s, 3H; MeCON), 0.92 (d, Jvic = 6.6 Hz, 3H; Me2CH), 0.89 (d, Jvic = 6.7 Hz, 3H; Me2CH); 13C NMR (CD3CN, 62.9 MHz): δ = 182.9 (C=S), 173.7, 171.3, 171.2, 170.6, 170.5 (5×C=O), 160.0 (Ar-C-O), 145.7 (Ar-C-SO2), 137.9, 137.0, 134.1, 132.5 (4×Ar-C), 130.2-127.8 (Ar-CH), 116.3 (2×Ar-CH), 83.2 (C-1), 73.4 (C-5), 73.2 (C-3), 69.5 (C-4), 69.8 (ArCH2O), 66.6 (CHNSO2), 62.8 (C-6), 53.7 (C-2), 45.2 CH2NSO2), 44.4 (CH2NH), 29.1 (CHMe2), 23.0 (MeCON), 20.9 (3×MeCOO), 20.3, 19.6 (Me2CH); elemental analysis calcd (%) for C41H49ClN4O13S2: C 54.39, H 5.45, N 6.19; found: C 54.37, H 5.43, N 6.17.

General procedure for the synthesis of deprotected carboxylic acids 3, 5, 7, 9 and 11

A solution of the appropriate protected carboxylic acid (2, 4, 6, 8 and 10, 0.1 mmol) in MeOH (1 mL) was treated with NH3-MeOH 7N (1 mL) and the solution was stirred at room temperature until the starting compound was completely reacted (TLC, CHCl3/MeOH 8:2, 20-24 h). The solution was coevaporated with toluene (4×10 mL) under diminished pressure and the crystallization (MeOH/EtOAc) or trituration of crude product with Et2O afforded pure carboxylic acids 3, 5, 7, 9 and 11.

Carboxylic acid (3). The trituration with Et2O afforded pure 3 (94% yield) as a white solid; Rf = 0.25 (CHCl3/MeOH 8:2); m.p. 131-133°C; [⍰]D23 = +20.3 (c=1.01 in MeOH); 1H NMR (CD3OD-D2O, 250.12 MHz): δ = 7.96-7.92 (m, 2H; 2×Ar-H), 7.81-7.75 (m, 2H; 2×Ar-H), 7.64 (m, 2H; 2×Ar-H), 7.49-7.39 (m, 3H; 3×Ar-H), 4.20-3.25 (m, 12H; H-1, H-2, H-3, H-4, H-5, H-6a, H-6b, CHNSO2, CH2NSO2, CH2NHCS), 2.19 (m, 1H; Me2CH), 1.93 (s, 3H; MeCON), 0.96 (m, 6H; Me2CH); 13C NMR (CD3OD/D2O, 62.9 MHz): δ = 183.1 (C=S), 174.9,174.8 (2×C=O), 146.7 (Ar-C-SO2), 140.3, 139.0 (2×Ar-C), 129.9-128.1 (Ar-CH), 84.3 (C-1), 79.4 (C-5), 75.7 (C-3), 71.7 (C-4), 66.8 (CHN), 62.5 (C-6), 55.9 (C-2), 46.1, 44.4 (CH2NH, CH2NSO2), 29.5 (CHMe2), 22.8 (MeCON), 20.6, 19.9 (Me2CH); elemental analysis calcd (%) for C28H38N4O9S2: C 52.65, H 6.00, N 8.77; found: C 52.63, H 6.01, N 8.76.

Carboxylic acid (5). The trituration with Et2O afforded pure 5 (88% yield) as a white solid; Rf = 0.07 (CHCl3/MeOH 8:2); m.p. 114-116°C; [⍰]D23 = -4.0 (c=1.08 in CHCl3); 1H NMR (CD3OD/D2O, 250.12 MHz): δ = 7.99-7.94 (m, 2H; 2×Ar-H), 7.78-7.74 (m, 2H; 2×Ar-H), 7.68-7.65 (m, 2H; 2×Ar-H), 7.49-7.38 (m, 3H; 3×Ar-H), 4.39 (d, J1,2 = 8.5 Hz, 1H; H-1), 4.08-3.78 [m, 5H; H-6b, CH2NSO2, CHNSO2, CH2O (1H)], 3.76-3.59 [m, 4H; H-2, H-6a, CH2O (1H), CH2NH (1H)], 3.58-3.30 [m, 6H; H-3, H-4, H-5, CH2NH (1H), CH2NH (2H)], 1.98 (s, 3H; MeCOON), 1.75 (m, 2H; CH2), 0.98 (bd, Jvic = 6.8 Hz, 3H; Me2CH), 0.90 (bs, 3H; Me2CH); 13C NMR (CD3OD/D2O, 62.9 MHz): δ = 179.8 (C=S), 174.2 174.1 (2×C=O), 146.4 (Ar-C-SO2), 140.4, 140.2 (2×Ar-C), 130.1-128.2 (Ar-CH), 102.5 (C-1), 77.5 (C-5), 75.8 (C-3), 71.9 (C-4), 70.7 (CHNSO2), 67.9 (CH2O), 62.5 (C-6), 57.2 (C-2), 43.5, 43.0, 42.8 (CH2NSO2, 2×CH2NH), 30.0 (Me2CH), 29.8 (CH2), 23.2 (MeCON), 20.4 (2×Me2CH); elemental analysis calcd (%) for C31H44N4O10S2: C 53.43, H 6.36, N 8.04; found: C 53.41, H 6.33, N 8.01.

Carboxylic acid (7). The crystallization (MeOH/EtOAc) afforded pure 7 (67% yield) as a white solid; Rf = 0.13 (CHCl3/MeOH 8:2); m.p. 115-117°C; [⍰]D23 = -10.2 (c=1.2 in MeOH); 1H NMR (CD3OD/D2O, 250.12 MHz): δ = 7.87-7.27 (m, 10H; 9×Ar-H, CH-triazole), 5.87 (bs, 1H; H-1), 4.35-3.15 (m, 9H; H-2, H-3, H-4, H-5, H-6a, H-6b,

CH2N, CHN,), 2.08 (m, 1H; Me2CH), 1.82 (s, 3H; MeCOON), 0.90-0.78 (m, 6H; Me2CH); 13C NMR (CD3OD/D2O, 62.9 MHz): δ = 176.6,173.6 (2×C=O), 146.4 (Ar-C-SO2, C-triazole), 140.4, 139.7 (2×Ar-C), 130.4-128.5 (Ar-CH), 121.8 (CH-triazole), 87.8 (C-1), 80.8 (C-5), 75.4 (C-3), 70.9 (C-4), 62.0 (CHN), 61.9 (C-6), 56.7 (C-2), 40.4 (CH2NSO2), 25.4 (CHMe2), 23.1 (MeCON), 21.0, 20.0 (Me2CH); elemental analysis calcd (%) for C28H35N5O9S: C 54.45, H 5.71, N 11.34; found: C 54.43, H 5.70, N 11.32.

Carboxylic acid (9). The trituration with Et2O afforded pure 9 (70% yield) as a white solid; Rf = 0.17 (CHCl3/MeOH 8:2); m.p. 156-157°C; [⍰]D23 = +12.3 (c=1.08 in MeOH); 1H NMR (CD3OD/D2O, 250.12 MHz): δ = 7.87-7.27 (m, 10H; 9×Ar-H, CH-triazole), 4.32 (d, J1,2 = 8.3 Hz, 1H; H-1), 4.25-3.10 (m, 13H; H-2, H-3, H-4, H-5, H-6a, H-6b, CH2NSO2, CHN, CH2N, CH2O), 2.18-203. (m, 3H; CH2, Me2CH), 1.89 (s, 3H; MeCOON), 1.02-0.81 (m, 6H; Me2CH);13C NMR (CD3OD/D2O, 62.9 MHz): δ = 175.3 170.5 (2×C=O), 146.3 (Ar-C-SO2, C-triazole), 140.4 (2×Ar-C), 130.3-129.1 (Ar-CH), 128.3 (CH-triazole), 102.7 (C-1), 77.9 (C-5), 75.9 (C-3), 72.0 (C-4), 66.9 (CH2O), 66.7 (CHN), 62.7 (C-6), 57.3 (C-2), 47.9 (CH2N), 41.8 (CH2NSO2), 30.7 (CH2), 30.6 (CHMe2), 23.4-22.0 (MeCON, Me2CH); elemental analysis calcd (%) for C31H41N5O10S: C 55.10, H 6.12, N 10.36; found: C 55.08, H 6.10, N 10.34.

Carboxylic acid (11). The trituration with Et2O afforded pure 11 (79% yield) as a white solid; Rf = 0.10 (CHCl3/MeOH 8:2); m.p. 134-136°C; [⍰]D23= +27.7 (c=0.85 in CHCl3); 1H NMR (CD3OD/D2O, 250.12 MHz): δ = 7.98-7.82 (m, 2H; 2×Ar-H), 7.77-7.73 (m, 2H; 2×Ar-H), 7.66-7.62 (m, 2H; 2×Ar-H), 7.45-7.38 (m, 4H; 4×Ar-H), 7.12-7.08 (m, 2H; 2×Ar-H), 5.12 (s, 2H; ArCH2O), 4.05-3.31 (m, 12H; H-1, H-2, H-3, H-4, H-5, H-6a, H-6b, CHNSO2, CH2NSO2, CH2NHCS), 2.18 (m, 1H, Me2CH), 1.97 (s, 3H; MeCON), 1.03-0.97 (m, 6H; Me2CH); 13C NMR (CD3OD/D2O, 62.9 MHz): δ = 181.7 (C=S), 174.8, 173.0 (2×C=O), 160.4 (Ar-C-O), 146.2 (Ar-C-SO2), 138.4, 137.3, 134.5, 133.1 (4×Ar-C), 129.6-127.8 (Ar-CH), 116.5 (2×Ar-CH), 84.5 (C-1), 78.9 (C-5), 75.8 (C-3), 71.8, (C-4), 70.2 (CHNSO2), 68.7 (ArCH2O), 62.6 (C-6), 56.1 (C-2), 45.9, 44.8 (CH2NH, CH2NSO2), 29.6 (Me2CH), 22.8 (MeCON), 20.7, 19.9 (Me2CH); elemental analysis calcd (%) for C35H43ClN4O10S2: C 53.94, H 5.56, N 7.19; found: C 53.91, H 5.53, N 7.17.

MMPs inhibition assays. Recombinant human MMP-14 catalytic domain was a kind gift from Prof. Gillian Murphy (Department of Oncology, University of Cambridge, U.K.). Pro-MMP-1, pro-MMP-2 and pro-MMP-9 were purchased from Calbiochem (Merck-Millipore). Pro-MMP-12 was purchased by R&D Systems. Proenzymes were activated immediately prior to use with p-aminophenylmercuric acetate (2 mM APMA for 1 h at 37 °C for MMP-2, 2 mM APMA for 2 h at 37 °C for MMP-1, and 1 mM APMA for 1 h at 37 °C for MMP-9). Pro-MMP-12 was autoactivated by incubating in Fluorometric Assay Buffer (FAB: Tris 50 mM, pH 7.5, NaCl 150 mM, CaCl2 10 mM, Brij-35 0.05%, and DMSO 1%) for 30 h at 37 °C. For assay measurements, the inhibitor stock solutions (DMSO, 10 mM) were further diluted in FAB. Activated enzyme (final concentrations of 0.56 nM for MMP-2, 1.3 nM for MMP-9, 1.0 nM for MMP-14cd, 2.0 nM for MMP-1, 2.3 nM for MMP-12) and inhibitor solutions were incubated in the assay buffer for 3 h at 25 °C. After the addition of 200 μM solution of the fluorogenic substrate Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH2 (Bachem) for all the enzymes in DMSO (final concentration of 2 μM for all enzymes), the hydrolysis was monitored every 10 s for 15 min, recording the increase in fluorescence (λex = 325 nm, λem = 400 nm) with a Molecular Devices SpectraMax Gemini XPS 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). Control wells lack inhibitor. The MMP inhibition activity was expressed in relative fluorescent units (RFU). Percent of inhibition was calculated from control reactions without the inhibitor. IC50 was determined using the formula vi/v0 = 1/(1 + [I]/IC50), where vi is the initial velocity of substrate cleavage in the presence of the inhibitor at concentration [I] and vo is the initial velocity in the absence of the inhibitor. Results were analyzed using SoftMax Pro software and and Prism Software version 5.0 (GraphPad Software, Inc., La Jolla, CA).

Protein preparation. The expression and purification for the catalytic domains used in the crystallographic studies, MMP-9 (MMP9wt) and MMP12 (hMMP12wt) and the active site glutamate to glutamine mutant hMMP12 (E219Q), has been described elsewhere.[28] In brief, the synthetic gene used for the catalytic

domain of human MMP-9 comprises residues Gly106–Gly215 and Gln391–Gly444, without the fibronectin domains, those for MMP-12. Plasmids were propagated in the Escherichia coli strain XL1-Blue and the recombinant catalytic domains expressed in E. coli cells BL21 (DE3 star). After induction, the cells were harvested by centrifugation and the pellets re-suspended, the cell suspension, disrupted, centrifuged and then redissolved for refolding and the protein purified and dialysed as previously described.[28] Acetohydroxamic acid (AHA) in the range 10-120 mM was added to prevent self-degradation during concentration.

Crystallization. The crystallization trials were carried out by sitting vapour diffusion with 1 µL equivolumetric drops of protein-substrate and reservoir solution using CrysChem plates stored in a cooled incubator at 20° C. Initial screening for the MMP-12 glycoconjugate complexes was carried out with five precipitant solutions: 27% polyethylene glycol 10,000 (PEG-10K), 0.2 M imidazole malate, pH 8.5; 45% (PEG-4K), 0.2 M imidazole piperidine, pH 8.5; 17% PEG-20K, 0.2 M imidazole malate, pH 8.5, 250 mM NaCl; 27% PEG-10K, 150 mM imidazole piperidine, pH 8.5 and 17% PEG-20K, 250 mM NaCl, 100 mM Tris-HCl, pH 10.0 used in the crystallization of other MMP-12 ligand complexes but with insufficient precipitating power for the MMP-12 glycoconjugate complexes. These precipitants were supplemented with 5-20% dioxane (Table 1S, see the Supporting Information). Crystals remain small and crystal growth is so slow that to avoid excessive protein degradation the E219Q MMP-12 mutant was used instead of the more active wild type enzyme for several MMP-12 complexes. Crystals for the trigonal polymorph of MMP-9 in complex with compound 2, were obtained from 40% monomethyl PEG 5,000 (MPEG-5K), 100 mM bicine, pH 7.25 (Table 1S, see the Supporting Information) conditions similar to those used for co-crystals with the hydroxamate-based inhibitor ARP101.[29]

Cryoprotection and data collection. The crystals used for data collection were picked up with a litho-loop from the crystallization tray and transferred to cryoprotectant solutions, which in the case of MMP-9 also contained 1 mM compound 2 (Table 1S, see the Supporting Information) to prevent the loss of the ligand during the cryo-soak phase of the experiment. The cryoprotectant solutions were prepared with mixed components either from 40% v/v CryoSolTM or CryoProtXTM (Molecular Dimensions, UK) solutions, 50% v/v precipitant and 10% v/v buffer as previously described.[30] Crystals were soaked in these solutions (Table 1S, see the Supporting Information) for a few seconds, then picked up into a loop and finally flash-cooled in liquid nitrogen using magnetic SPINE compatible cryo-vials for data collection at the ESRF synchrotron facility or transferred to Unipucks (MiTeGen, USA) for robotic mounting. Data were collected either on beamlines Proxima 1 or Proxima 2A[31] at the Soleil synchrotron Facility (St. Aubin, France) or at The ESRF synchrotron facility (Grenoble, France) on the user-operated beamline ID23-2 or dispatched to the fully automatic ID30A beamline. In all cases data were collected from a single crystal at 100 K with MxCube[32] and reduced using XDS[33] and XSCALE using the script “xdsme” (https://github.com/legrandp/xdsme).

Crystallographic structure determination. The structures were solved by molecular replacement using phaser[34] or MOLREP[35] and refined using REFMAC5[36] and Phenix[37]. The MMP-9 crystal belongs to the space group P3221 diffracting to 1.59 Å resolution.

Model building and electron density interpretation. The ligands were built with the CCP4 ligand sketcher[38] and fitted into the difference electron density (weighted Fo-Fc) calculated and displayed using COOT.[39] The electronic density for the inhibitor portion of the compounds is of excellent quality while the sugar moiety is poorly defined for most of the copies of the ligand in the asymmetric unit.The presence of the sugar moiety on the inhibitor shifts the preference for the P21212 space group with a single molecule in the asymmetric unit to a monoclinic P21 cell with two molecules in the asymmetric unit, a=39.1 Å b=62.9 Å c=63.7 Å, β=102.5° for the MMP-12●2 complex or four molecules in a larger cell a=63.7 Å b=63.1 Å c=78.9 Å, β=103.1° for the MMP-12●3 and with similar cell parameters for the other complexes. The packing of the MMP-12 catalytic domain in the larger cell is related to that in the smaller one. A strong pseudo-translational non-crystallographic (NCS) operation relates the two cells with a Patterson off-origin peak that is 38.6 % of the

origin peak. The sugar moiety of the inhibitor is positioned at a crystal contact and is the likely cause of the larger cell.

Acknowledgements

The allocation of synchrotron beamtime is acknowledged with gratitude. We are also grateful to Dr. Didier Nurizzo on beamline ID23-2 and Dr. Matthew Bowler on MASSIF-1 at the ESRF synchrotron facility, to Dr. Tatiana Isabet on beamline Proxima-1 and Dr. William Shepard on the Proxima-2A beamline at the SOLEIL synchrotron for help on the beamlines and with data collection and processing. This study was supported by the Italian Ministry of Education, University and Research (MIUR) within the frame of the COFIN national project anno 2010–2011 - prot. 2010JMAZML_008 and Italian Ministry of Health (5×1000 Funds 2012).

Keywords: drug discovery • MMP-12 inhibitors • metalloproteins • glycoconjugates • carbohydrate.

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Figure captions

Figure 1. Structure of the reference compound **1** and its glycoconjugated analogues **2-11.**

Figure 2. a) Molecular surface of MMP-9 with 2 (PDB accession code 5I12) bound showing the positioning of the sugar moiety. **b)** Molecular surface of MMP-12 with **2** (PDB accession code 5IOL) in the same orientation. The acetylated sugar moiety fits well in a pocket on the molecular surface. The sugar portion of the compound is in poor electron density in both MMP-9 and MMP-12 indicating that the interactions it makes with either protein are weak and unlikely to contribute to the enthalpy substantially. **c)** Comparison of the binding of compound 1 to MMP-12 (PDB_id=4H84)12 and 1-1 bound to MMP-9 (PDB_id=4HMA)12, to show that bind in a similar manner to the two different MMPs. **d)** Comparison of the binding of 1 and 2 to MMP-12 to show the change in position of the acetylated glucosamine of 2 compared to the phenyl ring of 1. **e)** Comparison of the binding of compounds 1-1 and 2 to MMP-9 to show that the sugar portion follows the same “natural” binding tendency evidenced by the phenyl of 1-1.

Figure 3. a) Superimpositions of compounds **1** (yellow), **2** (cyan) and **7** (pink) (PDB accession code 5I4O) showing their relative positions in MMP-12 catalytic domain. **b)** Compounds **2** and **3** (green) (PDB accession code 5I3M) with the more flexible thioureido linker place their sugar moiety in a totally different orientation compared to those with the triazole linker. **c)** Compounds **6** (ochre) (PDB accession code 5I2Z), **7** and **8** (white) (PDB accession code 5I43), with the triazole linker extend along the same path. The only noticeable difference with increasing linker length is a worse definition of the sugar in the electron density implying greater mobility.

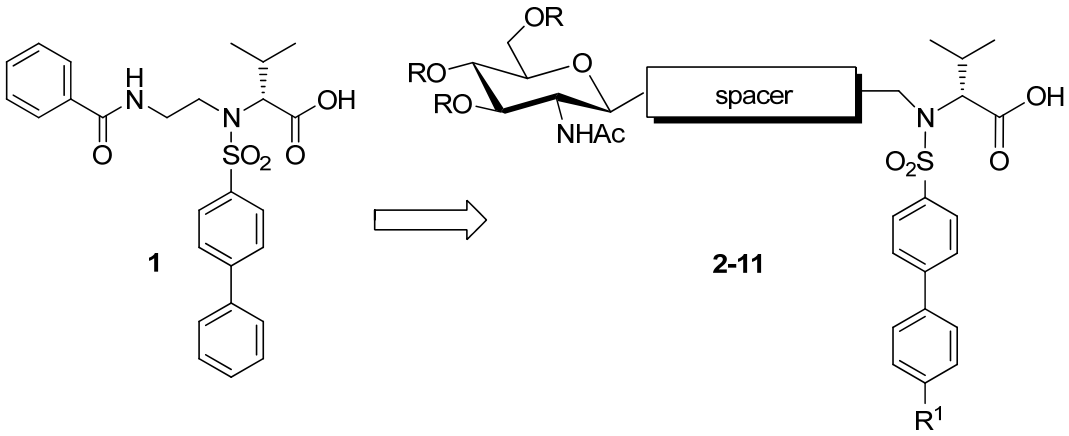

Figure 1

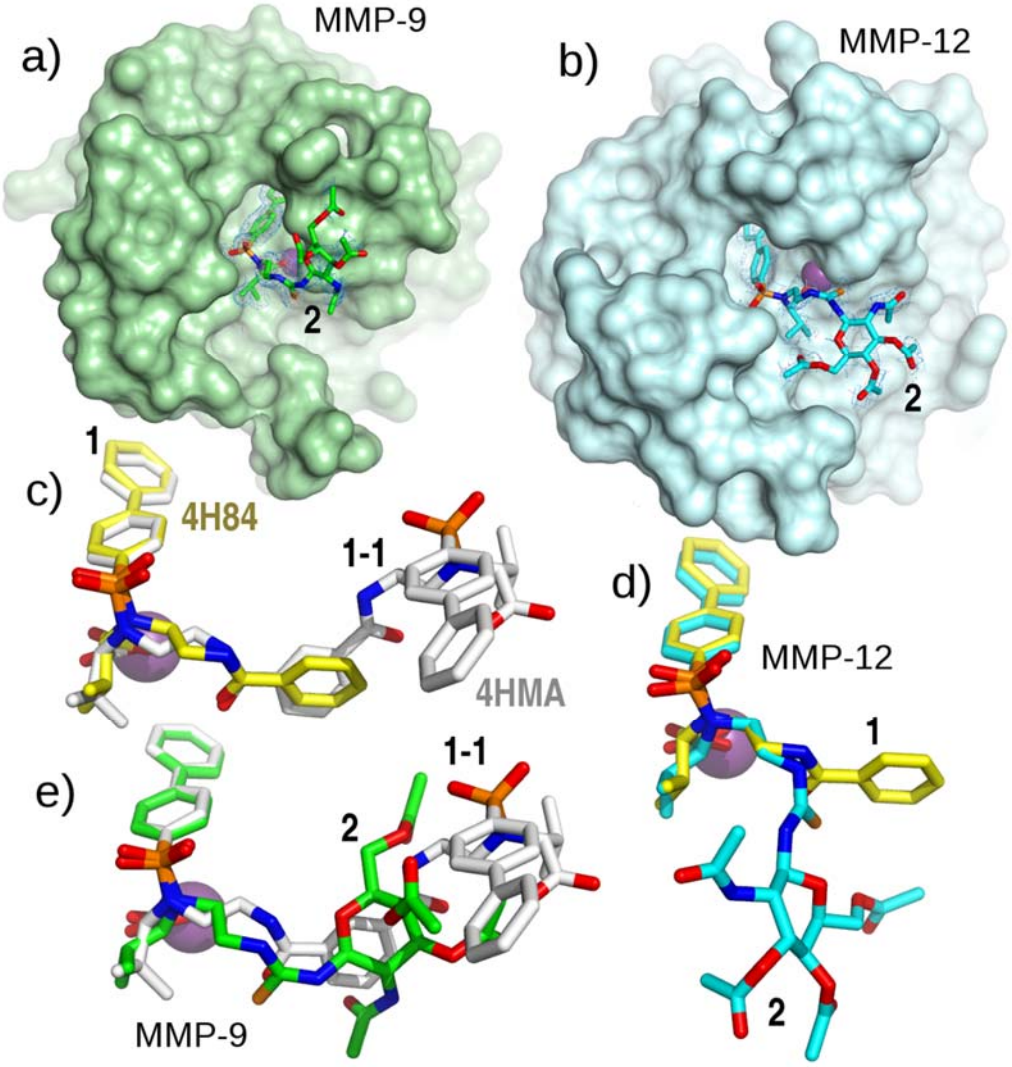

Figure 2

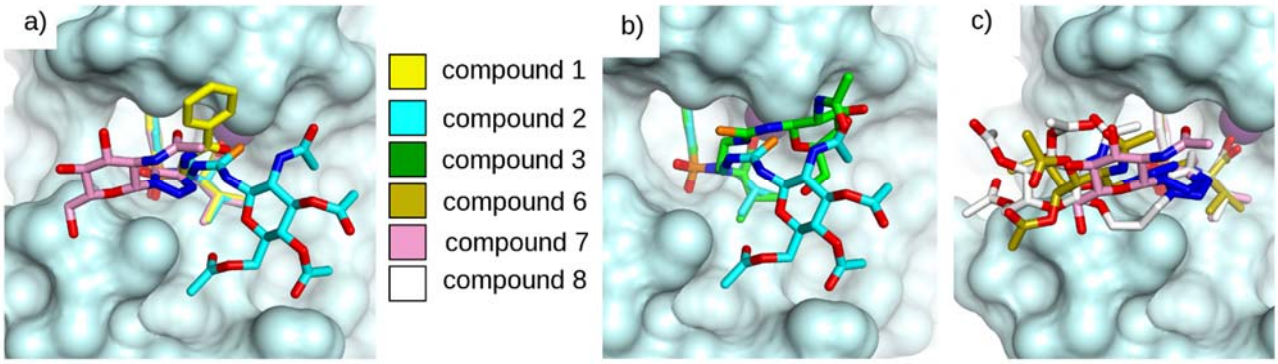
a)
compound 1
compound 2
compound 3
compound 6
compound 7
compound 8
b)
c)

Figure 3