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Synthesis and biological evaluation of imidazole thioacetanilides as novel non-nucleoside HIV-1 reverse transcriptase inhibitors

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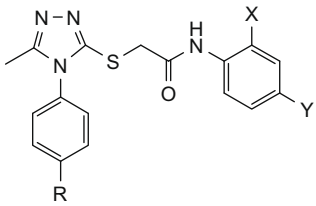
ABSTRACT

A series of 2-(1-aryl-1*H*-imidazol-2-ylthio)acetamide [imidazole thioacetanilide (ITA)] derivatives were synthesized and evaluated as potent inhibitors of human immunodeficiency virus type-1 (HIV-1). Among them, the most potent HIV-1 inhibitors were **4a5** (EC_{50} = 0.18 μM), and **4a2** (EC_{50} = 0.20 μM), which were more effective than the lead compound **L1** (EC_{50} = 2.053 μM) and the reference drugs nevirapine and delavirdine. The preliminary structure–activity relationship (SAR) of the newly synthesized congeners is discussed.

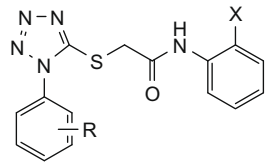
1. Introduction

The reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1) is a key target for inhibition of HIV-1 replication. The RT can be inhibited by two classes of drug belonging either to the nucleoside (or nucleotide) reverse transcriptase inhibitors (N(t)RTIs) or to the non-nucleoside reverse transcriptase inhibitors (NNRTIs). HIV-1 NNRTIs have become key components in the combination regimens of anti-HIV therapy.[1–4] Since the first report of sulfanyltriazole/tetrazoles (**L1–L4**, Fig. 1) as potent HIV-1 NNRTIs, these novel substituted scaffolds have excited great interest in the development of novel NNRTIs, because of their high potency and low toxicity against HIV-1 wild-type and resistant strains.[5–11] We recently reported that 1,2,3-thiadiazole efficiently behaves as a triazole/tetrazole surrogate (Fig. 2).[12] Therefore, it is likely that the five-membered heterocycle portion in these inhibitors could simply be acting as a scaffold which orients the pharmacophores into the proper geometry for binding to RT. This conclusion of preliminary SAR analysis was consistent with the results from molecular modeling studies.[11] However, recent research has also revealed that there are differences in the electronic and conformational contribution of the heterocyclic group to the binding of the inhibitors with the HIV-1 RT.[13] Therefore, further modification of this region will provide ample opportunity for the discovery of the next generation of HIV-1 NNRTIs.

In continuation of our research work on understanding the role of the five-membered heterocycles in the binding of the inhibitors to the RT and finding potent HIV replication inhibitors, a novel series of imidazole thioacetanilide (ITA) derivatives was designed and synthesized based on the general principle of bioisosterism in medicinal chemistry.[14–16] The triazole, tetrazole or 1,2,3-thiadiazole moieties in the corresponding lead compounds were replaced by the imidazole ring in the ITA analogues (Fig. 2), the other fragments which were considered to be necessary for conserving anti-



L1. R=CH_3, X=Y=H,
EC_{50}= 2.053μM(WT)
L2. R=H, X=Br, Y=CH_3
EC_{50}= 0.1 μM(WT)
EC_{50}= 1.3 μM(K103N/Y181C)



L3. R=2,4,6-TriMe, X=Cl
IC_{50}= 5 nM (WT)
L4. R-Ph=1-naphthalene, X=NO_2
IC_{50} = 9.5 nM(WT)
IC_{50} = 766 nM(K103N/Y181C)

Figure 1. Sulfanyltriazole and sulfanyltetrazole lead compounds.[5,6,8–10]

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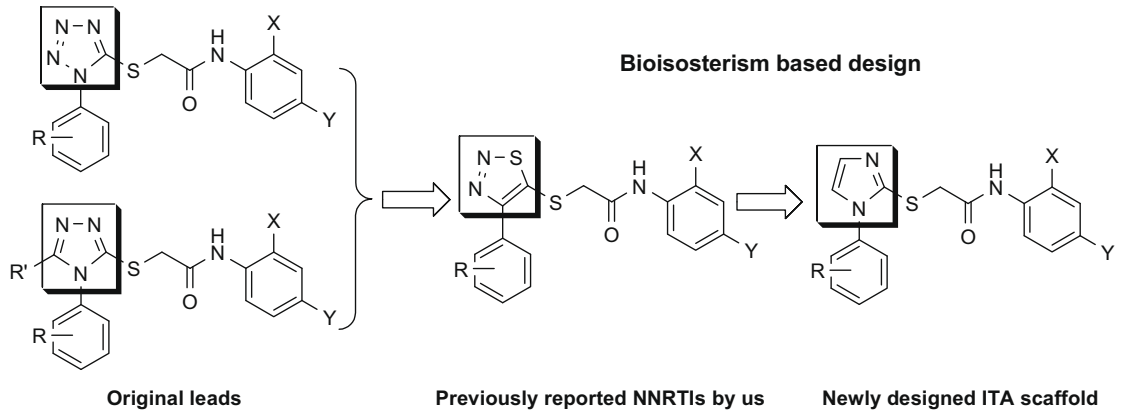


Figure 2. Bioisosterism based design of imidazole thioacetanilide (ITA) scaffold.

HIV-1 activity, such as the 'S–CH_2–CO–NH' linker and the 2-substituted anilides,[17] were left unchanged. Here, the synthesis and the anti-HIV evaluation of the designed ITAs (**4a1–4d6**) in MT-4 cell culture, are reported.

2. Results and discussion

2.1. Chemistry

The title compounds (series **4a**–**4d**) were prepared as shown in Scheme 1 according to the reported method.[18,19] Condensation of commercially available or self-made[20] aryl isothiocyanates (**1a**–**1d**) with 2,2-dimethoxyethanamine afforded the corresponding *N*-substituted thioureas (**2a**–**2d**), which was cyclized in the presence of 5 M HCl under reflux to give the *N*-1 substituted 2-mercapto-imidazoles (**3a**–**3d**). It must be pointed out that the by-product 5-methoxy-*N*-aryl-4,5-dihydrothiazol-2-amine (**3′**) was also formed during cyclization, which was consistent with the results previously reported.[19] The final imidazole thioacetanilides (**4a1**–**4d6**) were synthesized by reaction of intermediates **3a**–**3d** with suitable 2-chloro-*N*-aryl-substituted acetamides in good yield. All synthesized compounds were characterized by NMR, MS, and IR. Both analytical and spectral data of all the compounds are in full agreement with the proposed structures. For example, the ^{1}H NMR spectra of the synthesized compounds revealed the cyclization of imidazole, supported by two protons resonating as double singlets at about 7.3 and 7.2 ppm, respectively, with coupling constant (*J*) about 1.2 Hz. In addition, the ^{1}H chemical shift of the SCH_2 group was at about 4.00 ppm.

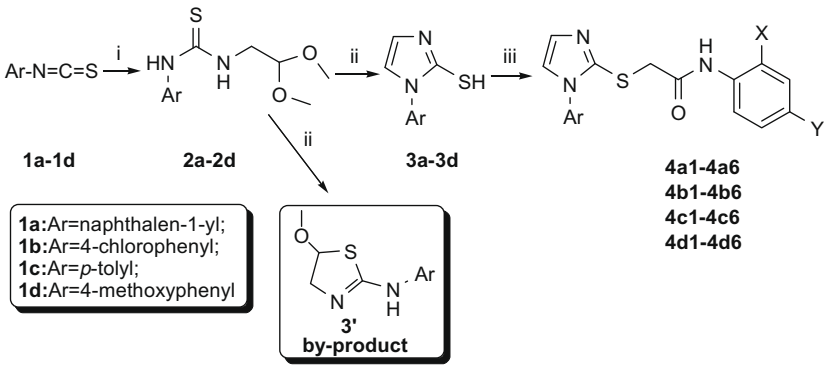
2.2. Anti-HIV evaluation

All of the newly synthesized imidazole thioacetanilides (**4a1–4d6**) were first evaluated for their anti-HIV activity and cytotoxicity in MT-4 cells infected with wild-type HIV-1 strain IIIB and HIV-2 strain ROD. The results, expressed as CC_{50}, EC_{50} and SI, are summarized in Table 1 together with those of nevirapine (NVP), delaviridine (DLV), efavirenz (EFV) and zidovudine (azidothymidine, AZT) as reference drugs for comparative purposes.

As shown in Table 1, most of the test compounds inhibited HIV-1 replication in a lower micromolar concentration range and none of the compounds were active against HIV-2. The most potent HIV-1 inhibitors were **4a5** (EC_{50} = 0.18 μM, CC_{50} = 28.81 μM, SI = 162), and **4a2** (EC_{50} = 0.20 μM, CC_{50} = 35.24 μM, SI = 170). The EC_{50} values of these two compounds were lower than those of the lead compound **L1** (EC_{50} = 2.053 μM) and of the reference drugs NVP and DLV. Other compounds, **4a3**, **4a4**, **4c5**, and **4a1**, also showed higher anti-HIV-1 potency (EC_{50} = 0.64, 0.73, 1.03, and 1.78 μM, respectively) compared with the respect to that of lead compound derivative **L1**, indicating that the imidazole is an acceptable isosteric replacement for the triazole, tetrazole, or 1,2,3-thiadiazole in the lead compounds.

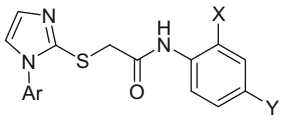
On the basis of the chemical structure and the fact that these compounds inhibit HIV-1, but not HIV-2 replication, these molecules can be proposed to act as genuine NNRTIs.

SAR analysis showed that analogues **4a** with naphthalen-1-yl at *N*-1 of the imidazole ring were found to be the most potent series, and the active sequence of the aryl groups was as follows: naphthalen-1-yl > 4-methylphenyl, 4-chlorophenyl > 4-methoxyphenyl. Therefore, the aryl linked to the imidazole core is crucial



Scheme 1. Reagents and conditions: (i) 2,2-dimethoxyethanamine, EtOH/petroleum ether; (ii) 5 M HCl; (iii) $ClCH_2CONHPh$, Na_2CO_3 or NaOH, EtOH.

Table 1
Anti-HIV activities, cytotoxicities and selectivity indices of 2-(1-aryl-1*H*-imidazol-2-ylthio)acetamides derivatives (series **4a**–**4d**)



No.	Ar	X	Y	EC_{50}(μM)[a]		CC_{50} (μM)[b]	SI[c]	
				HIV-1 III_B	HIV-2 ROD		HIV-1 III_B	HIV-2 ROD
4a1	Naphthalen-1-yl	F	H	**1.78 ± 0.26**	>34.66	34.66 ± 3.23	**20**	<1
4a2	Naphthalen-1-yl	Cl	H	**0.20 ± 0.08**	>35.24	35.24 ± 1.02	**170**	<1
4a3	Naphthalen-1-yl	Br	H	**0.64 ± 0.23**	>30.52	30.52 ± 0.98	**48**	<1
4a4	Naphthalen-1-yl	Br	Me	**0.73 ± 0.09**	>29.36	29.36 ± 2.81	**40**	<1
4a5	Naphthalen-1-yl	NO_2	H	**0.18 ± 0.05**	>28.81	28.81 ± 3.83	**162**	<1
4a6	Naphthalen-1-yl	Me	H	**2.46 ± 0.91**	>34.62	34.62 ± 2.12	**14**	<1
4b1	4-Chlorophenyl	F	H	**43.81 ± 0.58**	>345.48	>345.48	>8	×1
4b2	4-Chlorophenyl	Cl	H	⩾11.71	>330.44	>330.44	×1	×1
4b3	4-Chlorophenyl	Br	H	**15.80 ± 4.26**	>295.72	>300.2	**>19**	×1
4b4	4-Chlorophenyl	Br	Me	⩾55.18	>204.03	220.72 ± 10.62	⩽4	<1
4b5	4-Chlorophenyl	NO_2	H	**4.86 ± 1.60**	>321.48	>321.48	**>66**	×1
4b6	4-Chlorophenyl	Me	H	**35.35 ± 4.14**	>159.50	159.50 ± 9.86	**5**	<1
4c1	*p*-Tolyl	F	H	>37.87	>37.87	37.87 ± 4.48	<1	<1
4c2	*p*-Tolyl	Cl	H	**4.97 ± 0.64**	>33.67	33.67 ± 2.82	**7**	<1
4c3	*p*-Tolyl	Br	H	**5.15 ± 0.52**	>39.27	39.27 ± 8.58	**8**	<1
4c4	*p*-Tolyl	Br	Me	>80.78	>80.78	80.78 ± 88.87	⩽6	<1
4c5	*p*-Tolyl	NO_2	H	**1.03 ± 0.19**	>130.70	130.70 ± 21.36	**126**	<1
4c6	*p*-Tolyl	Me	H	**30.91 ± 8.68**	>147.29	147.29 ± 25.72	**5**	<1
4d1	4-Methoxyphenyl	F	H	>41.97	>41.97	⩾41.97	<or×1	<or×1
4d2	4-Methoxyphenyl	Cl	H	**32.28 ± 11.07**	>334.35	>334.35	**>10**	×1
4d3	4-Methoxyphenyl	Br	H	⩾18.36	>298.82	>298.82	>or×16	×1
4d4	4-Methoxyphenyl	Br	Me	>25.77	>25.77	25.77 ± 24.59	<1	<1
4d5	4-Methoxyphenyl	NO_2	H	**17.85 ± 1.40**	>160.97	160.97 ± 8.25	**>9**	<1
4d6	4-Methoxyphenyl	Me	H	**154.62 ± 62.61**	>353.67	>353.67	**>2**	×1
NVP[d]				0.208		>15.02	>72	
DLV[d]				0.320		>3.827	>12	
EFV[d]				0.00440		>6.336	>1434	
AZT[d]				0.0151		>93.55	>6192	

[a] EC_{50}: concentration of compound required to achieve 50% protection of MT-4 cell against HIV-1-induced cytotoxicity, as determined by the MTT method.
[b] CC_{50}: concentration required to reduce the viability of mock-infected cells by 50%, as determined by the MTT method.
[c] SI: selectivity index (CC_{50}/ EC_{50}). The SI values: ×1 stand for not active.
[d] The antiviral properties of these compounds were previously described.[12]

for the antiviral activity, and agrees with previous SARs findings in 1,2,3-thiadiazole thioacetanilides series.[12] Probably, the hydrophobicity of the aryl group helps to improve the binding affinity (π–π interaction) between the active binding site and the inhibitors, and thus enhance the biological activity.

Just as SAR of 1,2,3-thiadiazoles made in our previous studies,[12] the nature of the substituents at *ortho*-position of phenyl ring of the anilide moiety essentially influences the anti-HIV-1 activity. As shown in Table 1, the anti-HIV-1 activity of the same series increased mainly as the *ortho*-substitution of the anilide moiety was replaced by a methyl, fluorine atom, bromine atom, chlorine atom or nitro. It should highlighted that the 2-nitro substitution on the phenyl ring furnished the most potent compounds of the four series (as shown in Table 1).

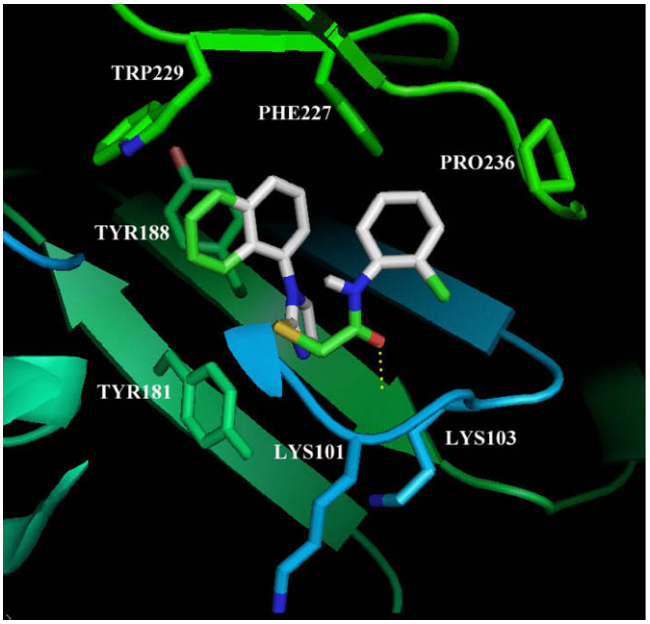
In addition, when the methyl group is introduced to the *para*-position of the anilide moiety, the bioactivity was decreased or completely lost (**4a3/4a4, 4b3/4b4, 4c3/4c4**). It is noteworthy that the introduction of a naphthalen-1-yl group (series **4a**) enhanced the biological activity, but led to increased cytotoxicity, thus providing important information for further design of novel analogues.

2.3. Molecular modeling analysis

In order to investigate the binding mode of our newly synthesized compounds, molecular modeling study was performed by means of Autodock Vina for docking. Compound **4a2** was chosen to be docked into the NNRTIs binding pocket (NNIBP) of HIV-1 RT. Three-dimensional coordinates of the HIV-1 RT/GW564511 (benzophenone based NNRTI) complex (Brookhaven Protein Data Bank entry 3DLG) were used as the input structure for docking calculations because of the high degree of similarity between sulfanyltriazole/tetrazoles and benzophenones.[11] Default parameters were used as described in the Autodock Vina manual unless otherwise specified. The theoretical binding mode of **4a2** to the NNIBP is shown in Figure 3.

Results showed that the naphthalene ring of **4a2** fits into the aromatic-rich binding pocket, surrounded by the aromatic side chains of Tyr188, Phe227, and Trp229. Detailed analysis of the binding mode showed that one phenyl ring (green color) is parallel to the Tyr188 side chain, giving rise to a positive π-stacking interaction. The inhibitor's amide carbonyl forms a key hydrogen bond with the backbone N–H of Lys103. The anilide moiety of **4a2** is close to Pro236, and the 4-substituent points toward the solvent exposed region. Therefore, 4-substituent allows hydrophilic groups as the preferred substituents, which can explain the SAR. As illustrated in Figure 3, the compound **4a2** binds in a 'kinked' conformation which involves a rotation about the 'S–CH_2–CO–NH' dihedral angle from 180° in the fully extended free-state conformation to almost 0° in the bound state. The imidazole is orthogonal to the naphthalene ring, which orients the pharmacophores into the proper geometry for binding.

In summary, the results of the molecular modeling analysis supported the SARs elucidation of our newly designed and synthesized compounds. Further optimization of imidazole analogues will take into account these aspects in further design attempts.



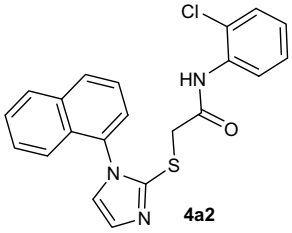


Figure 3. Model of **4a2** docked into the RT non-nucleoside binding site (PDB code: 3DLG) using Autodock Vina [http://vina.scripps.edu]. The docking result of **4a2** is showed by PyMOL[http://pymol.sourceforge.net].

3. Conclusions

The bioassay results show that our bioisosterism approach has led to the development of imidazole analogues as novel and potent anti-HIV agents. Among them, the most potent HIV-1 inhibitors were **4a5** (EC_{50} = 0.18 μM), and **4a2** (EC_{50} = 0.20 μM), which were more effective than the lead compound **L1** (EC_{50} = 2.053 μM) and the reference drugs NVP and DLV. Preliminary SAR results for the newly synthesized congeners and docking studies are presented, expecting to provide the foundation for the rational modification of novel azole thioacetanilides and accelerate the discovery of more potent and selective NNRTIs.

4. Experimental

4.1. Chemistry

All melting points were determined on a micromelting point apparatus and are uncorrected. Infrared spectra (IR) were recorded with a Nexus 470FT-IR Spectrometer. NMR spectra were obtained on a Bruker Avance-600 NMR-spectrometer in the indicated solvents. Chemical shifts are expressed in δ units and TMS as internal reference. Mass spectra were taken on a LC Autosampler Device: Standard G1313A instrument. TLC was performed on Silica Gel GF254 for TLC (Merck) and spots were visualized by iodine vapors or by irradiation with UV light (254 nm). Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of the reaction solutions involved the use of rotary evaporator at reduced pressure.

4.2. General procedure for the synthesis of 2-(1-(naphthalen-1-yl)-1*H*-imidazol-2-ylthio)acetamides (4a1–4a6)

To a stirred solution of 1-isothiocyanatonaphthalene (**1a,** 9.26 g, 50 mmol) in ethyl alcohol (100 ml) was added dropwise the 2,2-dimethoxyethanamine (5.3 g, 50 mmol) at room temperature. After further stirring for 30 min, the formed white solid was filtered, washed with cold ethyl alcohol (2 × 15 ml) and dried to give 1-(2,2-dimethoxyethyl)-3-(naphthalen-1-yl)thiourea (**2a**), which was used directly in the next reaction without any further purification. The crude product was boiled with 50 ml of 5 M hydrochloric acid for 3 h under reflux. The reaction mixture was cooled, alkalized to pH 8 with 4 M NaOH solution, and then filtered. The filtrate was acidified with 4 M hydrochloric acid, the precipitated white solid was collected, washed with water and dried under vacuum to give 1-(naphthalen-1-yl)-1*H*-imidazole-2-thiol (**3a**) as key intermediate (white powder), which was pure enough to be used in the following step. Yield: 27.8%.

To a mixture of **3a** (0.45 g, 2 mmol) in ethyl alcohol (30 ml) was added sodium hydroxide (0.08 g, 2 mmol) followed by various 2-chloro-*N*-(substituted aromatic group)acetamides (2 mmol). The solution was stirred at room temperature for 5–10 h (checked by TLC). The solvent was removed under reduced pressure, and the residue was diluted with dichloromethane (30 ml) and washed with water (3 × 30 ml) and brine, and dried with anhydrous Na_2SO_4. The mixture solution was filtered off, and the solvent was removed in vacuo. The residue was purified by recrystallisation from ethanol to yield the title compounds (**4a1**–**4a6**).

4.2.1. *N*-(2-Fluorophenyl)-2-(1-(naphthalen-1-yl)-1*H*-imidazol-2-ylthio)acetamide (4a1)

White needle crystals, yield: 48.5%. Mp: 133–135 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 11.23 (s, 1H, NH), 8.36 (t, 1H), 7.99 (d, 1H, J = 8.4 Hz), 7.95 (d, 1H, J = 8.4 Hz), 7.58–7.01 (m, 8H), 7.35 (d, 1H, J = 1.2 Hz, imidazol-H), 7.21 (d, 1H, J = 1.2 Hz, imidazol-H), 3.93 (s, 2H, S–CH_2). IR (KBr, cm^{-1}): 3177 (ν_{NH}), 1683 ($\nu_{C=O}$), 1615, 1550, 1492, 1440, 1511 (δ_{NH}). ESI-MS: *m/z* 380.0 (M+2), 755.6 (2M+1). $C_{21}H_{16}FN_3OS$ (377.1).

4.2.2. *N*-(2-Chlorophenyl)-2-(1-(naphthalen-1-yl)-1*H*-imidazol-2-ylthio)acetamide (4a2)

White needle crystals, yield: 54.8%. Mp: 125–127 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.59 (s, 1H, NH), 8.31 (d, 1H, J = 7.8 Hz), 8.02 (d, 1H, J = 8.4 Hz), 7.98 (d, 1H, J = 7.8 Hz), 7.60–7.05 (m, 9H), 3.98 (dd, 2H, S–CH_2); ^{13}C NMR ($CDCl_3$, ppm) δ: 167.88 (C=O), 144.28 (N=C–N), 135.40, 134.25, 132.48, 130.30, 129.60, 129.29, 129.06, 128.42, 127.86, 127.37, 127.11, 125.33, 125.18, 124.78, 124.13, 124.04, 122.63, 122.65, 36.69 (S–CH_2); IR (KBr, cm^{-1}): 3197 (ν_{NH}), 1683 ($\nu_{C=O}$), 1592, 1538, 1469, 1439. ESI-MS: *m/z* 394.8 (M+1). $C_{21}H_{16}ClN_3OS$ (393.07).

4.2.3. *N*-(2-Bromophenyl)-2-(1-(naphthalen-1-yl)-1*H*-imidazol-2-ylthio)acetamide (4a3)

White needle crystals, yield: 46.7%. Mp: 140–142 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.38 (s, 1H, NH), 8.24 (d, 1H, J = 8.4 Hz), 7.99 (d, 1H, J = 8.4 Hz), 7.95 (d, 1H, J = 8.4 Hz), 7.58–6.96 (m, 8H), 7.38 (d, 1H, J = 1.2 Hz, imidazol-H), 7.20 (d, 1H, J = 1.2 Hz, imidazol-H), 4.00 (s, 2H, S–CH_2). IR (KBr, cm^{-1}): 3187 (ν_{NH}), 1684 ($\nu_{C=O}$), 1590, 1533, 1467, 1437. ESI-MS: *m/z* 439.0 (M+1), 442.3 (M+4), 877.4 (2M+1). $C_{21}H_{16}BrN_3OS$ (437.02).

4.2.4. *N*-(2-Bromo-4-methylphenyl)-2-(1-(naphthalen-1-yl)-1*H*-imidazol-2-ylthio)acetamide (4a4)

White needle crystals, yield: 51.6%. Mp: 138–140 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.27 (s, 1H, NH), 8.07 (d, 1H, J = 8.4 Hz), 7.99 (d, 1H, J = 7.8 Hz), 7.95 (d, 1H, J = 7.8 Hz), 7.56 (m, 2H), 7.49 (m, 2H), 7.38 (m, 2H), 7.31 (d, 1H, J = 1.2 Hz, imidazol-H), 7.19 (d, 1H, J = 1.2 Hz, imidazol-H), 7.10 (d, 1H, J = 8.4 Hz), 3.97 (s, 2H, S–CH_2), 2.30 (s, 3H, CH_3). IR (KBr, cm^{-1}): 3195 (ν_{NH}), 1688 ($\nu_{C=O}$), 1582, 1532, 1467, 1440. ESI-MS: *m/z* 452.9 (M+), 456.1 (M+3), 905.4 (2M+1). $C_{22}H_{18}BrN_3OS$ (451.04).

4.2.5. 2-(1-(Naphthalen-1-yl)-1*H*-imidazol-2-ylthio)-*N*-(2-nitrophenyl)acetamide (4a5)

Yellow brown cubic crystals, yield: 68.1%. Mp: 167–169 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 11.43 (s, 1H, NH), 8.59 (d, 1H, J = 2.4 Hz), 8.11 (d, 1H, J = 2.4 Hz), 7.99–7.17 (m, 9H), 7.32 (d, 1H, J = 1.2 Hz, imidazol-H), 7.19 (d, 1H, J = 1.2 Hz, imidazol-H), 4.05 (s, 2H, S–CH_2). IR (KBr, cm^{-1}): 3156 (ν_{NH}), 1708($\nu_{C=O}$), 1660, 1584, 1553, 1503 (ν_{NO2}), 1488, 1440, 1347 (ν_{NO2}). ESI-MS: *m/z* 404.1 (M+), 407.3 (M+3), 809.4 (2M+1). $C_{21}H_{16}N_4O_3S$ (404.09).

4.2.6. 2-(1-(Naphthalen-1-yl)-1*H*-imidazol-2-ylthio)-*N*-*o*-tolylacetamide (4a6)

White needle crystals, yield: 45.2%. Mp: 143–145 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.37 (s, 1H, NH), 8.01 (d, 1H, J = 8.4 Hz), 7.96 (d, 1H, J = 7.8 Hz), 7.95 (d, 1H, J = 7.8 Hz), 7.56 (m, 2H), 7.53 (t, 1H), 7.49 (d, 1H, J = 7.2 Hz), 7.35 (d, 1H, J = 7.8 Hz), 7.26 (d, 1H, J = 1.2 Hz, imidazol-H), 7.22 (d, 1H, J = 1.2 Hz, imidazol-H), 7.19 (d, 2H, J = 8.4 Hz), 7.05 (t, 1H), 3.86 (s, 2H, S–CH_2), 2.35 (s, 3H, CH_3). IR (KBr, cm^{-1}): 3235 (ν_{NH}), 1677 ($\nu_{C=O}$), 1587, 1541, 1512, 1456, 1437. ESI-MS: *m/z* 376.6 (M+3), 747.6 (2M+1). $C_{22}H_{19}N_3OS$ (373.12).

4.3. General procedure for the synthesis of 2-(1-(4-chlorophenyl)-1*H*-imidazol-2-ylthio) acetamides (4b1–4b6)

With a similar procedure for compounds **4a1–4a6**, the 2-(1-(4-chlorophenyl)-1*H*-imidazol-2-ylthio)acetamides (**4b1–4b6**) were synthesized starting from 1-chloro-4-isothiocyanatobenzene **1b** (50 mmol). Compound 1b was carried out according to the reported procedure.[20]

4.3.1. 2-(1-(4-Chlorophenyl)-1*H*-imidazol-2-ylthio)-*N*-(2-fluorophenyl)acetamide (4b1)

White needle crystals, yield: 45.8%. Mp: 131–133 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 11.13 (s, 1H, NH), 8.35 (m, 1H, Ph′H), 7.47 (d, 2H, J = 8.4 Hz, PhH), 7.31 (d, 2H, J = 8.4 Hz, PhH), 7.23 (d, 1H, J = 1.2 Hz, imidazol-H), 7.12 (d, 1H, J = 1.2 Hz, imidazol-H), 7.10 (m, 3H, Ph′H), 3.90 (s, 2H, S–CH_2). IR (KBr, cm^{-1}): 3188 (ν_{NH}), 1679 ($\nu_{C=O}$), 1625, 1559, 1507, 1490, 1457. ESI-MS: *m/z* 363.6 (M+2), 365.2 (M+2). $C_{17}H_{13}ClFN_3OS$ (361.05).

4.3.2. *N*-(2-Chlorophenyl)-2-(1-(4-chlorophenyl)-1*H*-imidazol-2-ylthio)acetamide (4b2)

White needle crystals, yield: 48.9%. Mp: 143–145 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.54 (s, 1H, NH), 8.33 (d, 1H, J = 7.8 Hz), 7.46 (d, 2H, J = 8.4 Hz, PhH), 7.35 (dd, 1H, J_1 = 1.2 Hz, J_2 = 8.4 Hz, PhH), 7.32 (d, 2H, J = 8.4 Hz, PhH), 7.25 (dt, 1H), 7.20 (s, 1H), 7.11 (d, 1H, J = 1.2 Hz), 7.03 (dt, 1H), 3.98 (s, 2H, S–CH_2); ^{13}C NMR ($CDCl_3$, ppm) δ:167.87 (C=O), 142.61 (N=C–N), 135.40, 134.96, 134.81, 129.90, 129.58, 127.28, 126.35, 124.72, 123.74, 122.52, 122.38, 36.76 (S–CH_2); IR (KBr, cm^{-1}): 3211 (ν_{NH}), 1705 ($\nu_{C=O}$), 1597, 1541, 1512, 1489, 1442. ESI-MS: *m/z* 379.3 (M+1). $C_{17}H_{13}Cl_2N_3OS$ (377.02).

4.3.3. *N*-(2-Bromophenyl)-2-(1-(4-chlorophenyl)-1*H*-imidazol-2-ylthio)acetamide (4b3)

White needle crystals, yield: 54.3%. Mp: 169–171 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.25 (s, 1H, NH), 8.23 (d, 1H, J = 8.4 Hz, Ph′H), 8.23 (d, 1H, J = 8.4 Hz, Ph′H), 7.47 (d, 2H, J = 8.4 Hz, PhH), 7.32 (d, 2H, J = 8.4 Hz, PhH), 7.28(d, 1H, J = 7.8 Hz, PhH), 7.21 (d, 1H, J = 1.2 Hz, imidazol-H), 7.12 (d, 1H, J = 1.2 Hz, imidazol-H), 6.97 (t, 1H, Ph′H), 4.02 (s, 2H, S–CH_2). IR (KBr, cm^{-1}) 3198 (ν_{NH}), 1681 ($\nu_{C=O}$), 1594, 1538, 1510, 1492, 1438. ESI-MS: *m/z* 422.6 (M+1), 425.4 (M+3). $C_{17}H_{13}BrClN_3OS$ (420.97).

4.3.4. *N*-(2-Bromo-4-methylphenyl)-2-(1-(4-chlorophenyl)-1*H*-imidazol-2-ylthio)acetamide (4b4)

White needle crystals, yield: 37.6%. Mp: 147–149 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.17 (s, 1H, NH), 8.08 (d, 1H, J = 2.4 Hz, Ph′H), 7.46 (d, 2H, J = 8.4 Hz, PhH), 7.35 (s, 1H, Ph′H), 7.32 (d, 2H, J = 8.4 Hz, PhH), 7.20 (d, 1H, J = 1.2 Hz, imidazol-H), 7.11 (d, 1H, J = 1.2 Hz, imidazol-H), 7.09 (m, 1H, Ph′H), 3.98 (s, 2H, S–CH_2), 2.88 (s, 3H, CH_3). IR (KBr, cm^{-1}) 3133 (ν_{NH}), 1682 ($\nu_{C=O}$), 1590, 1539, 1511, 1492, 1442. ESI-MS: *m/z* 437.2 (M+2). $C_{18}H_{15}BrClN_3OS$ (434.98).

4.3.5. 2-(1-(4-Chlorophenyl)-1*H*-imidazol-2-ylthio)-*N*-(2-nitrophenyl)acetamide(4b5)

Light yellow needle crystals, yield: 38.7%. Mp: 193–195 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 11.41 (s, 1H, NH), 8.59 (d, 1H, J = 2.4 Hz, Ph′H), 8.12 (dd, 1H, J = 2.4 Hz, J = 1.2 Hz, Ph′H), 7.62 (dt, 1H, Ph′H), 7.47 (d, 2H, J = 8.4 Hz, PhH), 7.36 (d, 2H, J = 8.4 Hz, PhH), 7.21 (d, 1H, J = 1.2 Hz, imidazol-H), 7.19 (m, 1H, Ph′H), 7.12 (d, 1H, J = 1.2 Hz, imidazol-H), 4.07 (s, 2H, S–CH_2). IR (KBr, cm^{-1}) 3156 (ν_{NH}), 1708 ($\nu_{C=O}$), 1660, 1584, 1553, 1503 (δ_{NO2}), 1488, 1440, 1347 (δ_{NO2}). ESI-MS: *m/z* 390.7 (M+1), 392.1 (M+2). $C_{17}H_{13}ClN_4O_3S$ (388.04).

4.3.6. 2-(1-(4-Chlorophenyl)-1*H*-imidazol-2-ylthio)-*N*-*o*-tolylacetamide (4b6)

White needle crystals, yield: 41.2%. Mp: 164–166 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.25 (s, 1H, NH), 7.96 (d, 1H, J = 8.4 Hz, Ph′H), 7.47 (d, 2H, J = 8.4 Hz, PhH), 7.32 (d, 2H, J = 8.4 Hz, PhH), 7.20 (d, 1H, J = 1.2 Hz, imidazol-H), 7.17 (m, 2H, Ph′H), 7.13 (d, 1H, J = 1.2 Hz, imidazol-H), 7.04 (t, 1H, Ph′H), 3.90 (s, 2H, S–CH_2), 2.30 (s, 3H, CH_3). IR (KBr, cm^{-1}) 3200 (ν_{NH}), 1684 ($\nu_{C=O}$), 1584, 1551, 1507, 1488, 1434. ESI-MS: *m/z* 359.3 (M+2), 361.2 (M+4). $C_{18}H_{16}ClN_3OS$ (357.07).

4.4. General procedure for the synthesis of 2-(1-(*p*-tolyl)-1*H*-imidazol-2-ylthio) acetamides (4c1–4c6)

The starting material 1-isothiocyanato-4-methylbenzene was synthesized according to a known procedure.[20] A solution of 1-isothiocyanato-4-methylbenzene (**1c,** 7.46 g, 50 mmol) and 2,2-dimethoxyethanamine (5.3 g, 50 mmol) in petroleum ether (bp: 60–90 °C, 100 ml) was stirred at room temperature for 2.5 h. The formed white solid was filtered, washed with petroleum ether (2 × 20 ml) and dried in air to give 1-(2,2-dimethoxyethyl)-3-(*p*-tolyl)thiourea (**2c**), which was used directly without any further purification. The crude product was boiled with 50 ml of 5 M

hydrochloric acid for 3 h under reflux. The mixture was concentrated under reduced pressure. It was triturated with hot ethyl alcohol to give the compound 1-*p*-tolyl-1*H*-imidazole-2-thiol (**3c**) as white crystals, Yield: 32.7%.

A mixture of **3c** (0.38 g, 2 mmol), sodium carbonate (0.23 g, 2.1 mmol), and 2-chloro-*N*-(substituted aromatic group)acetamides (2 mmol) in *N*,*N*-dimethylformamide (10 ml) was stirred overnight. The resulting mixture was diluted with water and extracted with dichloromethane (30 ml). The organic layer was washed with water (3 × 30 ml), brine, and dried over Na_2SO_4. Removal of the solvent in vacuo and recrystallisation from ethanol afforded the title compounds (**4c1**–**4c6**).

4.4.1. *N*-(2-Fluorophenyl)-2-(1-*p*-tolyl-1*H*-imidazol-2-ylthio)acetamide (4c1)

White solid, yield: 88.0%. Mp: 115–117 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 11.31 (s, 1H, NH), 8.37 (d, 1H, *J* = 8.4 Hz, Ph′H), 7.28 (d, 2H, *J* = 8.4 Hz, PhH), 7.24 (d, 2H, *J* = 8.4 Hz, PhH), 7.21 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.12 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.10–6.99 (m, 3H, Ph′H), 3.88 (s, 2H, S–CH_2), 2.41 (s, 3H, CH_3). IR (KBr, cm^{-1}): 3194 (ν_{NH}), 1669 ($\nu_{C=O}$), 1617, 1548, 1508, 1493, 1449. ESI-MS: *m/z* 342.3 (M+1). $C_{18}H_{16}FN_3OS$ (341.1).

4.4.2. *N*-(2-Chlorophenyl)-2-(1-*p*-tolyl-1*H*-imidazol-2-ylthio)acetamide (4c2)

White solid, yield: 70.1%. Mp: 105–107 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.68 (s, 1H, NH), 8.33 (d, 1H, *J* = 8.4 Hz, Ph′H), 7.34 (d, 1H, *J* = 8.4 Hz, Ph′H), 7.28 (d, 2H, *J* = 8.4 Hz, PhH), 7.26 (m, 1H, Ph′H), 7.23 (d, 2H, *J* = 8.4 Hz, PhH), 7.17 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.11 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.01 (dt, 1H, Ph′H), 3.96 (s, 2H, S–CH_2), 2.41 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, ppm) δ: 168.17, 142.58, 138.97, 135.54, 134.01, 130.20, 129.26, 127.35, 124.87, 124.62, 123.82, 122.69, 122.43, 36.79, 21.15. IR (KBr, cm^{-1}): 3221 (ν_{NH}), 1698 ($\nu_{C=O}$), 1578, 1539, 1515, 1490, 1439. ESI-MS: *m/z* 358.3 (M+1). $C_{18}H_{16}ClN_3OS$ (357.07).

4.4.3. *N*-(2-Bromophenyl)-2-(1-*p*-tolyl-1*H*-imidazol-2-ylthio)-acetamide (4c3)

Colorless needle crystals, yield: 46.5%. Mp: 128–130 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.45 (s, 1H, NH), 8.25 (d, 1H, *J* = 8.4 Hz, Ph′H), 7.52 (m, 1H, Ph′H), 7.29 (d, 2H, *J* = 8.4 Hz, PhH), 7.25 (d, 1H, *J* = 8.4 Hz, Ph′H), 7.24 (d, 2H, *J* = 8.4 Hz, PhH), 7.18 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.12 (d, 1H, *J* = 1.2 Hz, imidazol-H), 6.96 (dt, 1H, Ph′H), 3.97 (s, 2H, S–CH_2), 2.41 (s, 3H, CH_3). IR (KBr, cm^{-1}): 3432 (ν_{NH}), 1696 ($\nu_{C=O}$), 1595, 1519, 1438, 1303, 825, 746. ESI-MS: *m/z* 402.3 (M+1), 404.3 (M+3). $C_{18}H_{16}BrN_3OS$ (401.02).

4.4.4. *N*-(2-Bromo-4-methylphenyl)-2-(1-*p*-tolyl-1*H*-imidazol-2-ylthio)acetamide (4c4)

White needle crystals, yield: 74.1%. Mp: 138–140 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.33 (s, 1H, NH), 8.08 (d, 1H, *J* = 8.4 Hz, Ph′H), 7.35 (s, 1H, Ph′H), 7.28 (d, 2H, *J* = 8.4 Hz, PhH), 7.24 (d, 2H, *J* = 8.4 Hz, PhH), 7.17 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.11 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.09 (d, 1H, *J* = 8.4 Hz, Ph′H), 3.95 (s, 2H, S–CH_2), 2.41 (s, 3H, CH_3), 2.28 (s, 3H, CH_3). IR (KBr, cm^{-1}) 3130 (ν_{NH}), 1690 ($\nu_{C=O}$), 1588, 1541, 1515, 1489, 1448. ESI-MS: *m/z* 416.3 (M+1), 418.3 (M+3). $C_{19}H_{18}BrN_3OS$ (415.04).

4.4.5. *N*-(2-Nitrophenyl)-2-(1-*p*-tolyl-1*H*-imidazol-2-ylthio)acetamide (4c5)

Light yellow powder, yield: 61.1%. Mp: 146–148 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 11.50 (s, 1H, NH), 8.60 (d, 1H, *J* = 8.4 Hz, Ph′H), 8.10 (d, 1H, *J* = 8.4 Hz, Ph′H), 7.28 (d, 2H, *J* = 8.4 Hz, PhH), 7.23 (d, 2H, *J* = 8.4 Hz, PhH), 7.19 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.11 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.08 (m, 2H, Ph′H), 4.03 (s, 2H, S–CH_2), 2.41 (s, 3H, CH_3). IR (KBr, cm^{-1}): 3289 (ν_{NH}), 1693 ($\nu_{C=O}$), 1610, 1584, 1502 (ν_{NO2}), 1439, 1336 (ν_{NO2}), 1273, 1146, 824, 743. ESI-MS: *m/z* 369.3 (M+1). $C_{18}H_{16}N_4O_3S$ (368.09).

4.4.6. *N*-*o*-Tolyl-2-(1-*p*-tolyl-1*H*-imidazol-2-ylthio)acetamide (4c6)

White crystals, yield: 59.3%. Mp: 144–146 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.39 (s, 1H, NH), 7.97 (d, 1H, *J* = 7.8 Hz, Ph′H), 7.28 (d, 2H, *J* = 8.4 Hz, PhH), 7.24 (d, 2H, *J* = 8.4 Hz, PhH), 7.20 (m, 1H, Ph′H), 7.17 (m, 1H, Ph′H), 7.13 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.12 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.03 (m, 1H, Ph′H), 3.88 (s, 2H, S–CH_2), 2.42 (s, 3H, CH_3), 2.31 (s, 3H, CH_3). IR (KBr, cm^{-1}): 3244 (ν_{NH}), 1674 ($\nu_{C=O}$), 1593, 1554, 1518, 1459, 819, 761, 730. ESI-MS: *m/z* 338.4 (M+1). $C_{19}H_{19}N_3OS$ (337.12).

4.5. General procedure for the synthesis of 2-(1-(4-methoxyphenyl)-1H-imidazol-2-ylthio) acetamides (4d1–4d6)

With a similar procedure for compounds **4c1–4c6**, the 2-(1-(4-methoxyphenyl)-1*H*-imidazol-2-ylthio)acetamides (**4d1–4d6**) were synthesized starting from 1-isothiocyanato-4-methoxybenzene 1d (50 mmol). Compound 1d was obtained according to the reported procedure.[20]

4.5.1. *N*-(2-Fluorophenyl)-2-(1-(4-methoxyphenyl)-1*H*-imidazol-2-ylthio)acetamide (4d1)

White needle crystals, yield: 28.1%. Mp: 125–127 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 11.27 (s, 1H, NH), 8.37 (t, 1H, Ph′H), 7.28 (d, 2H, *J* = 8.4 Hz, PhH), 7.20 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.12 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.10–7.02 (m, 3H, Ph′H), 6.98 (d, 2H, *J* = 8.4 Hz, PhH), 3.88 (s, 2H, S–CH_2), 3.86 (s, 3H, OCH_3). IR (KBr, cm^{-1}): 3248 (ν_{NH}), 1693 ($\nu_{C=O}$), 1624, 1554, 1520, 1458, 1327, 1259, 839, 753. ESI-MS: *m/z* 358.3 (M+1). $C_{18}H_{16}FN_3O_2S$ (357.09).

4.5.2. *N*-(2-Chlorophenyl)-2-(1-(4-methoxyphenyl)-1*H*-imidazol-2-ylthio)acetamide (4d2)

White needle crystals, yield: 46.9%. Mp: 121–123 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.67 (s, 1H, NH), 8.33 (d, 1H, *J* = 8.4 Hz, Ph′H), 7.35 (d, 1H, *J* = 7.8 Hz, Ph′H), 7.27 (d, 2H, *J* = 8.4 Hz, PhH), 7.25 (m, 1H, Ph′H), 7.17 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.09 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.02 (m, 1H, Ph′H), 6.98 (d, 2H, *J* = 8.4 Hz, PhH), 3.96 (s, 2H, S–CH_2), 3.86 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$, ppm) δ: 168.18, 159.78, 142.85, 135.56, 129.33, 129.27, 129.22, 127.36, 126.55, 124.61, 123.80, 122.92, 122.42, 114.71, 55.59, 36.74. IR (KBr, cm^{-1}): 3431 (ν_{NH}), 1700 ($\nu_{C=O}$), 1597, 1518, 1442, 1254, 837, 749. ESI-MS: *m/z* 374.3 (M+1). $C_{18}H_{16}ClN_3O_2S$ (373.07).

4.5.3. *N*-(2-Bromophenyl)-2-(1-(4-methoxyphenyl)-1*H*-imidazol-2-ylthio)acetamide (4d3)

White needle crystals, yield: 24.0%. Mp: 117–119 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.41 (s, 1H, NH), 8.25 (d, 1H, *J* = 7.8 Hz, Ph′H), 7.52 (dd, 1H, *J* = 1.2 Hz, *J* = 8.4 Hz, Ph′H), 7.28 (m, 3H, 1 × Ph′H and 2 × PhH), 7.18 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.09 (d, 1H, *J* = 1.2 Hz, imidazol-H), 6.96 (m, 3H, 1 × Ph′H and 2 × PhH), 3.97 (s, 2H, S–CH_2), 3.86 (s, 3H, OCH_3). IR (KBr, cm^{-1}): 3428 (ν_{NH}), 1694 ($\nu_{C=O}$), 1594, 1520, 1437, 1256, 1027, 839, 747. ESI-MS: *m/z* 418.4 (M+1). $C_{18}H_{16}BrN_3O_2S$ (417.01).

4.5.4. *N*-(2-Bromo-4-methylphenyl)-2-(1-(4-methoxyphenyl)-1*H*-imidazol-2-ylthio)acetamide (4d4)

White needle crystals, yield: 63.8%. Mp: 138–140 °C. ^{1}H NMR ($CDCl_3$, ppm) δ: 10.28 (s, 1H, NH), 8.07 (d, 1H, *J* = 8.4 Hz, Ph′H), 7.35 (s, 1H, Ph′H), 7.27 (d, 2H, *J* = 8.4 Hz, PhH), 7.17 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.11 (d, 1H, *J* = 1.2 Hz, imidazol-H), 7.09 (m, 1H, Ph′H), 6.98 (d, 2H, *J* = 8.4 Hz, PhH), 3.97 (s, 2H, S–CH_2),

3.86 (s, 3H, OCH_3), 2.29 (s, 3H, CH_3). IR (KBr, cm^{-1}): 3203 (ν_{NH}), 1690 ($\nu_{C=O}$), 1521, 1127, 1030, 841, 815, 739. ESI-MS: *m/z* 432.3 (M+1). $C_{19}H_{18}BrN_3O_2S$ (431.03).

4.5.5. 2-(1-(4-Methoxyphenyl)-1*H*-imidazol-2-ylthio)-*N*-(2-nitrophenyl)acetamide (4d5)

Light yellow solid, yield: 32.6%. Mp: 139–141 °C. ^1H NMR ($CDCl_3$, ppm) δ: 11.49 (s, 1H, NH), 8.61 (d, 1H, J = 7.8 Hz, Ph′H), 8.11 (d, 1H, J = 8.4 Hz, Ph′H), 7.61 (dt, 1H, Ph′H), 7.30 (d, 2H, J = 8.4 Hz, PhH), 7.18 (m, 2H, imidazol-H and Ph′H), 7.10 (d, 1H, J = 1.2 Hz, imidazol-H), 6.97 (d, 2H, J = 8.4 Hz, PhH), 4.02 (s, 2H, S–CH_2), 3.86 (s, 3H, OCH_3). IR (KBr, cm^{-1}): 3290 (ν_{NH}), 1690 ($\nu_{C=O}$), 1503 ($\nu_{as\ NO2}$), 1428, 1336 ($\nu_{s\ NO2}$), 1271, 1249. ESI-MS: *m/z* 385.4 (M+1). $C_{18}H_{16}N_4O_4S$ (384.09).

4.5.6. 2-(1-(4-Methoxyphenyl)-1*H*-imidazol-2-ylthio)-*N*-*o*-tolylacetamide (4d6)

White needle crystals, yield: 70.8%. Mp: 142–144 °C. ^1H NMR ($CDCl_3$, ppm) δ: 10.37 (s, 1H, NH), 7.96 (d, 1H, J = 7.8 Hz, Ph′H), 7.26 (d, 2H, J = 8.4 Hz, PhH), 7.20 (dt, 1H, Ph′H), 7.17 (d, 1H, J = 7.8 Hz, Ph′H), 7.13 (d, 1H, J = 1.2 Hz, imidazol-H), 7.10 (d, 1H, J = 1.2 Hz, imidazol-H), 7.04 (dt, 1H, Ph′H), 6.98 (d, 2H, J = 8.4 Hz, PhH), 3.89 (s, 2H, S–CH_2), 3.86 (s, 3H, OCH_3), 2.31 (s, 3H, CH_3). IR (KBr, cm^{-1}): 3245 (ν_{NH}), 1673($\nu_{C=O}$), 1592, 1558, 1518, 1251, 838, 755, 735. ESI-MS: *m/z* 354.1 (M+1). $C_{19}H_{19}N_3O_2S$ (353.12).

4.6. Anti-HIV activity assays

Evaluation of the antiviral activity of the compounds against HIV-1 strain III_B and HIV-2 strain (ROD) in MT-4 cells was performed using the MTT assay as previously described.[21,22] Stock solutions (10 × final concentration) of test compounds were added in 25-μl volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock- and HIV-infected cells at the beginning of each experiment. Serial fivefold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman instruments, Fullerton, CA). Untreated control HIV- and mock-infected cell samples were included for each sample.

HIV-1(III_B)[23] or HIV-2 (ROD)[24] stock (50 μL) at 100–300 $CCID_{50}$ (cell culture infectious dose) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of test compound on uninfected cells in order to assess the cytotoxicity of the test compound. Exponentially growing MT-4 cells[25] were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6×10^5 cells/mL, and 50-μL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically by the MTT assay.

The MTT assay is based on the reduction of yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a blue-purple formazan that can be measured spectrophotometrically. The absorbances were read in an eight-channel computer-controlled photometer (Multiscan Ascent Reader, Labsystems, Helsinki, Finland), at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of tree wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD540) of the mock-infected control sample by 50%. The concentration achieving 50% protection from the cytopathic effect of the virus in infected cells was defined as the 50% effective concentration (EC_{50}).

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