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Bioorganic & Medicinal Chemistry 12 (2004) 2441–2450
Bioorganic & Medicinal Chemistry

New *N*-arylsulfonyl-*N*-alkoxyaminoacetohydroxamic acids as selective inhibitors of gelatinase A (MMP-2)

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Received 12 December 2003; accepted 30 January 2004

Abstract—New *N*-arylsulfonyl-substituted alkoxyaminoaceto hydroxamic acid derivatives of types **8** and **10** designed as oxa-analogues of known sulfonamide-based MMPi of types **2** and **7** were synthesized and tested for their inhibitory activities on some matrix metalloproteinases. The combination of a biphenylsulfonamide group with oxyamino oxygen in the pharmacophoric central skeleton of sulfonamide-based MMPi obtained in the new sulfonamides **10** seems to be able to give selectivity for MMP-2 over MMP-1. The most potent derivative of this type, **10a**, shows similar anti-invasive properties to the analogue reference drug CGS27023A, **2**, in an in vitro model of invasion on matrigel, carried out on cellular lines of fibrosarcoma HT1080 (tumoural cells over-expressing MMP-2 and MMP-9).

1. Introduction

During tumour progression, an increased expression of certain enzymes belonging to the family of matrix metalloproteinases (MMPs), which are believed to be involved in metastatic tumour dispersion and angiogenesis, accompanies the passage from a benign to a malignant phenotype.[1–4] These metallo (zinc) endopeptidases are produced and secreted as inactive zymogens in the extracellular matrix by the tumour cells themselves or by surrounding stromal cells stimulated by the nearby tumour. The pro-enzyme activation occurs by means of other proteases [i.e., uPa and/or MMPs, especially of a membrane-associated type, such as MT1-MMP (MMP14)]. Normally, the homeostasis of MMPs is maintained by their tissue inhibitors TIMPs, but in cancer progression, there is often an uncontrolled over-expression of MMPs.[5] Two of them, MMP-2 (gelatinase A; EC 3.4.24.24) and MMP-9 (gelatinase B; EC 3.4.24.35), seem to play an important role in these processes, considering that they are involved in metastatic tumour dispersion and angiogenesis.[6–9] Indirect confirmation of this fact may be found in recent studies on MMP-2- and MMP-9-deficient mice, which have been shown to be resistant to cancer growth and metastasis diffusion, without the development of any other abnormalities.[10,11] All these data, taken together, indicate that gelatinase A and B may represent an important target to develop new potential antimetastatic and antiangiogenic drugs.[12,13]

In the past few years, some potent 'broad spectrum' MMP inhibitors (MMPi) have been proposed and tested against tumours.[14] Some of them have entered clinical trials, but at present none of them is on the market. In actual fact, some trials with these MMPi were carried out on tumour forms, which do not express these enzymes, thus leading to disappointing results.[15] As they were developed as cytostatic agents, they may be used in

Keywords: Gelatinase A inhibitors; MMP-2 selective; Sulfonamide-based MMP inhibitors.
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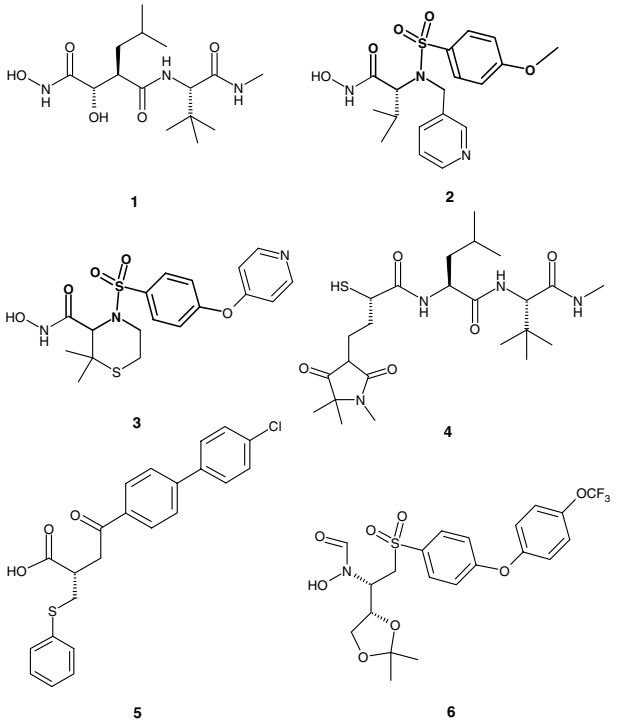
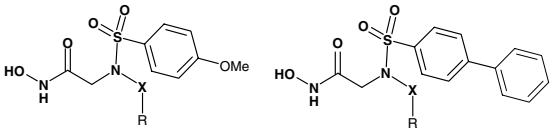
doi:10.1016/j.bmc.2004.01.047

a prolonged therapy, and therefore their bioavailability and slow long-term toxicity are very important.[16] Compounds such as Marimastat **1**, CGS-27023A **2**, Prinomastat (AG-3340) **3** and BMS-275291 **4** have shown a severe musculoskeletal syndrome, with fibroproliferative effects in the joint capsule of the knees.[17–19] These effects are thought to be linked to an impairment of normal tissue remodelling governed by the MMP-1 and/or by sheddases such as TNF-α convertase.[20] For these reasons, a lack of activity with respect to MMP-1 is considered to be an important factor in reducing some of the side effects found for 'nonselective' MMPi such as AG-3340, **3**.[21] Recently some synthetic MMPi, such as, for example, BAY 12-9566 (**5**) and the retrohydroxamate-based derivative ABT-518 (**6**), have shown a high inhibitory potency versus gelatinases A and B, with good indices of selectivity.[22,23] Also in the class of the sulfonamido aminoacids, in addition to potent nonselective gelatinase A and B inhibitors such as CGS 27023A (**2**) and its related analogue AG-3340 (**3**), some more selective MMPi have recently been identified.[22,24–28]



Starting from the hypothesis that in many classes of peptide-mimic enzyme inhibitors, even small structural modifications may significantly influence their potency and/or selectivity, we planned the synthesis of new arylsulfonamido compounds, taking as a structural model CGS 27023A, **2**, whose molecular frame is partially included also in the structure of the more active AG-3340 **3** ($IC_{50} = 83$ pM on MMP-2).

In order to carry out a preliminary screening on the principal MMPs, we synthesized the already known *N*-(4-methoxyphenyl)sulfonyl-*N*-isobutyl-acetohydroxamic acid **7a**[25] and its *N*-biphenylsulfonamido analogue **9a**, which may represent structurally simplified analogues of compounds of type **3**, and their oxa-analogues **8a** and **10a**, in which the methylenic carbon of their isobutyl group is replaced by an oxygen atom. This structural modification, already tested in other classes of enzyme inhibitors,[29] inserts into the molecular backbone of **7a** and **9a** a new functionality (the oxyaminic oxygen) able to act as a hydrogen bond acceptor, which may strengthen the interaction of the new compounds (**8a** and **10a**) with the active site of the MMPs, thus improving their biopharmacological properties. The choice of the *p*-methoxyphenyl (for **7**, **8**) or the biphenyl (for **9**, **10**) groups was made considering that many of the known MMPi contain in their molecular structures a phenyl ring substituted in the *para* position with more or less hindered groups, especially of an aromatic or heteroaromatic nature.

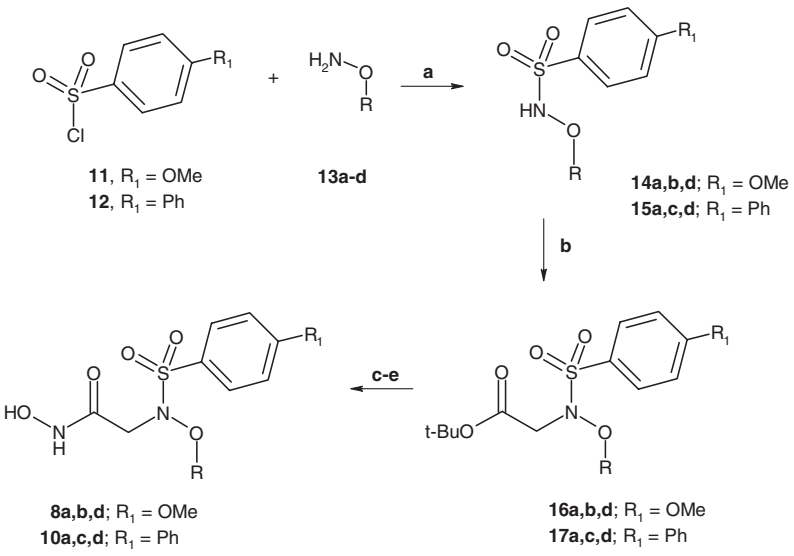


a, R = i-Pr; **b**, R = Et; **c**, R = Allyl; **d**, R = *p*-BnO-Bn

The use of 'lengthened chains' on the sulfonamide group, which is present in many of the sulfonamides studied, seems to give these known inhibitors selectivity for MMP-2 and MMP-9, accommodating these linear chains into the S1′ pocket of MMP-2 and MMP-9, which is described as a long, almost rectilinear canal.[30–35] According to our hypothesis, the combination of the two groups, the oxyaminic oxygen and the biphenyl group, in consideration of the differences in the S1′ and S2′ pockets existing between MMP-2 and MMP-1, might influence the selectivity for MMP-2 over MMP-1 of type **10** sulfonamides without modifying their inhibitory potency significantly. Finally, in order to evaluate the effects on their inhibitory properties of the replacement of the isopropyl group of **8a** and **10a** with other substituents with a different steric hindrance and lipophilicity, some of their ethyl (**8b**), allyl (**10c**) and *p*-benzyloxybenzyl (**8d** and **10d**) analogues were also synthesized.

2. Chemistry

The synthetic route to the *N*-arylsulfonyl-*N*-alkoxyaminoacetohydroxamic acids **8a**,**b**,**d** and **10a**,**c**,**d** is reported in Scheme 1. *para*-Substituted arylsulfonyl chlorides **11** and **12** were coupled with the appropriate *O*-alkylhydroxylamines **13a**–**d** in the presence of *N*-methylmorpholine to give the corresponding *O*-alkylsulfonamides **14a**,**b**,**d** and **15a**,**c**,**d**. Reaction of **14** and **15** with *tert*-butylbromoacetate yielded the corresponding *tert*-butyl esters **16a**,**b**,**d** and **17a**,**c**,**d**. The deprotection of **16** and **17**, followed by the reaction with *O*-(*tert*-butyl-



Scheme 1. Reagents and conditions: (a) NMM, anhyd THF, rt; (b) $BrCH_2CO_2$–Bu^t, Cs_2CO_3, Bu_4NHSO_4, anhyd DMF, rt, 3 days; (c) TFA, anhyd CH_2Cl_2, 0 °C, 5 h; (d) $TBDMSONH_2$, EDCl, anhyd CH_2Cl_2, 0–25 °C, 24 h; (e) TFA, anhyd CH_2Cl_2, 0 °C, 5 h.

dimethylsilyl)hydroxylamine in the presence of EDCI gave the O-silylated hydroxamates **20a**,**b**,**d** and **21a**,**c**,**d**, which were transformed by acid cleavage into the corresponding hydroxamic acids **8a**,**b**,**d** and **10a**,**c**,**d**. The carba-analogues of **8a** and **10a** (**7a** and **9a**) were prepared using a known synthetic method.[36]

3. MMP inhibition

Table 1 shows the inhibitory indices (IC_{50}) towards some of the principal MMPs of the *N*-arylsulfonyl-*N*-substituted acetohydroxamic acids **7**–**10** and of the reference drugs CGS 27023A (**2**) and Ag-3340 (**3**).[6]

For the more interesting compounds and reference drugs **2**, **3**, selectivity indices for MMP-2 over the other MMPs studied are also reported, expressed as ratios of their inhibitory indices (Table 1). On MMP-2, the two isobutylsulfonamides **7a** and **9a** show a good potency, with an IC_{50} near to 10 nM; the reference drug **2** shows a slightly lower inhibitory activity ($IC_{50} = 20$ nM) while Ag-3340 (**3**) is more active by two orders of magnitude ($IC_{50} = 0.083$ nM). On the same MMP-2, while the oxa-analogue of **7a**, compound **8a**, proves to be less active by

Table 1. Inhibitory activity of **7a**, **8a**,**b**,**d**, **9a**, **10a**,**c**,**d**, **2** and **3** towards some of the principal MMPs and, for the more active compounds, the MMP-2 selectivity[a] (in parentheses)

Compound	X	R	IC_{50} (nM)[6]				
			MMP-1	MMP-2	MMP-3	MMP-7	MMP-9
7a	CH_2	*i*-Pr	220 (32)[a]	6.9	118 (17)[a]		2.9 (0.4)[a]
8a	O	*i*-Pr	4800 (1.3)[a]	3600	7800 (2.2)[a]		3350 (0.9)[a]
8b	O	Et		9600			2200 (0.2)[a]
8d	O	*p*-Bn–O–Bn		2100			1830 (0.4)[a]
9a	CH_2	*i*-Pr	3270 (251)[a]	13	6500 (500)[a]		34 (2.6)[a]
10a	O	*i*-Pr	>50,000 (>4000)[a]	12	4500 (375)[a]	>50,000 (>4000)[a]	200 (17)[a]
10c	O	Allyl	13,000 (394)[a]	33	7800 (236)[a]		520 (16)[a]
10d	O	*p*-Bn–O–Bn	>50,000 (>4000)[a]	41	10,000 (244)[a]		640 (16)[a]
	2, CGS27023A		55 (2.8)[a]	20	22 (1.1)[a]	100 (5)[a]	8 (0.4)[a]
	3, Ag-3340		8.2 (99)[a]	0.083	0.23 (2.8)[a]	54 (650)[a]	0.26 (3.1)[a]

[a] Selectivity for MMP-2 over each of the other MMPs, is expressed as the ratio of the IC_{50} value for MMPn over the value for MMP-2.

more than two orders of magnitude than **7a**, the oxa-analogue of **9a**, compound **10a**, shows an inhibitory activity ($IC_{50} = 12$ nM) practically identical to that of **9a** ($IC_{50} = 13$ nM). Among the other *N*-alkyloxy sulfonamides, while the *p*-methoxyphenyl substituted ones **8b**,**d** show an inhibitory activity similar to that of the corresponding *O*-*i*-Pr homologue (**8a**), the *p*-biphenyl substituted ones (**10c**,**d**) are slightly less active than their *O*-*i*-Pr homologue **10a**. Also at the level of MMP-9, the isobutyl derivative **7a** appears to be the most active of the sulfonamidic compounds ($IC_{50} = 2.9$ nM), while its oxa-homologue is the least active ($IC_{50} = 3350$ nM). Also the isobutyl derivative **9a** shows a good inhibitory activity ($IC_{50} = 34$ nM) while its oxa-homologue **10a** exhibits an inhibitory activity six times lower ($IC_{50} = 200$ nM). On the same enzyme, the *p*-MeO substituted compounds **8b**,**d** prove to be slightly more active than their homologue **8a**, whereas the *p*-Ph-substituted ones **10c** and **10d** are slightly less active than their homologue **10a**. On MMP-9, Ag-3340 **3** shows a sub-nanomolar inhibitory activity ($IC_{50} = 0.26$ nM). At the level of MMP-1, among the sulfonamido derivatives **7**–**10**, only the isobutyl one **7a** shows an IC_{50} in the sub-micromolar range, while the other compounds are scarcely active, with an IC_{50} ranging from 3270 nM of **9a** to 13,000 nM of **10c**, or practically inactive, with an $IC_{50} > 5 \times 10^4$ nM (**10a**,**d**). On the same MMP-1, the reference drugs **2** and **3** are potent inhibitors, with IC_{50} values in the nanomolar range. On MMP-3, all compounds **7**–**10**, with the only exception of **7a**, which shows an IC_{50} value of 118 nM, are very poor inhibitors (IC_{50} values >4 μM), while the two reference drugs **2** and **3** are very potent (IC_{50} in the nanomolar or sub-nanomolar range, respectively). The compound, which is most active on MMP-2 (**10a**) appears to be practically inactive on MMP-7, while the two reference drugs **2** and **3** show a good inhibitory activity. An analysis of the selectivity indices reported in parentheses in Table 1 for the *N*-alkoxy-*N*-sulfonamides **8a**, **10a**,**c**,**d** and the reference compounds **2** and **3** shows that **10a** and **10d** are the ones with the highest selectivity profile (MMP-1/MMP-2 ratio >4000 for both compounds, MMP-3/MMP-2 ratios of 375 and 244 for **10a** and **10d**, respectively, MMP-7/MMP-2 ratio >4000 for **10a** and MMP-9/MMP-2 ratios of 17 and 16 for **10a** and **10d**, respectively). The *N*-isobutyl-sulfonamidic compounds **7a** and **9a** (similar in their inhibitory potency towards MMP-2 to **10a**, **10c** and **10d**) show a lower MMP-1/MMP-2 selectivity ratio that is 32 and 251, respectively. On the MMPs tested, the reference compound **2** appears to be practically devoid of any selectivity; also Ag-3340 **3** proves to be devoid of MMP-3/MMP-2 and MMP-9/MMP-2 selectivity, but proves to be more selective, with respect to CGS 27023A **2**, towards the other MMPs (MMP-1/MMP-2 and MMP-7/MMP-2 ratios of 99 and 650, respectively).

4. Cellular studies

The most potent and MMP-2 selective of the new inhibitors, the biphenylsulfonamide **10a**, was tested in an in vitro model of invasion on matrigel, carried out on cellular lines of fibrosarcoma HT1080 (tumoural cells that over-express MMP-2 and MMP-9).[37] Figure 1 shows the effects of **10a** (Fig. 1a) and of drug **2** (Fig. 1b) on the invasion process, in comparison with the control results (Fig. 1c). Normally HT1080 cells plated on matrigel in complete medium, rapidly organize into 'cord-like structures' with an 'inhibitor-insensitive' behaviour, indicating the lack of toxic effects. From these colonies some small elongations, representing the invasive cells, can be observed (red arrows). Both compounds (**10a** and **2**), at the same concentration of 50 nM, show a significant reduction in the total number of invasive elongations observed in controls, thus suggesting similar anti-invasive properties (see red arrows in Fig. 1a and b and compare their amount with the control in Fig. 1c).

A quantification of the inhibitory effect in the matrigel test of **10a** and of drug **2** is shown in Figure 2.

5. Conclusions

The replacement of the methylenic carbon atom linked to the sulfonamidic nitrogen of **7a** and **9a** with an oxygen atom has a negative effect at the level of all the MMPs studied. This fact can be seen in their structurally related sulfonamides **8a** and **10a**. The only exceptions are the biphenyl-substituted compounds **9a** and **10a**, where the oxygenated analogue **10a** maintains the same inhibitory activity as the corresponding methylenic substituted **9a** at the level of MMP-2. In these type **10** oxy-substituted compounds, also the other two biphenyl homologues of **10a**, the derivatives **10c** and **10d**, appear to possess a good ability to inhibit MMP-2. As a con-

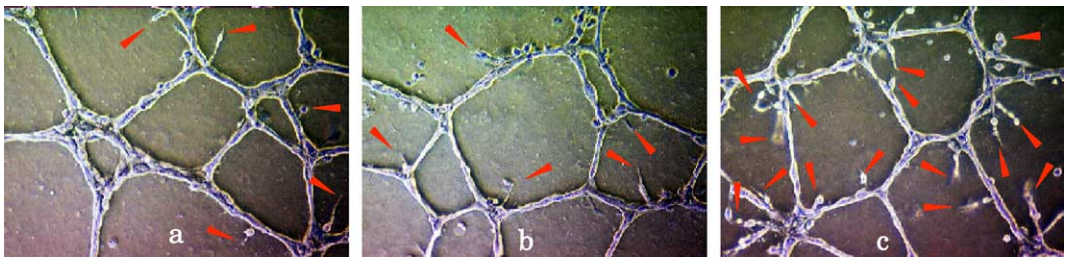


Figure 1. In vitro inhibition of HT1080 sarcoma cells growth on matrigel by **10a** (a), CGS 27023A, **2** (b) and control (c).

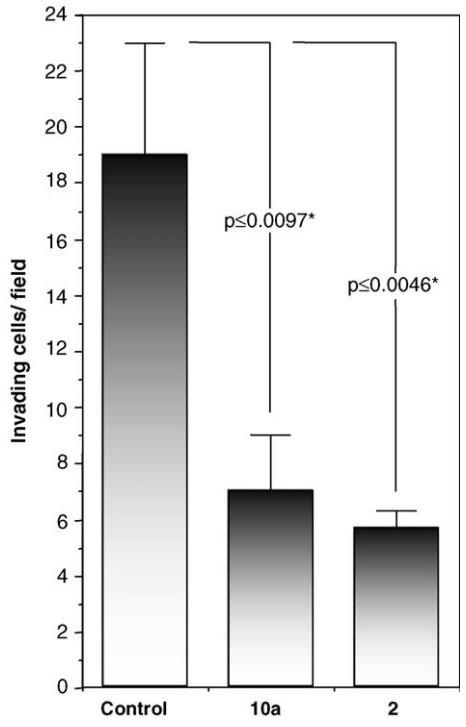


Figure 2. Means of invasive elongations/field for **10a** and for **2** as compared to the control. *Student's test.

sequence, for the more lipophilic biphenyl-substituted molecules of type **10**, it may be hypothesized that the presence of the oxyaminic group may give to these types of sulfonamide-based inhibitors an appreciable MMP-2 selectivity, in particular with respect to MMP-1. The MMP-2 selectivity of type **10** compounds seems to be interesting, even when compared with that of the reference model compounds CGS 27023A (**2**) and Ag-3340 (**3**), which are poorly selective.

Finally, the selective and potent *N*-biphenylsulfonyl-*N*-isopropoxy-aminoacetohydroxamic acid **10a** proves to be effective also in an in vitro model of invasion on matrigel carried out on cellular lines of fibrosarcoma HT1080 (tumoural cells over-expressing MMP-2).

In conclusion, the results of this study seem to indicate a possible structural requirement to be taken into consideration in the design of new sulfonamido-based MMPi useful in the control of tumoural invasion and angiogenesis. In addition, compounds structurally related to **10** may be useful tools in the study of the role of the single MMPs in certain pathological processes.[11–13,15]

6. Experimental

6.1. General methods

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Infrared (IR) spectra for comparison of compounds were recorded on a Mattson 1000 FTIR spectrometer. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Gemini 200 (200 MHz) in a ca. 2% solution of $CDCl_3$ or DMSO-d_6 for all compounds. Peak positions are given in parts per million (ppm, δ units). The proton magnetic resonance assignments were established on the basis of the expected chemical shifts and the multiplicity of the signals. Mass spectra were recorded on a HP-5988 A spectrometer using a direct injection probe and an electron beam energy of 70 eV. Reactions were routinely monitored by thin-layer chromatography (TLC) on 0.25 mm silica gel plates (Merck $60F_{254}$) and hydroxamic acids were visualized with $FeCl_3$ aqueous solution. Flash chromatography or preparative medium pressure liquid chromatography (MPLC) were carried out through glass columns containing 40–63 μm silica gel (Macherey-Nagel Silica Gel 60) The MPLCs were performed using a chromatographic apparatus consisting of a Buchi 681 pump, a Knauer differential refractometer detector and a Philips PM 8220 pen recorder. Solvents and reagents were obtained from commercial sources in the appropriate grade and were used without further purification unless otherwise indicated. Reactions were run under an argon atmosphere. Elemental analyses were carried out by our analytical laboratory and were consistent with theoretical values to within ±0.4%.

6.1.1. General procedure for the preparation of sulfonamides (14a,b,d, 15a,c,d). A solution of the sulfonyl chloride **9** or **10** (7.0 mmol) in anhydrous THF (18.0 mL) was added dropwise to a stirred and cooled (0 °C) solution of the appropriate *O*-alkyl hydroxylamine hydrochloride **11a–d** (7.0 mmol) and *N*-methylmorpholine (1.5 mL, 14 mmol) in anhydrous THF (18.0 mL). After 30 min under these conditions, the reaction mixture was allowed to rest at rt for 4 days and was then diluted with H_2O (100 mL) and extracted with EtOAc (100 mL) giving, after work-up, crude residues, which were purified by flash chromatography on silica gel to yield **14a**,**b**,**d**, **15a**,**c**,**d** as pure solids.

6.1.1.1. *N*-Isopropoxy-4-methoxybenzenesulfonamide (14a). The title compound was prepared from *O*-isopropylhydroxylamine hydrochloride **13a** and 4-methoxybenzenesulfonyl chloride **11** following the general procedure. Compound **14a** was used without further purification: a white solid (65%); mp 93–95 °C; ^{1}H NMR ($CDCl_3$): δ 1.17 (d, $J = 6.2$ Hz, 6H), 3.88 (s, 3H), 4.23 (septet, $J = 6.2$ Hz, 1H), 6.67 (s, 1H), 6.98–7.02 (m, 2H), 7.83–7.87 (m, 2H). Anal. ($C_{10}H_{15}NO_4S$) C, H, N.

6.1.1.2. *N*-Ethyloxy-4-methoxybenzenesulfonamide (14b). The title compound was prepared from *O*-ethyloxyhydroxylamine hydrochloride **13b** and 4-methoxybenzenesulfonyl chloride **11** following the general procedure. Compound **14b** was used without further purification: a white solid (76%); mp 64–66 °C; ^{1}H NMR ($CDCl_3$): δ 1.18 (t, $J = 7.1$ Hz, 3H), 3.88 (s, 3H), 4.01 (q,

$J = 7.1$ Hz, 2H), 6.89 (s, 1H), 6.98–7.02 (m, 2H), 7.83–7.88 (m, 2H). Anal. ($C_9H_{13}NO_4S$) C, H, N.

6.1.1.3. *N*-Benzyloxy-benzyloxy-4-methoxybenzenesulfonamide (14d). The title compound was prepared from *O*-benzyloxy-benzyloxyhydroxylamine hydrochloride **13d** and 4-methoxybenzenesulfonyl chloride **11** following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane–ethyl acetate 2/1 v/v to give **14d** as a pale yellow solid (46%): $R_f = 0.27$; ^{1}H NMR ($CDCl_3$): δ 3.87 (s, 3H), 4.90 (s, 2H), 5.07 (s, 2H), 6.89 (s, 1H), 6.92–7.01 (m, 4H), 7.27–7.42 (m, 7H), 7.83–7.88 (m, 2H). Anal. ($C_{21}H_{21}NO_5S$) C, H, N.

6.1.1.4. *N*-Isopropoxy-1,1′-biphenyl-4-sulfonamide (15a). The title compound was prepared from *O*-isopropylhydroxylamine hydrochloride **13a** and 1,1′-biphenyl-4-sulfonyl chloride **12** following the general procedure. Compound **15a** was used without further purification: a white solid (83%); mp 156–158 °C; ^{1}H NMR ($CDCl_3$) δ: 1.21 (d, $J = 6.2$ Hz, 6H), 4.3 (septet, $J = 6.2$ Hz, 1H), 6.78 (s, 1H), 7.42–7.63 (m, 5H), 7.72–7.77 (m, 2H), 7.96–8.01 (m, 2H). Anal. ($C_{15}H_{17}NO_3S$) C, H, N.

6.1.1.5. *N*-Allyloxy-1,1′-biphenyl-4-sulfonamide (15c). The title compound was prepared from *O*-allylhydroxylamine hydrochloride **13c** and 1,1′-biphenyl-4-sulfonyl chloride **12** following the general procedure. The crude reaction mixture was purified by flash chromatography on silica gel using hexane–ethyl acetate 3/1 v/v to give **15c** as a pale yellow solid (37%): $R_f = 0.37$; mp 116–118 °C; ^{1}H NMR ($CDCl_3$) δ: 4.50 (d, $J = 7.3$ Hz, 2H), 5.25–5.37 (m, 2H), 5.80–6.10 (m, 1H), 6.98 (s, 1H), 7.42–7.64 (m, 5H), 7.73–7.78 (m, 2H), 7.97–8.02 (m, 2H). Anal. ($C_{15}H_{15}NO_3S$) C, H, N.

6.1.1.6. *N*-Benzyloxy-benzyloxy-1,1′-biphenyl-4-sulfonamide (15d). The title compound was prepared from *O*-benzyloxy-benzyloxyhydroxylamine hydrochloride **13d** and 1,1′-biphenyl-4-sulfonyl chloride **12** following the general procedure. Recrystallization of the crude product from isopropanol provided **15d** as a white solid (68%): mp 176–177 °C; ^{1}H NMR (DMSO-d_6) δ: 4.83 (s, 2H), 5.09 (s, 2H), 6.97–7.01 (m, 2H), 7.25–7.51 (m, 10H), 7.72–7.76 (m, 2H), 7.91 (m, 4H), 10.47 (s, 1H). Anal. ($C_{26}H_{23}NO_4S$) C, H, N.

6.1.2. General procedure for the preparation of *tert*-butyl esters (16a,b,d, 17a,c,d). A solution of the appropriate sulfonamide (**13**, **14**) (1 mmol) in anhydrous DMF (3.0 mL) was treated with *tert*-butylbromoacetate (1.2 mmol), caesium carbonate (1 mmol) and tetrabutylammonium hydrogensulfate (1 mmol). The reaction mixture was stirred for 3 days at rt, then diluted with H_2O (20.0 mL) and extracted three times with ethyl acetate (20.0 mL) giving, after work-up, crude residues, which were purified by flash chromatography on silica gel to yield **16a**,**b**,**d**, **17a**,**c**,**d** as pure solids.

6.1.2.1. *tert*-Butyl{isopropoxy[(4-methoxyphenyl)sulfonyl]amino}acetate (16a). The title compound was prepared from **14a** and *tert*-butylbromoacetate following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane–ethyl acetate 3/1 v/v to give **16a** as an oil (52%): $R_f = 0.44$; ^{1}H NMR ($CDCl_3$): δ 1.23 (d, $J = 6.2$ Hz, 6H), 1.46 (s, 9H), 3.58 (s, 2H), 3.88 (s, 3H), 4.52 (septet, $J = 6.2$ Hz, 1H), 6.99–7.04 (m, 2H), 7.78–7.82 (m, 2H). Anal. ($C_{16}H_{25}NO_6S$) C, H, N.

6.1.2.2. *tert*-Butyl{ethyloxy[(4-methoxyphenyl)sulfonyl]amino}acetate (16b). The title compound was prepared from **14b** and *tert*-butylbromoacetate following the general procedure. The crude product **16b** was used without further purification: a yellow gel (76%); ^{1}H NMR ($CDCl_3$): δ 1.15 (t, $J = 7.1$ Hz, 3H), 1.45 (s, 9H), 3.58 (s, 2H), 3.88 (s, 3H), 4.31 (q, $J = 7.1$ Hz, 2H), 7.00–7.04 (m, 2H), 7.77–7.82 (m, 2H). Anal. ($C_{15}H_{23}NO_6S$) C, H, N.

6.1.2.3. *tert*-Butyl{benzyloxybenzyloxy[(4-methoxyphenyl)sulfonyl]amino}acetate (16d). The title compound was prepared from **14d** and *tert*-butylbromoacetate following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane–ethyl acetate 7/2 v/v to give **16d** as a yellow solid (32%): $R_f = 0.25$; ^{1}H NMR ($CDCl_3$): δ 1.47 (s, 9H), 3.61 (s, 2H), 3.84 (s, 3H), 5.06 (s, 2H), 5.25 (s, 2H), 6.92–6.98 (m, 4H), 7.29–7.40 (m, 7H), 7.75–7.80 (m, 2H). Anal. ($C_{27}H_{31}NO_7S$) C, H, N.

6.1.2.4. *tert*-Butyl[(1,1′-biphenyl-4-ylsulfonyl)(isopropoxy)amino]acetate (17a). The title compound was prepared from **15a** and *tert*-butylbromoacetate following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane–ethyl acetate 7/1 v/v to give **17a** as a white solid (34%): $R_f = 0.25$; mp 70–72 °C; ^{1}H NMR ($CDCl_3$): δ 1.26 (d, $J = 6.2$ Hz, 6H), 1.47 (s, 9H), 3.64 (br s, 2H), 4.58 (septet, $J = 6.2$ Hz, 1H), 7.42–7.64 (m, 5H), 7.74–7.78 (m, 2H), 7.91–7.95 (m, 2H). Anal. ($C_{21}H_{27}NO_5S$) C, H, N.

6.1.2.5. *tert*-Butyl[(1,1′-biphenyl-4-ylsulfonyl)(allyloxy)amino]acetate (17c). The title compound was prepared from **15c** and *tert*-butylbromoacetate following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane–ethyl acetate 5/1 v/v to give **17c** as a white solid (64%): $R_f = 0.38$; mp 95–97 °C; ^{1}H NMR ($CDCl_3$): δ 1.46 (s, 9H), 3.68 (br s, 2H), 4.82 (d, $J = 6.2$ Hz, 2H), 5.21–5.37 (m, 2H), 5.84–6.10 (m, 1H), 7.43–7.64 (m, 5H), 7.74–7.78 (m, 2H), 7.91–7.95 (m, 2H). Anal. ($C_{21}H_{25}NO_5S$) C, H, N.

6.1.2.6. *tert*-Butyl[(1,1′-biphenyl-4-ylsulfonyl)(benzyloxybenzyloxy)amino]acetate (17d). The title compound was prepared from **15d** and *tert*-butylbromoacetate following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane–ethyl acetate 4/1 v/v to give **17d** as a white solid

(60%): $R_f = 0.37$; mp 105–107 °C; ^{1}H NMR ($CDCl_3$): δ 1.48 (s, 9H), 3.69 (br s, 2H), 5.06 (s, 2H), 5.30 (s, 2H), 6.93–6.98 (m, 2H), 7.32–7.59 (m, 12H), 7.68–7.72 (m, 2H), 7.89–7.93 (m, 2H). Anal. ($C_{32}H_{33}NO_6S$) C, H, N.

6.1.3. General procedure for the preparation of carboxylic acids (18a,b,d, 19a,c,d). Trifluoroacetic acid (4.4 mL, 57 mmol) was added dropwise to a stirred and cooled (0 °C) solution of the appropriate *tert*-butyl ester **15**,**16** (1 mmol) in freshly distilled (P_2O_5) dichloromethane (3.4 mL). The solution was stirred for 5 h at 0 °C and the solvent was then removed in vacuo to give pure **18a**,**b**,**d**, **19a**,**c**,**d** as solids.

6.1.3.1. {Isopropoxy[(4-methoxyphenyl)sulfonyl]amino}-acetic acid (18a). The title compound was prepared from **16a** following the general procedure. The crude product **18a** was used without further purification: a yellow oil (99%); ^{1}H NMR ($CDCl_3$): δ 1.23 (d, $J = 6.2$ Hz, 6H), 3.74 (s, 2H), 3.89 (s, 3H), 4.52 (septet, $J = 6.2$ Hz, 1H), 7.01–7.05 (m, 2H), 7.80–7.83 (m, 2H). Anal. ($C_{12}H_{17}NO_6S$) C, H, N.

6.1.3.2. {Ethyloxy[(4-methoxyphenyl)sulfonyl]amino}-acetic acid (18b). The title compound was prepared from **16b** following the general procedure. The crude product **18b** was used without further purification: a yellow oil (99%); ^{1}H NMR ($CDCl_3$): δ 1.15 (t, $J = 7.1$ Hz, 3H), 3.76 (s, 2H), 3.89 (s, 3H), 4.28 (q, $J = 7.1$ Hz, 2H), 7.01–7.06 (m, 2H), 7.79–7.83 (m, 2H). Anal. ($C_{11}H_{15}NO_6S$) C, H, N.

6.1.3.3. {Benzyloxybenzyloxy[(4-methoxyphenyl)sulfonyl]amino}acetic acid (18d). The title compound was prepared from **16d** following the general procedure. The crude product **18d** was used without further purification: a yellow solid (96%); ^{1}H NMR ($CDCl_3$): δ 3.78 (s, 2H), 3.85 (s, 3H), 5.04 (s, 2H), 5.21 (s, 2H), 5.40 (br s, 1H), 6.92–7.00 (m, 4H), 7.28–7.40 (m, 7H), 7.77–7.81 (m, 2H). Anal. ($C_{23}H_{23}NO_7S$) C, H, N.

6.1.3.4. [(Isopropoxy)(1,1′-biphenyl-4-ylsulfonyl)amino]-acetic acid (19a). The title compound was prepared from **17a** following the general procedure. The crude product **19a** was used without further purification: a yellow solid (86%); mp 125–127 °C; ^{1} H NMR ($CDCl_3$): δ 1.26 (d, $J = 6.2$ Hz, 6H), 3.82 (s, 2H), 4.58 (septet, $J = 6.2$ Hz, 1H), 7.43–7.64 (m, 5H), 7.76–7.80 (m, 2H), 7.92–7.96 (m, 2H). Anal. ($C_{17}H_{19}NO_5S$) C, H, N.

6.1.3.5. [(Allyloxy)(1,1′-biphenyl-4-ylsulfonyl)amino]-acetic acid (19c). The title compound was prepared from **17c** following the general procedure. The crude product **19c** was used without further purification: a pale yellow solid (92%); mp 150–152 °C; ^{1}H NMR ($CDCl_3$): δ 3.86 (s, 2H), 4.81 (d, $J = 6.2$ Hz, 2H), 5.23–5.37 (m, 2H), 5.81–6.01 (m, 1H), 7.43–7.63 (m, 5H), 7.76–7.80 (m, 2H), 7.92–7.96 (m, 2H). Anal. ($C_{17}H_{17}NO_5S$) C, H, N.

6.1.3.6. [(Benzyloxybenzyloxy)(1,1′-biphenyl-4-ylsulfonyl)amino]acetic acid (19d). The title compound was prepared from **17d** following the general procedure. The crude product was purified by chromatography on silica gel using hexane–ethyl acetate 2/1 v/v to give **19d** as a solid (28%); mp 62–63 °C; ^{1}H NMR ($CDCl_3$): δ 3.83 (s, 2H), 5.01 (s, 2H), 5.25 (s, 2H), 6.90–6.94 (m, 2H), 7.29–7.57 (m, 12H), 7.65–7.70 (m, 2H), 7.87–7.91 (m, 2H). Anal. ($C_{28}H_{25}NO_6S$) C, H, N.

6.1.4. General procedure for the preparation of *O*-TBDMS acid hydroxyamides 20a,b,d, 21a,c,d. 1-[3-(Dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (EDCI) was added portionwise (1 mmol) to a stirred and cooled (0 °C) solution of the carboxylic acid **18**, **19** (1 mmol) and *O*-(*tert*-butyldimethylsilyl-hydroxylamine (1 mmol) in freshly distilled (P_2O_5) dichloromethane (18 mL). After stirring at rt for 20 h, the mixture was washed with water (20 mL) and the organic phase was dried and evaporated in vacuo. The residue was purified by flash chromatography to yield pure silyl esters **20a**,**b**,**d**, **21a**,**c**,**d** as oils.

6.1.4.1. 2-{Isopropoxy[(4-methoxyphenyl)sulfonyl]-amino}acetic acid (*tert*-butyldimethylsilyl)hydroxyamide (20a). The title compound was prepared from **18a** following the general procedure. The crude oil **20a** (75%) was used without further purification; ^{1}H NMR ($CDCl_3$): δ 0.20 (s, 6H), 0.97 (s, 9H), 1.22 (d, $J = 6.2$ Hz, 6H), 3.66 (s, 2H), 3.89 (s, 3H), 4.41 (septet, $J = 6.2$ Hz, 1H), 7.01–7.05 (m, 2H), 7.78–7.82 (m, 2H), 8.59 (br s, 1H). Anal. ($C_{18}H_{32}N_2O_6SSi$) C, H, N.

6.1.4.2. 2-{Ethyloxy[(4-methoxyphenyl)sulfonyl]amino}-acetic acid (*tert*-butyldimethylsilyl)hydroxyamide (20b). The title compound was prepared from **18b** following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane–ethyl acetate 1/1 v/v to give **20b** as a yellow oil (20%): ^{1}H NMR ($CDCl_3$): δ 0.20 (s, 6H), 0.97 (s, 9H), 1.19 (t, $J = 7.1$ Hz, 3H), 3.62 (s, 2H), 3.89 (s, 3H), 4.21 (q, $J = 7.1$ Hz, 2H), 6.98–7.01 (m, 2H), 7.77–7.82 (m, 2H). Anal. ($C_{17}H_{30}N_2O_6SSi$) C, H, N.

6.1.4.3. 2-{Benzyloxybenzyloxy[(4-methoxyphenyl)sulfonyl]amino}acetic acid (*tert*-butyldimethylsilyl)hydroxyamide (20d). The title compound was prepared from **18d** following the general procedure. The crude oil **20d** (67%) was used without further purification; ^{1}H NMR ($CDCl_3$): δ 0.17 (s, 6H), 0.97 (s, 9H), 3.57 (s, 2H), 3.86 (s, 3H), 5.06 (s, 2H), 5.09 (s, 2H), 6.95–7.00 (m, 4H), 7.31–7.41 (m, 7H), 7.76–7.81 (m, 2 H), 8.16 (br s, 1H). Anal. ($C_{29}H_{38}N_2O_7SSi$) C, H, N.

6.1.4.4. [(Isopropoxy)(1,1′-biphenyl-4-ylsulfonyl)amino]-acetic acid (*tert*-butyldimethylsilyl)hydroxyamide (21a). The title compound was prepared from **19a** following the general procedure. The crude oil **21a** (58%) was used without further purification; ^{1}H NMR ($CDCl_3$): δ 0.20 (s, 6H), 0.90 (s, 9H), 1.26 (d, $J = 6.2$ Hz, 6H), 3.71 (s, 2H), 4.50 (septet, $J = 6.2$ Hz, 1H), 7.42–7.64 (m, 5H),

7.76–7.80 (m, 2H), 7.92–7.96 (m, 2H). Anal. ($C_{23}H_{34}N_2O_5SSi$) C, H, N.

6.1.4.5. [(Allyloxy)(1,1′-biphenyl-4-ylsulfonyl)amino]-acetic acid (*tert*-butyldimethylsilyl)hydroxyamide (21c). The title compound was prepared from **19c** following the general procedure. The crude pale yellow oil **21c** (81%) was used without further purification; ^{1}H NMR ($CDCl_3$): δ 0.10 (s, 6H), 0.91 (s, 9H), 3.76 (s, 2H), 4.69 (d, $J = 6.2$ Hz, 2H), 5.33–5.43 (m, 2H), 5.80–6.00 (m, 1H), 7.46–7.64 (m, 5H), 7.76–7.79 (m, 2H), 7.91–7.95 (m, 2H). Anal. ($C_{23}H_{32}N_2O_5SSi$) C, H, N.

6.1.4.6. [(Benzyloxybenzyloxy)(1,1′-biphenyl-4-ylsulfonyl)amino]acetic acid (*tert*-butyldimethylsilyl)hydroxyamide (21d). The title compound was prepared from **19d** following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane–ethyl acetate 5/2 v/v to give **21d** as a yellow oil (9%): $R_f = 0.35$; ^{1}H NMR ($CDCl_3$): δ 0.18 (s, 6H), 0.96 (s, 9H), 3.63 (s, 2H), 5.06 (s, 2H), 5.14 (s, 2H), 6.97–7.01 (m, 2H), 7.32–7.53 (m, 10H), 7.56–7.61 (m, 2H), 7.71–7.75 (m, 2H), 7.89–7.94 (m, 2H), 8.11 (s, 1H). Anal. ($C_{34}H_{40}N_2O_6SSi$) C, H, N.

6.1.5. General procedure for the preparation of acid hydroxyamides (8a,b,d, 10a,c,d). Trifluoroacetic acid (4.4 mL, 57 mmol) was added dropwise to a stirred and cooled solution (0 °C) of **20a**,**b**,**d**, or **21a**,**c**,**d** (1 mmol) in freshly distilled (P_2O_5) dichloromethane (3.4 mL). The solution was stirred under these reaction conditions for 5 h and the solvent was removed in vacuo to give **8a**,**b**,**d**, **10a**,**c**,**d** as solids.

6.1.5.1. 2-{Isopropoxy[(4-methoxyphenyl)sulfonyl]-amino}-*N*-hydroxyacetamide (8a). The title compound was prepared from **20a** following the general procedure. Recrystallization from $CHCl_3$/hexane gave **8a** as a white solid (67%): mp 62–64 °C; ^{1}H NMR (DMSO-d_6): δ 1.12 (d, $J = 6.2$ Hz, 6H), 3.74 (s, 2H), 3.87 (s, 3H), 4.29 (septet, $J = 6.2$ Hz, 1H), 7.17–7.22 (m, 2H), 7.76–7.81 (m, 2H), 10.69 (s, 1H). EI-MS m/z: 319 (1, M+1), 276 (2), 171 (36), 107 (22), 45 (100). Anal. ($C_{12}H_{18}N_2O_6S$) C, H, N.

6.1.5.2. 2-{Ethyloxy[(4-methoxyphenyl)sulfonyl]amino}-*N*-hydroxyacetamide (8b). The title compound was prepared from **20b** following the general procedure. The crude product was purified by preparative thin-layer chromatography using $CHCl_3$–MeOH 9/1 as the eluent to give **8b** as a yellow solid (3%): ^{1}H NMR ($CDCl_3$): δ 1.16 (t, $J = 6.9$ Hz, 3H), 3.66 (s, 2H), 3.88 (s, 3H), 4.18 (q, $J = 6.9$ Hz, 2H), 7.00–7.05 (m, 2H), 7.77–7.82 (m, 2H). EI-MS m/z: 171 (84.19), 107 (67.69), 77 (100), 44 (88.67). Anal. ($C_{11}H_{16}N_2O_6S$) C, H, N.

6.1.5.3. 2-{Benzyloxybenzyloxy[(4-methoxyphenyl)sulfonyl]amino}-*N*-hydroxyacetamide (8d). The title compound was prepared from **20d** following the general procedure. Recrystallization from $CHCl_3$/hexane gave **8d** as a white solid (62%): mp 140–142 °C; ^{1}H NMR (DMSO-d_6): δ 3.62 (s, 2H), 3.87 (s, 3H), 4.93 (s, 2H), 5.10 (s, 2H), 6.96–7.00 (m, 2H), 7.14–7.41 (m, 9H), 7.73–7.78 (m, 2H), 10.70 (s, 1H). EI-MS m/z: 214 (1.95), 171 (8.14), 107 (8.86), 91 (100), 44 (34.18). Anal. ($C_{23}H_{24}N_2O_7S$) C, H, N.

6.1.5.4. 2-[(Isopropoxy)(1,1′-biphenyl-4-ylsulfonyl)-amino]-*N*-hydroxyacetamide (10a). The title compound was prepared from **21a** following the general procedure. Recrystallization from diethyl ether/hexane provided **10a** as white solid: mp 99–101 °C (25%); ^{1}H NMR ($CDCl_3$): δ 1.25 (d, $J = 6.2$ Hz, 6H), 3.79 (s, 2H), 4.48 (septet, $J = 6.2$ Hz, 1H), 7.44–7.64 (m, 5H), 7.76–7.80 (m, 2H), 7.91–7.96 (m, 2H). EI-MS m/z: 365 (2, M+1), 321 (8), 217 (51), 152 (100), 43 (55). Anal. ($C_{17}H_{20}N_2O_5S$) C, H, N.

6.1.5.5. 2-[(Allyloxy)(1,1′-biphenyl-4-ylsulfonyl)amino]-*N*-hydroxyacetamide (10c). The title compound was prepared from **21c** following the general procedure. The crude product was purified by flash chromatography on silica gel using $CHCl_3$–MeOH 9/1 v/v as the eluent. Recrystallization from diethyl ether/hexane gave **10c** as a white solid (57%): mp 133–135 °C; ^{1}H NMR (DMSO-d_6): δ 3.49 (s, 2H), 4.56 (d, $J = 6.2$ Hz, 2H), 5.20–5.32 (m, 2H), 5.81–5.91 (m, 1H), 7.50–7.57 (m, 3H), 7.76–7.80 (m, 2H), 7.89–8.03 (m, 4H), 9.04 (s, 1H), 10.69 (s, 1H). EI-MS m/z: 363 (1, M+1), 322 (7), 217 (46), 152 (100), 41 (62). Anal. ($C_{17}H_{18}N_2O_5S$) C, H, N.

6.1.5.6. 2-[(Benzyloxybenzyloxy)(1,1′-biphenyl-4-ylsulfonyl)amino]-*N*-hydroxyacetamide (10d). The title compound was prepared from **21d** following the general procedure. Recrystallization from diethyl ether/hexane gave **10d** as yellow solid (94%): mp 75–77 °C; ^{1}H NMR ($CDCl_3$): δ 3.67 (br s, 2H), 5.04 (s, 2H), 5.11 (s, 2H), 6.95–6.98 (m, 2H), 7.33–7.57 (m, 12H), 7.67–7.71 (m, 2H), 7.87–7.91 (m, 2H). Anal. ($C_{28}H_{26}N_2O_6S$) C, H, N.

6.1.5.7. *N*-Isobutyl-1,1′-biphenyl-4-sulfonamide (22). Isobutylamine **23** (4.7 mL, 47.4 mmol) was dissolved in $CHCl_3$ (36 mL), and the solution was cooled to 0 °C. 1,1′-Biphenyl-4-sulfonyl chloride **11** (4.0 g, 15.8 mmol) was added to this solution. The reaction mixture was stirred at rt for 1 h and then refluxed for 1 h. After being cooled back to rt, the reaction mixture was washed three times with 4 N hydrochloric acid, twice with water and once with brine, and then it was dried (Na_2SO_4), and the solvent was evaporated to give **22** as a solid (4.0 g, 87%): mp 88–90 °C; ^{1}H NMR ($CDCl_3$): δ 0.89 (d, $J = 6.8$ Hz, 6H), 1.74 (m, 1H), 2.81 (dd, $J = 6.6$ and 6.8 Hz, 2H), 4.47 (t, 1H), 7.41–7.53 (m, 3H), 7.58–7.64 (m, 2H), 7.69–7.75 (m, 2 H), 7.89–7.94 (m, 2H). Anal. ($C_{16}H_{19}NO_2S$) C, H, N.

6.1.5.8. Ethyl[isobutyl-1,1′-biphenyl-4-sulfonylamino]-acetate (24). Sodium hydride (0.27 g of a 60% oil dispersion, 6.9 mmol) was suspended in THF (17.6 mL). A solution of sulfonamide **22** (2.0 g, 6.9 mmol) also in THF

(70.7 mL) was added, and the reaction mixture was stirred for 30 min at rt. Then ethyl bromoacetate (1.15 mL, 10.35 mmol) was added, and the reaction mixture was stirred overnight at rt. The reaction was quenched with a small amount of water, and all the solvent was removed. The crude mixture was partitioned between ethyl acetate and water, the aqueous phase was extracted several times with ethyl acetate, the combined organic layers were dried (Na_2SO_4) and the solvent was evaporated. The crude product recrystallized from hexane provided **24** as a solid (1.24 g, 48%): mp 70–72 °C; ^{1}H NMR ($CDCl_3$): δ 0.91 (d, $J = 6.6$ Hz, 6H), 1.16 (t, $J = 7.1$ Hz, 3H), 1.86 (m, 1H), 3.09 (d, $J = 7.5$ Hz, 2H), 3.99–4.10 (m, 4H), 7.40–7.54 (m, 3H), 7.57–7.63 (m, 2H), 7.66–7.73 (m, 2H), 7.84–7.91 (m, 2H). Anal. ($C_{20}H_{25}NO_4S$) C, H, N.

6.1.5.9. 2-[Isobutyl-1,1′-biphenyl-4-sulfonyl-amino]-*N*-hydroxyacetamide (9a). Ester **24** (1.23 g, 3.28 mmol) was dissolved in methanol (50 mL). Hydroxylamine hydrochloride (0.46 g, 6.56 mmol) was added to this solution, followed by the addition of sodium methoxide, freshly prepared from sodium (0.226 g, 9.8 mmol) dissolved in methanol (5.82 mL). The reaction mixture was stirred overnight at rt. The reaction was worked up by partitioning between dilute hydrochloric acid (pH = 3) and ethyl acetate. The aqueous phase was extracted well with ethyl acetate, the combined organic layers were dried and the solvent was evaporated. The product was purified by flash chromatography on silica gel using hexane–ethyl acetate 1/1 v/v and then was triturated with hexane/diethyl ether to give **9a** (0.47 g, 40%) as a solid; mp 150–152 °C; ^{1}H NMR (DMSO-d_6): 0.77 (d, $J = 6.4$ Hz, 6H), 1.84 (m, 1H), 3.05 (d, $J = 7.3$ Hz, 2H), 3.68 (s, 2H), 7.41–7.53 (m, 3H), 7.70–7.73 (m, 2H), 7.77–7.81 (m, 2H), 7.89–7.93 (m, 2H). Anal. ($C_{18}H_{22}N_2O_4S$) C, H, N.

6.2. Matrix metalloproteinase activation and fluorimetric assay (MMP-1, -2, -3, -7 and -9).[6,38]

Type I collagenase (pro-MMP-1, native enzyme derived from cell culture of human rheumatoid synovial fibroblasts), stromelysin (pro-MMP-3, human recombinant) and matrilysin (pro-MMP-7, human recombinant) was purchased from Calbiochem. Recombinant human progelatinases A (pro-MMP-2) and B (pro-MMP9) secreted by transfected mouse myeloma cells were prepared in our laboratory. Proenzymes were activated immediately prior to use with *p*-aminophenylmercuric acetate (APMA 2 mM for 1 h at 37 °C for pro-MMP-1, APMA 2 mM for 1 h at 25 °C for pro-MMP-2 and pro-MMP-7; APMA 1 mM for 1 h at 37 °C for pro-MMP-9) and with trypsin 13 μg/mL for 15 min at 37 °C followed by soybean trypsin inhibitor (SBTI) 50 μg/mL for pro-MMP-3. For assay measurements, the stock solutions (100 mM) of the inhibitors in DMSO were further diluted, at seven different concentrations (1–1500 nM) for each MMP, as required in the fluorimetric assay buffer (FAB): Tris–HCl 50 mM, pH = 7.5, NaCl 150 mM, $CaCl_2$ 10 mM, Brij 35 0.05% and DMSO 1%. The activated enzyme (final concentration 155 pM for MMP-2, 2 nM for MMP-3, 178 pM for MMP-7, 728 pM for MMP-1 and 216 pM for MMP-9) and inhibitor solutions were incubated in the assay buffer for 4 h. The assay temperature was 25 °C. After the addition of 100 μM solution of the fluorogenic substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) in DMSO (final concentration 1 μM), the hydrolysis was monitored by continuously recording the increase in fluorescence (λ_{ex} 328 nm, λ_{em} 393 nm) using a Perkin–Elmer spectrofluorimeter LS 50B. Percent inhibitions were calculated from control reactions without the inhibitor. IC_{50} was determined using the formula: $V_i/V_o = 1/(1 + [I]/IC_{50})$, 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 GraFit.[39]

6.3. Growth on matrigel

Matrigel, a mixture of basement membrane components extracted from the EHS tumour,[37] was thawed at 4 °C in an ice-water bath and diluted in water to a final concentration of 14 mg/mL. A 0.3 mL volume of this solution was gently pipetted into a 13-mm-diameter tissue culture well (24-well plate). After one hour of incubation at 37 °C, the matrigel was completely polymerized, and 5×10^4 cells in 1 mL of complete medium were carefully seeded on top of the matrigel layer and treated with MMP inhibitors. Controls were treated with the carrier (DMSO) alone. The seeded plates were incubated at 37 °C in a 5% CO_2 humidified atmosphere, and were photographed after 24 h.

HT1080 sarcoma cells, plated on matrigel in complete medium, rapidly organize into a network of cord-like structures with an 'inhibitor-insensitive' behaviour, indicating the lack of toxic effects. During this phase, cells migrate on the surface of matrigel and proliferate, but do not invade the matrix. After 24 h, it is possible to note single or small groups of cells protruding from the established 'cords'; these structures, indicated in Figure 1 by red arrows, represent the starting of HT1080 invasive behaviour. Incubation with **10a** or **2** (50 nM) affected the formation of invading structures. Both molecules strongly reduced the invasion of matrigel, with a very low number of HT1080-derived invasive structures compared with untreated controls.

Acknowledgements

The authors would like to thank Mr. Christian Vanzetto, for his studies on cell cultures carried out at the Istituto Nazionale per la Ricerca sul Cancro, Genova.

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