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Synthesis and pharmacological evaluation of thieno[2,3-*b*]pyridine derivatives as novel c-Src inhibitors

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ABSTRACT

Among the recently investigated targets for cancer therapy is the c-Src non-receptor tyrosine kinase. Indeed research around deregulated activity of this enzyme has proven its role in tumor progression, while the beneficial effects of c-Src inhibitors in several pathological models has also been demonstrated.

We report here the preparation and pharmacological profile of a novel series of c-Src inhibitors that was elaborated around a 3-amino-thieno[2,3-*b*]pyridine discovered during an HTS campaign.

c-Src enzyme inhibition and c-Src inhibition were investigated in a series of related compounds derived from the initial hit. Molecular modeling as well as X-ray studies on one active compound allowed us to hypothesize on ligand orientation and interactions within the ATP hydrophobic pocket.

Design and synthesis of structural analogs then led to new ligands possessing quite efficient enzymatic and c-Src inhibition.

The structure–activity elements disclosed in this study shed light on the role played by substituents on the thienopyridine ring as well as the impact of other aromatic moieties in the molecule when interacting with the enzyme.

1. Introduction

The non-receptor tyrosine kinase c-Src belongs to a family of closely related kinases and is widely expressed in all cells types; while being highly regulated in normal cells, this enzyme has deregulated increased activity in several tumors especially colon, breast and lung cancers.[1] Research around increased activity of c-Src (found in normal cells in a low concentration inactive form), has revealed that deregulation of the enzyme can be linked to increased motility/invasiveness of tumor cells and to tumor progression.[2] Moreover, beneficial effects of c-Src inhibition in several pathological models (cell cycle arrest of tumor cell lines, growth inhibition of Src-transformed fibroblast derived tumors, metastasis inhibition in human tumor models) has been demonstrated.[3] As a result of preclinical studies it has also been claimed, that c-Src kinase inhibitors may act in synergy with hormonal and cytotoxic agents.[4] Metastatic bone disease occurring in many advanced solid tumor cancers has been also linked with c-Src kinase activity while in animal models bone metastases could be diminished by inhibition of c-Src kinase.[5]

The study of c-Src kinase has generated various inhibitors belonging to different chemical families,[3b] some of which reached more advanced stages (Fig. 1).

All of these compounds with the exception of KX2-391 compete for the ATP site and have shown in vivo inhibition in implanted tumors. AZD0530 and Dasatinib have shown activity only in orthotopic metastasis models.

In clinical studies side effects (gastro-intestinal, skin rash, cytopenia, pleural effusions) and toxicities, when Src inhibition dosage is too near to MTD, have been observed.[6g]

New inhibitors possessing maximal anti-tumor effects coupled to a better safety profile and therefore continued research in this area are still needed.

Our own work focused on inhibitors elaborated around a 3-amino-thieno(2,3-*b*)pyridine scaffold.

Indeed, an in house screening campaign (HTS, see Section 4) revealed that structure **9** (Fig. 2, Table 1) exhibits a modest inhibitory effect on the c-Src enzyme also showing cellular activity, while structure **10** (Fig. 2, Table 1), lacking the two methyl groups on the pyridine ring is devoid of any such activity. Therefore, we investigated more thoroughly the influence of the substitution (position and nature) on the pyridine moiety as well as the influence of replacing the naphthyl moiety by other aromatics.

The synthesis and the structure-activity elements resulting from the pharmacological evaluation of these compounds are described. For clarity of discussion only the data on a selected representative set of compounds are used to describe the structure–activity tendencies.

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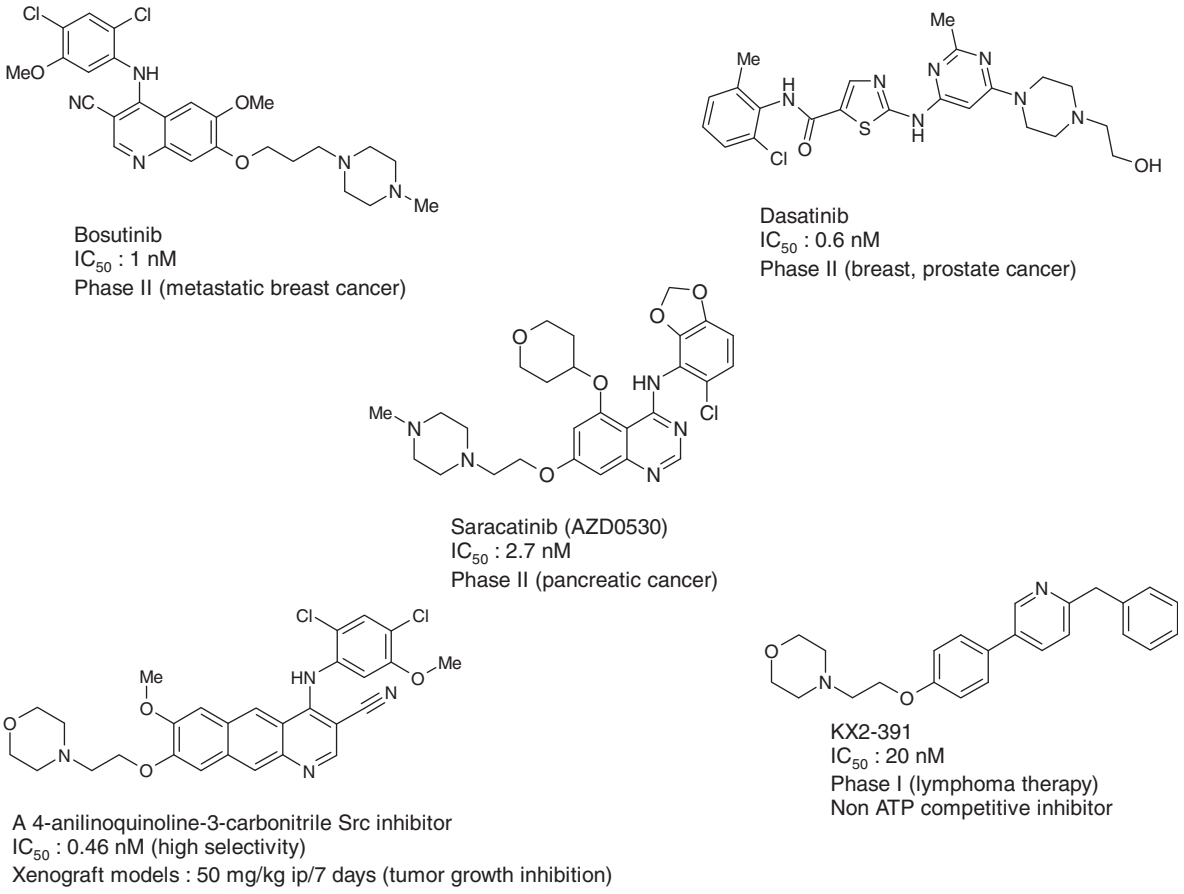


Figure 1. Known c-Src kinase inhibitors.

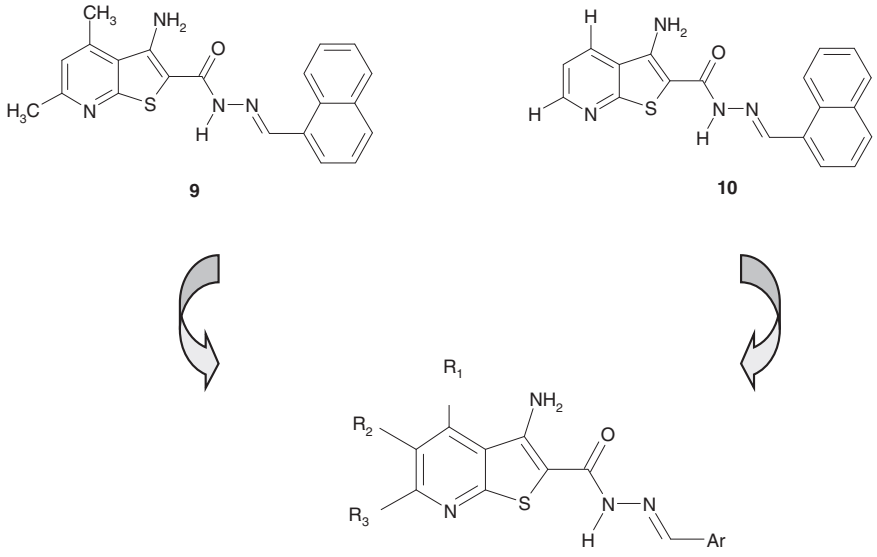


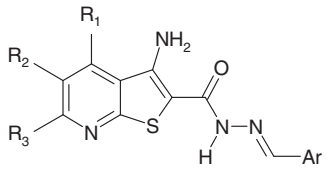
Figure 2. Thieno[2,3-*b*]pyridine based c-Src inhibitors.

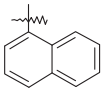
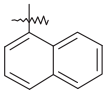
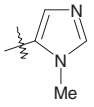
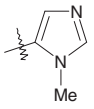
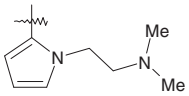
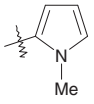
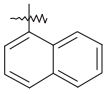
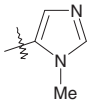
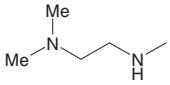
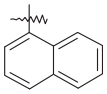
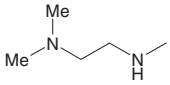
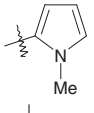
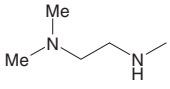
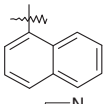
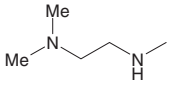
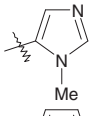
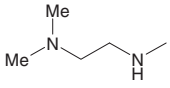
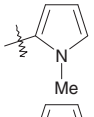
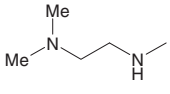
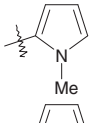
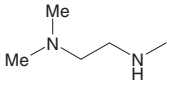
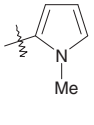
2. Results and discussion

2.1. Chemistry

The compounds investigated in this study were synthesized by one of the two methods depicted in Scheme 1 (paths A and B). The thieno[2,3-*b*]pyridine core[8] construction starts with the elaboration of the pyridine ring moiety obtained via a Guareschi-Thorpe[9] type reaction from the β-keto-ester **1**, either commercially available or readily prepared from the corresponding ester through a Claisen condensation. The subsequent chlorination reaction carried out on compounds **2** with phosphorus oxychloride, led to the 2,6-dichloropyridines **3** in good yields. The introduction of the side-chain R3 was performed by aromatic substitution reaction of *N*,*N*-dimethyl-ethylenediamine on derivatives **3**, thus yielding a mixture of two regioisomers, from which the desired and major

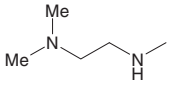
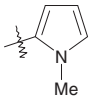
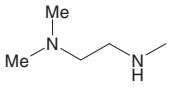
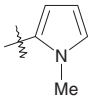
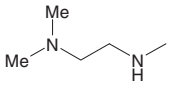
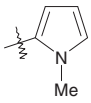
Table 1
Structure activity data



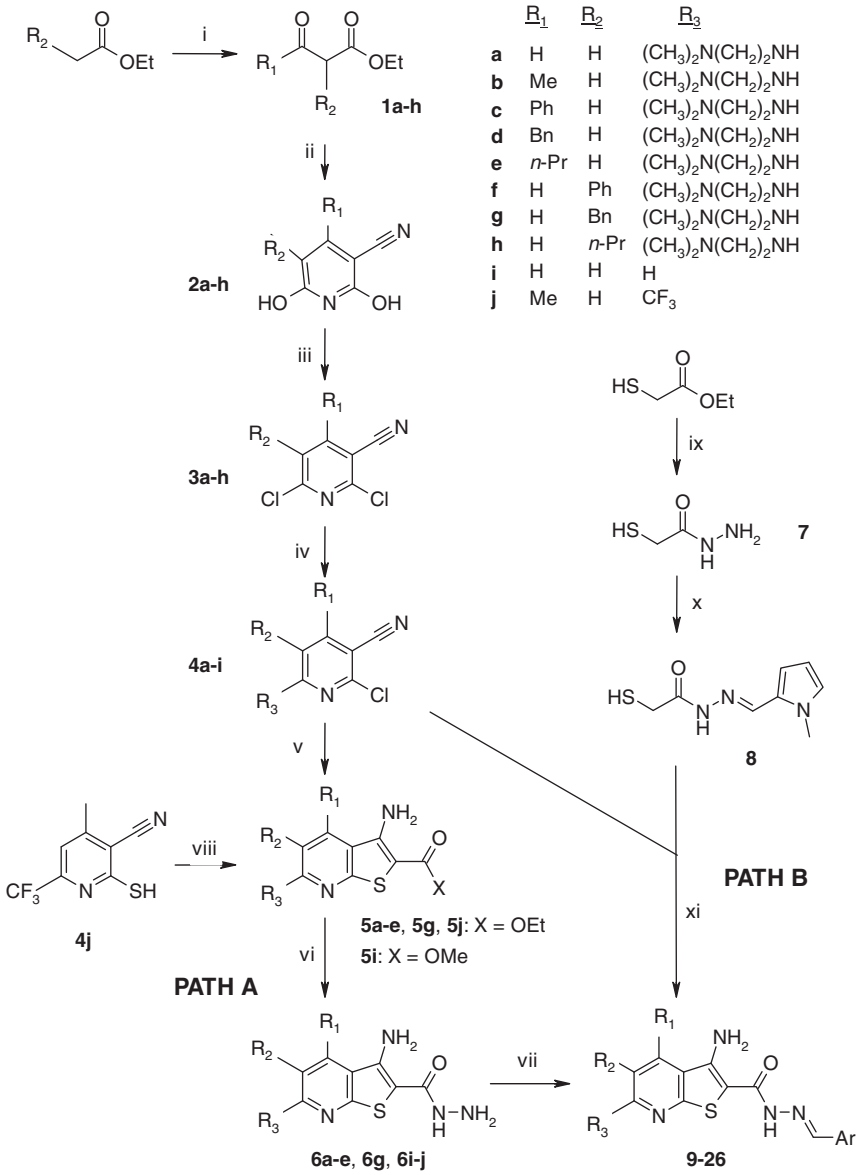
Compd	R_1	R_2	R_3	Ar	Src enzyme IC_{50}[a] (nM)	Src cells (μM)
9	Me	H	Me		269 ± 18[b]	10[c]
10	H	H	H		>10,000	NT[c]
11	Me	H	Me		769 ± 7.8	>10 μM[c]
12	H	H	H		1300 ± 275.8	NT[c]
13	Me	H	Me		>10,000	NT[c]
14	Me	H	Me		840 ± 28.6	10[d]
15	Me	H	CF_3		1340 ± 127.3	NT[c]
16	Me	H	CF_3		330 ± 38.2	>10[d]
17	Me	H			230 ± 31.5	>10[d]
18	Me	H			246 ± 25.9	10[d]
19	Ph	H			107 ± 40.3	3[d]
20	Ph	H			32 ± 17.3	3–10[d]
21	Ph	H			68 ± 13.8	3–10[d]
22	Bn	H			40 ± 12.2	3[d]
23	*n*-Pr	H			63 ± 8.6	10[d]

(*continued on next page*)

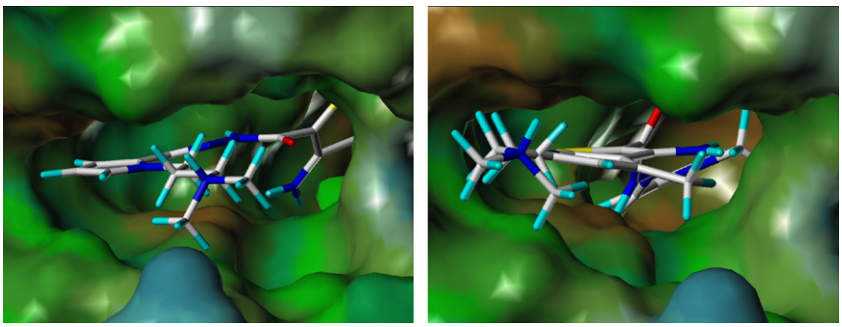
Table 1 (*continued*)

Compd	R_1	R_2	R_3	Ar	Src enzyme IC_{50}[a] (nM)	Src cells (μM)
24	H	Ph			4100 ± 99.7[d]	NT[e]
25	H	Bn			3870 ± 268.7	NT[e]
26	H	*n*-Pr			2080 ± 19.8	NT[e]

[a] The IC_{50} (at least two separate determinations was established using a sigmoidal dose–response (one site) curve and the variable slope parameters calculated on the means of activity data points. The 4-anilinoquinoline-3-carbonitrile[3b] (Fig. 1) was used as positive control and DMSO as negative control.
[b] ±SEM.
[c] Phospho—STAT3 Western blot assays (see Section 4).
[d] Phosphor—FAK Western blot assays (see Section 4).
[e] Not tested.



Scheme 1. Synthesis of thieno[2,3-*b*]pyridine derivatives. Reagents and conditions: (i) R_1CO_2Et, NaH, THF; (ii) $NCCH_2CONH_2$, KOH, EtOH, Δ; (iii) $POCl_3$, autoclave; (iv) R_3-H, EtOH; (v) $HSCH_2CO_2Et$, K_2CO_3, EtOH, Δ; (vi) $N_2H_4{\cdot}H_2O$, EtOH, Δ; (vii) Ar-CHO, EtOH, Δ; (viii) $ClCH_2CO_2Et$, DBU, THF; (ix) $N_2H_4{\cdot}H_2O$, EtOH, Δ; (x) *N*-methyl-2-pyrrolcarboxaldehyde, EtOH, Δ; (xi) K_2CO_3, EtOH, Δ.



A
Thienopyridine ring within
a hydrophobic pocket

B
Pyrrole ring within
a hydrophobic pocket

Figure 3. Docking solutions: 3A (compound **13**)—thienopyridine ring within a hydrophobic pocket; 3B (compound **18**)—pyrrole ring within a hydrophobic pocket.

isomer **4** was isolated (**4i** and **4j** being commercially available). Construction of the fused thiophene ring featuring the acylhydrazone side-chain bearing Ar, from compounds **4**, could be performed in two different ways, namely either a linear three-step sequence via **5** and **6** (Scheme 1, path A) or a convergent synthesis involving compound **8**, made aside in two steps[10] (Scheme 1, path B). The former sequence begins with the thiophene ring formation giving **5**, followed by an amide formation reaction yielding acylhydrazines **6**. Variations in the Ar group were performed in the final step using commercially available aldehydes Ar-CHO. Compound **9** (Table 1) has been synthesized by an external source and only analytical data are presented here.

2.2. Biology and molecular modeling

Pharmacological data (c-Src enzyme inhibition and cellular Src inhibition) obtained for derivatives bearing a naphthyl, imidazole or a pyrrole ring (compounds **9–26**) are summarized in Table 1. Replacement of the naphtyl substituent on the hydrazide function (compound **9**, Table 1) by an imidazole or a pyrrole substituent (compounds **11** and **14**, Table 1) leads to an approximate fourfold drop in c-Src enzyme inhibition. Compound **9** as well as compound **14** (a weaker inhibitor) demonstrate cellular Src inhibition, at 10 μM.

However, this is not the case for compound **11** (imidazole on the hydrazide function), that lacks cellular activity above 10 μM. No straightforward explanation concerning the above differences in cellular activity can be invoked but it might be speculated that differential solubility and partition play a role.

The impact of removing the methyl substitution on the thienopyridine ring (e.g., R1, R3) also depends on the nature of the aromatic ring on the hydrazide function. Thus a clear drop in c-Src inhibition is observed when comparing compounds **9** and **10** (Table 1) while the difference observed between compounds **11** and **12** is much lower.

Exchanging the methyl substituent in the 6 position (e.g., R_3), with a CF_3 group has a negative influence on the c-Src inhibition when compounds **9** and **15** (Table 1) are compared.

However no such effect is observed when the couple **11** and **16** (Table 1) is considered; actually a slight improvement in c-Src inhibition is observed in this case. Nevertheless, both **11** and **16** (Table 1) are devoid of cellular activity.

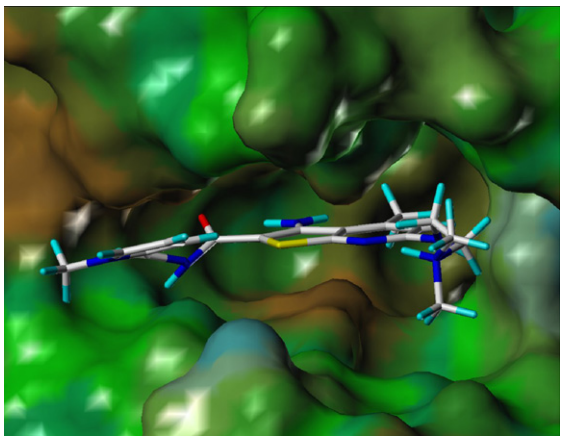
The presence of basic protonable groups in c-Src inhibitors (e.g., Fig. 1) is expected to increase solubility and might improve cellular activity. However in our case, comparison of compounds **9** and **17** shows that while the c-Src inhibitory activity is identical, the latter (possessing a *N*,*N*-dimethyl-ethylene-diamino substituent on the 6 position) is unexpectedly devoid of cellular activity. Compound **18**, possessing the same protonable basic side chain on the 6 position, exhibits a higher c-Src inhibitory potency than its analog lacking this side chain (compound **14**, Table 1); both **14** and **18** also exhibit cellular activities, at 10 μM. The c-Src enzymatic inhibition as well as cellular activities are completely abolished in compound **13** (Table 1), where the basic protonable chain substitutes the pyrrole nitrogen.

Docking protocols (see Section 4 for details) allowed the examination of ligand position and interaction within the ATP hydrophobic pocket, whose role in stabilizing the ligand-enzyme complex is well documented.[7]

Thus obtained docking solutions for compound **13** indicate an orientation whereby the dimethylaminoethyl substituted pyrrole points away from the hydrophobic pocket (Fig. 3A). Therefore it might be logically hypothesized that the interaction of the pyrrole ring with the hydrophobic pocket (precluded in this case by the basic side chain) is crucial for inhibitory activity.

Indeed, when this interaction can take place without impediment like in compound **18**, where the basic group is on the thienopyridine nucleus (Fig. 3B), a better c-Src inhibition is obtained. This kind of orientation whereby an aromatic ring (in this case a pyrrole) interacts with the hydrophobic pocket is found in the vast majority of ATP-site ligands that have been co-crystallized with c-Src kinase, and therefore the molecular modeling solution seemed coherent.

However, a co-crystallization study (PDB code, 3LTK, Fig. 4) performed with compound **18**, indicates that this ligand adopts within



Me(R_1) on thienopyridine ring points towards
a hydrophobic pocket

Figure 4. Co-crystallization of compound **18** with c-Src. Me (R_1) on thienopyridone ring points towards a hydrophobic pocket.

the hydrophobic pocket a different arrangement than that indicated in Figure 3B. Indeed, as can be seen from Figure 4, it is the methyl group on the 4 position of the thienopyridine that is directed towards the inside of the cavity; the basic chain (6 position of the thienopyridine) is outside this cavity and does not interfere with the interaction.

It might be also observed, that a substituent bigger than methyl on the pyrrole ring, would induce a negative effect on the interaction due to steric hindrance (the inactivity of compound **13** might be also deduced on the basis of this orientation). A few ATP-site ligands co-crystallized with c-Src, found in the Protein Data Bank display a non-aromatic substituent (always larger than methyl) in interaction with the hydrophobic pocket. Therefore due to the size of the ATP pocket it became obvious that introduction of larger hydrophobic substituents on the 4 position (replacing the methyl group) should have a positive impact on the c-Src inhibitory efficacy.

Indeed this hypothesis was verified by the synthesis and testing of compounds **19–23** (Table 1) bearing propyl or aromatic substituents on the 4 position of the thienopyridine ring (R_1). Indeed all these molecules exhibited better inhibition levels than the previously described compounds and also possessed cellular activities.

Interestingly analogs **24–26** possessing the same phenyl, benzyl or propyl substituents but on the 5 position (R_2) are devoid of inhibitory activity. From inspection of Figure 4 it can be clearly concluded that substituents on position 5 of the thienopyridine ring (R_2) introduce unfavorable steric interactions with the hydrophobic cavity, thus explaining the pharmacological result.

In order to exploit the promising potential of the thienopyridine ring, further modifications are investigated and will be reported in due time.

3. Conclusions

A new class of c-Src inhibitors, possessing a thieno[2,3-*b*]pyridine scaffold has been discovered. The initially found modest inhibitor could be elaborated into relatively potent new thienopyridines exhibiting both enzymatic and cellular c-Src inhibition activities.

The presence of a non polar substituent at R_1 (Ph, Bn, *n*-Pr, Me) on the thienopyridine ring enhances the interaction with the enzyme (hydrophobic pocket). The basic protonable side chain at R_3 improves cellular activity only when the Ar substituent is a pyrrole or imidazole ring. Also, this study points to the fact that the 4,6 relative positions of these substituents on the thienopyridine nucleus is crucial for c-Src activity.

4. Experimental section

4.1. Chemistry

All commercially available reagents and solvents were used without further purification. The purities of tested compounds were assessed by analytical HPLC (Alliance, Water apparatus equipped with a XTerra, MSC18 2.5 μM column, $H_2O/CH_3CN/CH_3SO_3H$ 100/25/1 gradient for 1 min then $CH_3CN/H_2O/CH_3SO_3H$ 100/25/1 gradient for 9 min, and were found >95%. Combustion analysis was performed on a Flash EA1112, Thermo apparatus, NMR spectra were performed on a Bruker 400 MHz at 300 K with tetramethylsilane (TMS) as an internal reference ($\delta = 0$). Chemical shifts (δ) are reported in parts per million (ppm). IR spectra were performed on a Bruker FTIR Tensor 27 apparatus and frequencies (ν) are expressed in cm^{-1}. Electrospray (ESI) high resolution mass spectra (HRMS) were obtained in positive mode using a quadrupole time-of-flight mass spectrometer (QToF2, Waters Micromass). All the compounds analyzed by ESI produced very abundant $[M+H]^+$ ions and, sometimes, in competition with $[M+Na]^+$ ions. Accurate mass measurements were obtained by the 'lock mass' technique. Some compounds were analyzed by electron impact (EI) using a single quadrupole mass spectrometer (DSQII, Thermo Fisher Scientific). In these cases, all EI mass spectra exhibit a molecular ion M^+. The co-crystallization studies on compound **18** have been custom-made by Novalix, Ilkirch, France (PDB code, 3LTK).

4.1.1. Ethyl 3-oxo-2-phenyl-propionate (1f)

Sodium hydride (60% dispersion in mineral oil, 11.0 g, 0.27 mol, 3 equiv) was added slowly to a solution of ethyl phenylacetate (14.5 mL, 0.09 mol, 1 equiv) and ethyl formate (150 mL) in anhydrous THF (220 mL). The reaction mixture was stirred mechanically overnight at room temperature. After completion of the reaction, the mixture was hydrolyzed slowly at 0 °C with a solution of 2 N HCl (150 mL). Followed an extraction with Et_2O (3 × 200 mL) and the organic layers were combined, dried over Na_2SO_4, filtered and concentrated in vacuum, leading to a yellow oil which was purified by distillation under reduced pressure (110 °C, 7 mbar) giving 9.61 g (55%) of **1f** as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 12.15 (d, 1H, OH), 7.4–7.2 (m, 6H), 4.30 (q, 2H), 1.30 (t, 3H). IR (neat) ν 3500–2500, 1736, 1655. MS (EI) M^+ *m/z* 192.1.

Compounds **1a–e** are commercially available. Compounds **1g**, **1h** have both been synthesized according to the procedure above but are not described herein since they are known from the literature.[11]

4.1.2. General procedure 1 (compound 2)

A suspension of potassium hydroxide (5.6 g, 0.10 mol, 1 equiv) in ethanol (50 mL) was added slowly to a solution of β-keto-ester **1** (0.10 mol, 1 equiv) and cyano-acetamide (0.10 mol, 1 equiv) in EtOH (50 mL). The mixture was stirred at reflux for 18 h. After cooling down, the formed precipitate was filtered off and made soluble in warm water (200 mL). Thereafter the solution was acidified using 30 mL of a solution of 4 N HCl giving a white precipitate which was filtered off and washed with water and cold diethyl ether. The solid residue was dried in vacuum to yield the desired substituted pyridine as a white powder. No further purification was needed.

Compounds **2a** and **2b** are commercially available.

4.1.2.1. 2,6-Dihydroxy-4-phenyl-nicotinonitrile (2c). The product was prepared according to the general procedure **1** and yielded 5.95 g (28%); ^1H NMR (400 MHz, DMSO-d_6) δ 12.6–11.8 (br s, 2H, OH), 7.50 (m, 5H), 5.64 (s, 1H). IR (neat) ν 3390, 3164, 2154, 2217. MS (EI): M^+ *m/z* 212.1.

4.1.2.2. 4-Benzyl-2,6-dihydroxynicotinonitrile (2d). The product was prepared according to the general procedure **1** and yielded 20.61 g (91%); ^1H NMR (400 MHz, DMSO-d_6) δ 12.10 (br s, 2H, OH), 7.32 (m, 2H), 7.28 (m, 3H), 5.50 (s, 1H), 3.89 (s, 2H). IR (neat) ν 3380, 2400, 2221, 1708, 1633. HRMS (ESI): calcd for $C_{13}H_{11}N_2O_2$ $[M+H]^+$ 227.0821, found 227.0840.

4.1.2.3. 2,6-Dihydroxy-4-propyl-nicotinonitrile (2e). The product was prepared according to the general procedure **1** and yielded 11.40 g (64%); ^1H NMR (400 MHz, DMSO-d_6) δ 13.0–11.5 (br s, 2H, OH), 5.60 (s, 1H), 2.50 (t, 2H), 1.59 (m, 2H), 0.91 (t, 3H). IR (neat) ν 3280, 2160, 2223. HRMS (ESI): calcd for $C_9H_{11}N_2O_2$ $[M+H]^+$ 179.0821, found 179.0846.

4.1.2.4. 2,6-Dihydroxy-5-phenyl-nicotinonitrile (2f). The product was prepared according to the general procedure **1** and yielded

8.28 g (39%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.0–7.5 (br s, 2H, OH), 7.75 (s, 1H), 7.55 (m, 2H), 7.30 (m, 2H), 7.20 (m, 1H). IR (neat) ν 3200, 2000, 2226, 1620, 1600. MS (EI): M^+ *m/z* 212.1.

4.1.2.5. 5-Benzyl-2,6-dihydroxynicotinonitrile (2g). The product was prepared according to the general procedure **1** and yielded 17.42 g (77%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.48 (s, 1H), 7.25 (d, 2H), 7.20 (t, 2H), 7.15 (t, 1H), 3.62 (s, 2H). IR (neat) ν 3500, 2300, 2216, 1625. HRMS (ESI); calcd for $C_{13}H_{11}N_2O_2$ $[M+H]^+$ 227.0821, found 227.0824.

4.1.2.6. 2,6-Dihydroxy-5-propyl-nicotinonitrile (2h). The product was prepared according to the general procedure **1** and yielded 7.13 g (40%). Compound used as such in the next step.

4.1.3. General procedure 2 (compound 3)

The dihydroxypyridine **2** (0.05 mol, 1 equiv), tetramethylammonium chloride (11.0 g, 0.10 mol, 2 equiv), toluene (100 mL) and phosphorus(III) oxychloride (23 mL, 0.25 mol, 5 equiv) were successively introduced into a high-pressure reactor. The reactor was tightly sealed and heated at 200 °C for 5 h under magnetic stirring. Afterwards, the reactor was cooled down, until the pressure was back to atmospheric, and unsealed carefully. The smoking reaction mixture was slowly poured into warm water (80 mL) and diluted with ethyl acetate (100 mL). The layers were separated and the organic one was washed with brine (50 mL). The aqueous layers were washed with ethyl acetate (2 × 80 mL). The resulting organic phases were combined, dried over Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by column chromatography to afford the desired dichloronicotinonitrile as a whitish solid.

Compounds **3a** and **3b** are commercially available.

4.1.3.1. 2,6-Dichloro-4-phenyl-nicotinonitrile (3c). The product was prepared according to the general procedure **2** and yielded 9.22 g (74%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (s, 1H), 7.72 (m, 2H), 7.60 (m, 3H). IR (neat) ν 3080, 2230. HRMS (ESI): calcd for $C_{12}H_7Cl_2N_2$ $[M+H]^+$ 248.9986, found 248.9971.

4.1.3.2. 4-Benzyl-2,6-dichloronicotinonitrile (3d). The product was prepared according to the general procedure **2** and yielded 7.37 g (56%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.38 (t, 2H), 7.31 (t, 1H), 7.23 (d, 2H), 7.11 (s, 1H), 4.19 (s, 2H). IR (neat) ν 3081, 2233, 1567, 1535. MS (EI): M^+ *m/z* 262.0.

4.1.3.3. 2,6-Dichloro-4-propyl-nicotinonitrile (3e). The product was prepared according to the general procedure **2** and yielded 9.46 g (88%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.26 (s, 1H), 2.81 (m, 2H), 1.75 (m, 2H), 1.03 (t, 3H). IR (neat) ν 3075, 2966, 2875, 2232, 1571, 1533. MS (EI) M^+: *m/z* 214.0.

4.1.3.4. 2,6-Dichloro-5-phenyl-nicotinonitrile (3f). The product was prepared according to the general procedure **2** and yielded 9.47 g, (76%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (s, 1H), 7.55–7.35 (m, 5H). IR (neat) ν 2237. MS (EI): M^+ *m/z* 248.0.

4.1.3.5. 5-Benzyl-2,6-dichloronicotinonitrile (3g). The product was prepared according to the general procedure **2** and yielded 10.26 g (78%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.60 (s, 1H), 7.4–7.2 (m, 5H), 4.10 (s, 2H). IR (neat) ν 2239. HRMS (ESI): calcd for $C_{13}H_9Cl_2N_2$ $[M+H]^+$ 263.0143, found 263.0152.

4.1.3.6. 2,6-Dichloro-5-propyl-nicotinonitrile (3h). The product was prepared according to the general procedure **2** and yielded 7.10 g (66%). Compound used as such in the next step.

4.1.4. General procedure 3 (compound 4)

The *N*,*N*-dimethylethylenediamine (0.20 mol, 5 equiv) was added to a solution of the appropriate dichloronicotinonitrile derivative (0.04 mol, 1 equiv) in ethanol (250 mL). After 48 h of stirring at room temperature, the solvent was removed under reduced pressure and the crude product was diluted back in ethyl acetate (100 mL). The solution was washed with water and brine (50 mL). Thereafter the combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue was purified by flash silica gel chromatography to separate the two formed regioisomers and isolate the desired isomer.

4.1.4.1. 2-Chloro-6-(2-dimethylamino-ethylamino)-4-methyl-nicotinonitrile (4b). The product was prepared according to the general procedure **3** and yielded 6.50 g (68%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.82 (br s, 1H, NH), 6.43 (s, 1H), 3.35 (m, 2H), 2.38 (t, 2H), 2.28 (br s, 3H), 2.15 (s, 6H). IR (neat) ν 3300–2600, 2215, 1612. HRMS (ESI): calcd for $C_{11}H_{16}ClN_4$ $[M+H]^+$ 239.1063, found 239.1074.

4.1.4.2. 2-Chloro-6-(2-dimethylamino-ethylamino)-4-phenyl-nicotinonitrile (4c). The product was prepared according to the general procedure **3** and yielded 8.78 g (73%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.00 (br s, 1H, NH), 7.52 (m, 5H), 6.60 (s, 1H), 3.40 (m, 2H), 2.42 (t, 2H), 2.18 (s, 6H). IR (neat) ν 3244, 2208. HRMS (ESI): calcd for $C_{16}H_{18}ClN_4$ $[M+H]^+$ 301.1220, found 301.1214.

4.1.4.3. 4-Benzyl-2-chloro-6-(2-dimethylamino-ethylamino)-nicotinonitrile (4d). The product was prepared according to the general procedure **3** and yielded 6.55 g (52%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.4–7.2 (m, 5H), 6.00 (s, 1H), 5.70 (br s, 1H, NH), 4.00 (s, 2H), 3.30 (m, 2H), 2.50 (t, 2H), 2.20 (s, 6H). IR (neat) ν 3260, 2216, 1601. HRMS (ESI): calcd for $C_{17}H_{20}ClN_4$ $[M+H]^+$ 315.1376, found 315.1387.

4.1.4.4. 2-Chloro-6-(2-dimethylamino-ethylamino)-4-propyl-nicotinonitrile (4e). The product was prepared according to the general procedure **3** and yielded 6.19 g (58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.15 (s, 1H), 5.80 (br s, 1H, NH), 3.40 (m, 2H), 2.65 (t, 2H), 2.52 (t, 2H), 2.25 (s, 6H), 1.70 (m, 2H), 1.00 (t, 3H). IR (neat) ν 3241, 2216, 1614. HRMS (ESI): calcd for $C_{13}H_{20}ClN_4$ $[M+H]^+$ 267.1376, found 267.1370.

4.1.4.5. 2-Chloro-6-(2-dimethylamino-ethylamino)-5-phenyl-nicotinonitrile (4f). The product was prepared according to the general procedure **3** and yielded 3.25 g (27%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.70 (s, 1H), 7.50 (t, 2H), 7.45 (t, 1H), 7.43 (d, 2H), 6.90 (t, 1H, NH), 3.42 (m, 2H), 2.38 (t, 2H), 2.12 (s, 6H). IR (neat) ν 3369, 2219. HRMS (ESI): calcd for $C_{16}H_{18}ClN_4$ $[M+H]^+$ 301.1220, found 301.1219.

4.1.4.6. 5-Benzyl-2-chloro-6-(2-dimethylamino-ethylamino)-nicotinonitrile (4g). The product was prepared according to the general procedure **3** and yielded 7.81 g (62%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.52 (s, 1H), 7.31 (t, 2H), 7.25 (m, 3H), 7.18 (t, 1H, NH), 3.80 (s, 2H), 3.41 (m, 2H), 2.37 (t, 2H), 2.12 (s, 6H). IR (neat) ν 3335, 2215, 1600. HRMS (ESI): calcd for $C_{17}H_{20}ClN_4$ $[M+H]^+$ 315.1376, found 315.1373.

4.1.4.7. 2-Chloro-6-(2-dimethylamino-ethylamino)-5-propyl-nicotinonitrile (4h). The product was prepared according to the general procedure **3** and yielded 3.84 g (36%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (s, 1H), 5.83 (br s, 1H, NH), 3.50 (m, 2H), 2.55 (m, 2H), 2.32 (t, 2H), 2.28 (s, 6H), 1.65 (m, 2H), 1.00 (t, 3H). HRMS (ESI): calcd for $C_{13}H_{20}ClN_4$ $[M+H]^+$ 267.1376, found 267.1371.

4.1.5. General procedure 4 (compound 5)

Potassium carbonate (1.52 g, 11 mmol, 2.2 equiv) was added to a solution of substituted nicotinonitrile (5 mmol, 1 equiv) and ethyl thioglycolate (1.2 mL, 11 mmol, 2.2 equiv) in ethanol (140 mL). The reaction mixture was stirred at 80 °C overnight. After cooling down, the observed precipitate was filtered off and washed with EtOH (40 mL). The solid residue was put in suspension in warm water (100 mL) and cooled down. The new precipitate was filtered off, washed consecutively with water (50 mL) and EtOH (2 × 50 mL) and dried in vacuum at 60 °C to afford a powder corresponding to the expected thienopyridine **5** with no further purification needed.

4.1.5.1. 3-Amino-6-(2-dimethylamino-ethylamino)-4-methyl-thieno[2,3-*b*]pyridine-2-carboxylic acid ethyl ester (5b). The product was prepared according to the general procedure **4** and yielded 1.52 g (94%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.05 (t, 1H, NH), 6.60 (br s, 2H, NH_2), 6.30 (s, 1H), 4.20 (q, 2H), 3.35 (m, 2H), 2.55 (s, 3H), 2.40 (t, 2H), 2.15 (s, 6H), 1.25 (t, 3H). IR (neat) ν 3377, 1643, 1611. HRMS (ESI): calcd for $C_{15}H_{23}N_4O_2S$ $[M+H]^+$ 323.1542, found 323.1530.

4.1.5.2. 3-Amino-6-(2-dimethylamino-ethylamino)-4-phenyl-thieno[2,3-*b*]pyridine-2-carboxylic acid ethyl ester (5c). The product was prepared according to the general procedure **4** and yielded 827 mg (43%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.57 (m, 3H), 7.45 (m, 2H), 7.30 (t, 1H, NH), 6.32 (s, 1H), 5.57 (br s, 2H, NH_2), 4.20 (q, 2H), 3.45 (m, 2H), 2.45 (t, 2H), 2.20 (s, 6H), 1.23 (t, 3H). IR (neat) ν 3493, 3389, 3350, 1644, 1588, 1501. HRMS (ESI): calcd for $C_{20}H_{25}N_4O_2S$ $[M+H]^+$ 385.1698, found 385.1686.

4.1.5.3. 3-Amino-4-benzyl-6-(2-dimethylamino-ethylamino)-thieno[2,3-*b*]pyridine-2-carboxylic acid ethyl ester (5d). The product was prepared according to the general procedure **4** and yielded 1.75 g (88%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.37 (m, 2H), 7.29 (m, 1H), 7.22 (m, 2H), 7.11 (br t, 1H, NH), 6.60 (s, 2H, NH_2), 6.01 (s, 1H), 4.32 (s, 2H), 4.20 (q, 2H), 3.32 (m, 2H), 2.37 (t, 2H), 2.13 (s, 6H), 1.24 (t, 3H). IR (neat) ν 3495, 3352, 3244, 1656. HRMS (ESI): calcd for $C_{21}H_{27}N_4O_2S$ $[M+H]^+$ 399.1855, found 399.1868.

4.1.5.4. 3-Amino-6-(2-dimethylamino-ethylamino)-4-propyl-thieno[2,3-*b*]pyridine-2-carboxylic acid ethyl ester (5e). The product was prepared according to the general procedure **4** and yielded 1.42 g (81%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.13 (s, 1H), 6.08 (br s, 2H, NH_2), 5.38 (br s, 1H, NH), 4.30 (q, 2H), 3.47 (m, 2H), 2.83 (t, 2H), 2.56 (t, 2H), 2.28 (s, 6H), 1.75 (m, 2H), 1.36 (t, 3H), 1.05 (t, 3H). IR (neat) ν 3520, 3366, 3248, 1663. HRMS (ESI): calcd for $C_{17}H_{27}N_4O_2S$ $[M+H]^+$ 351.1855, found 351.1855.

4.1.5.5. 3-Amino-5-benzyl-6-(2-dimethylamino-ethylamino)-thieno[2,3-*b*]pyridine-2-carboxylic acid ethyl ester (5g). The product was prepared according to the general procedure **4** and yielded 1.12 g (56%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.95 (s, 1H), 7.35–7.15 (m, 5H), 7.00 (s, 2H, NH_2), 6.35 (br t, 1H, NH), 4.20 (q, 2H), 3.85 (s, 2H), 3.40 (m, 2H), 2.40 (t, 2H), 2.15 (s, 6H), 1.25 (t, 3H). IR (neat) ν 3434, 3332, 1680, 1640. HRMS (ESI): calcd for $C_{21}H_{27}N_4O_2S$ $[M+H]^+$ 399.1855, found 399.1870.

4.1.5.6. 3-Aminothieno[2,3-*b*]pyridine-2-carboxylic acid methyl ester (5i). The product has been synthesized according to a procedure described in the literature[12] (and references cited therein) on a 0.125 mol scale, and isolated as a solid, 21 g (81%), mp = 195 °C.

4.1.5.7. 3-Amino-4-methyl-6-trifluoromethyl-thieno[2,3-*b*]pyridine-2-carboxylic acid ethyl ester (5j). A solution of 1,8-diazabicyclo[5.4.0]-undec-7-ene (7.0 mL, 0.05 mol, 1.5 equiv) in THF (10 mL) was added dropwise, over a period of 20 min, to a solution of the commercial 2-mercapto-4-methyl-6-trifluoromethyl-nicotinonitrile **4j** (6.77 g, 0.03 mol, 1 equiv) and ethyl 2-chloro-acetate (3.3 mL, 0.03 mol, 1 equiv) in THF 200 mL. The reaction mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure and the residue was diluted back in CH_2Cl_2 (300 mL). The organic layer was washed with water (2 × 200 mL), dried over Na_2SO_4, filtered and concentrated in vacuum till dryness yielding 9.27 g (98%) of a solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (s, 1H), 6.35 (br s, 2H, NH_2), 4.40 (q, 2H, CH_2), 2.75 (s, 3H), 1.40 (t, 3H). IR (neat) ν 3526, 3357, 1686, 1250, 1110. HRMS (ESI): calcd for $C_{12}H_{12}F_3N_2O_2S$ $[M+H]^+$ 305.0572, found 305.0555.

4.1.6. General procedure 5 (compound 6)

Hydrazine monohydrate (15 mL) was added to a suspension of **5** (3.5 mmol, 1 equiv) in ethanol (15 mL). The reaction mixture was heated at 80 °C overnight and cooled down in an ice bath giving a flaky precipitate that was filtered off and washed consecutively with cold water (2 × 20 mL) and cold ethanol (2 × 20 mL). The solid residue was then dried in vacuum to afford **6** as a powder.

4.1.6.1. 3-Amino-6-(2-dimethylamino-ethylamino)-4-methyl-thieno[2,3-*b*]pyridine-2-carboxylic acid hydrazide (6b). The product was prepared according to the general procedure **5** and yielded 928 mg (86%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.55 (m, 1H, NH), 6.85 (t, 1H, NH), 6.60 (m, 2H, NH_2), 6.25 (s, 1H), 4.25 (br s, 2H, NH_2), 3.35 (q, 2H), 2.55 (s, 3H), 2.40 (t, 2H), 2.15 (s, 6H). IR (neat) ν 3500, 2500, 1603, 1585. HRMS (ESI): calcd for $C_{13}H_{21}N_6OS$ $[M+H]^+$ 309.1498, found 309.1504.

4.1.6.2. 3-Amino-6-(2-dimethylamino-ethylamino)-4-phenyl-thieno[2,3-*b*]pyridine-2-carboxylic acid hydrazide (6c). The product was prepared according to the general procedure **5** and yielded 1.22 g (94%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (br s, 1H, NH), 7.55 (m, 3H), 7.40 (m, 2H), 7.10 (t, 1H, NH), 6.30 (s, 1H), 5.60 (br s, 2H, NH_2), 4.30 (br s, 2H, NH_2), 3.45 (q, 2H), 2.45 (t, 2H), 2.20 (s, 6H). IR (neat) ν 3487, 3040, 1614, 1586. HRMS (ESI): calcd for $C_{18}H_{23}N_6OS$ $[M+H]^+$ 371.1654, found 371.1658.

4.1.6.3. 3-Amino-4-benzyl-6-(2-dimethylamino-ethylamino)-thieno[2,3-*b*]pyridine-2-carboxylic acid hydrazide (6d). The product was prepared according to the general procedure **5** and yielded 1.18 g (88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.42 (s, 1H, NH), 7.32 (t, 2H), 7.22 (m, 3H), 6.75 (t, 1H, NH), 6.47 (s, 2H, NH_2), 6.08 (s, 1H), 4.31 (s, 2H), 4.24 (s, 2H, NH_2), 3.32 (q, 2H), 2.39 (t, 2H), 2.16 (s, 6H). IR (neat) ν 3500, 2500, 1602, 1590. HRMS (ESI): calcd for $C_{19}H_{25}N_6OS$ $[M+H]^+$ 385.1811, found 385.1820.

4.1.6.4. 3-Amino-6-(2-dimethylamino-ethylamino)-4-propyl-thieno[2,3-*b*]pyridine-2-carboxylic acid hydrazide (6e). The product was prepared according to the general procedure **5** and yielded 1.01 g (86%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.59 (s, 1H, NH), 6.89 (t, 1H, NH), 6.60 (s, 2H, NH_2), 6.30 (s, 1H), 4.29 (s, 2H, NH_2), 3.38 (q, 2H), 2.85 (dd, 2H), 2.40 (t, 2H), 2.19 (s, 6H), 1.63 (sext., 2H), 0.97 (t, 3H). IR (neat) ν 3500, 2400, 1602, 1588. HRMS (ESI) calcd for $C_{15}H_{25}N_6OS$ $[M+H]^+$: 337.1811, found: 337.1810.

4.1.6.5. 3-Amino-5-benzyl-6-(2-dimethylamino-ethylamino)-thieno[2,3-*b*]pyridine-2-carboxylic acid hydrazide (6g). The product was prepared according to the general procedure **5** and yielded 942 mg (70%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.50 (s, 1H, NH), 7.85 (s, 1H), 7.35–7.20 (m, 5H), 6.85 (m, 2H, NH_2), 6.20 (t, 1H, NH), 4.30 (s, 2H, NH_2), 3.85 (s, 2H), 3.40 (q, 2H), 2.40 (t, 2H), 2.10 (s, 6H). IR (neat) ν 3395, 3190, 1619, 1587. HRMS (ESI): calcd for $C_{19}H_{25}N_6OS$ $[M+H]^+$ 385.1811, found 385.1794.

4.1.6.6. 3-Aminothieno[2,3-*b*]pyridine-2-carboxylic acid hydrazide (6i). The reaction was carried out as described in the general procedure **5** but heated under microwave irradiations (10 min, 150 °C, 100 W) instead of classical thermic conditions. Quantitative yield. Compound described in the literature.[13]

4.1.7. Mercapto-acetic acid hydrazide (7)

To a solution of ethyl thioglycolate (11 mL, 0.10 mol, 1 equiv) in ethanol (30 mL) was added hydrazine monohydrate (5.8 mL, 0.12 mol, 1.2 equiv). The mixture was stirred at reflux overnight. The reaction progress was monitored by HPLC and, if necessary, small amounts of hydrazine (by portion of 0.2 equiv) were added until completion of the reaction. The reaction mixture was concentrated in vacuum yielding 9.65 mg (91%) of a yellow liquid corresponding to the expected acylhydrazine **7**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (br s, 1H, NH), 3.90 (dd, 2H, NH_2), 3.25 (s, 2H, CH_2), 1.95 (t, 1H, SH). MS (EI): M^+ *m/z* 106.0.

4.1.8. Mercapto-acetic acid (1-methyl-1*H*-pyrrol-2-yl-methylene) hydrazide (8)

To a solution of acylhydrazine **7** (8.00 g, 0.08 mol, 1 equiv) in ethanol (50 mL) was introduced the *N*-methyl-2-pyrrolcarboxaldehyde (8.10 mL, 0.08 mol, 1 equiv). The reaction mixture was stirred at 80 °C. After 4 h of stirring, the reaction was achieved, then the solvent was removed in vacuum giving a solid residue. The purification by crystallization from ethanol of the latter afforded 6.76 g (43%) of a white solid, corresponding to the *E* isomer of **8** (conformation determined by NOE enhancements). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.1 (s, 1H, NH), 8.0 (s, 1H), 6.92 (br s, 1H), 6.43 (dd, 1H), 6.05 (m, 1H), 3.80 (s, 3H), 3.5–3.2 (br s, 2H), 2.80 (br s, 1H, SH). IR (neat) ν 3320, 2700, 2530, 1651, 1617–1595. HRMS (ESI): calcd for $C_8H_{12}N_3OS$ $[M+H]^+$ 198.0701, found 198.0702.

4.1.9. Synthesis of compounds 9–26

4.1.9.1. General procedure A. Into a round-bottom flask, equipped with a condenser and containing a suspension of **6** (2.0 mmol, 1 equiv) in ethanol (15 mL), was introduced the aldehyde (2.2 mmol, 1.1 equiv) and two drops of concentrated HCl. The reaction mixture was stirred and heated at 80 °C for approximately 18 h. While cooling down the crude mixture in an ice bath, a precipitate was formed which was subsequently filtered off and washed with both water (2 × 10 mL) and cold ethanol (3 × 15 mL). The solid residue was then dried in vacuum at 60 °C.

4.1.9.2. General procedure B. Potassium carbonate (2.90 g, 2.1 equiv) was added to a solution of substituted nicotinonitrile **4** (0.01 mol, 1 equiv) and acylhydrazone **8** (0.01 mol, 1 equiv) in ethanol (90 mL). The reaction mixture was stirred and heated at 80 °C overnight. After cooling down, a precipitate was isolated by vacuum filtration. The precipitate was washed with water (2 × 20 mL) and cold ethanol (4 × 30 mL) to afford a yellow solid which was brought to dryness in vacuum at 60 °C.

4.1.9.3. 3-Amino-4,6-dimethyl-thieno[2,3-*b*]pyridine-2-carboxylic acid naphthalen-1-yl-methylene hydrazide (9). The product was prepared and supplied by outsourcing. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.40 (br s, 1H, NH), 8.85 (br s, 1H), 8.40 (br s, 1H), 8.20 (br s, 1H), 8.05 (m, 2H), 7.7–7.6 (2 t, 2H), 7.40 (br s, 2H, NH_2), 7.20 (m, 1H), 7.05 (s, 1H), 2.75 (s, 3H, CH_3), 2.55 (s, 3H, CH_3). IR (neat) ν 3466 (NH_2), 3301 (NH_2), 1629 (C=O), 1597, 1590. Anal. Calcd for $C_{21}H_{18}N_4OS$: C, 67.36; H, 4.85; N, 14.96; S, 8.56. Found: C, 67.51; H, 4.94; N, 14.75; S, 8.93. HRMS (ESI): calcd for $C_{21}H_{19}N_4OS$ $[M+H]^+$ 375.1279, found 375.1296.

4.1.9.4. 3-Amino-thieno[2,3-b]pyridine-2-carboxylic acid naphthalen-1-yl-methylene hydrazide (10). The product was prepared according to the general procedure A and afforded 300 mg (43%) of a yellow solid; mp >250 °C. ^{1}H NMR (300/500 MHz, DMSO-d_6) δ 11.40 (s, 1H, NH), 8.98 (s, 1H), 8.70 (d, 1H), 8.55 (d, 1H), 8.50 (br d, 2H), 8.20 (br d, 1H), 8.05 (m, 2H), 7.70 (m, 1H), 7.70–7.60 (m, 2H), 7.45 (dd, 1H). IR (neat) ν 3460, 2400, 1625, 1597, 1581. Anal. Calcd for $C_{19}H_{14}N_4OS$: C, 65.88; H, 4.07; N, 16.17; S, 9.26. Found: C, 65.64; H, 4.07; N, 15.56; S, 9.51. HRMS (ESI): calcd for $C_{19}H_{15}N_4OS$ $[M+H]^+$ 347.0966, found 347.0987.

4.1.9.5. 3-Amino-4,6-dimethyl-thieno[2,3-*b*]pyridine-2-carboxylic acid (3-methyl-3*H*-imidazol-4-yl-methylene) hydrazide (11). The product was prepared according to the general procedure A and led to 473 mg (72%) of a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.20 (br s, 1H, NH), 8.13 (br s, 1H), 7.78 (s 1H), 7.38 (s, 1H), 7.30 (br s, 2H, NH_2), 7.03 (s, 1H), 3.90 (s, 3H), 2.75 (s, 3H), 2.50 (s, 3H). IR (neat) ν 3434, 3305, 1632, 1598. Anal. Calcd for $C_{15}H_{16}N_6OS$: C, 54.86; H, 4.91; N, 25.59; S, 9.76. Found: C, 54.70; H, 5.11; N, 24.75; S, 9.54. HRMS (ESI): calcd. for $C_{15}H_{17}N_6OS$ $[M+H]^+$ 329.1185, found 329.1188.

4.1.9.6. 3-Amino-thieno[2,3-*b*]pyridine-2-carboxylic acid (3-methyl-3*H*-imidazol-4-yl-methylene) hydrazide (12). The product was prepared according to the general procedure A and gave 336 mg (56%) of a yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.25 (s, 1H, NH), 8.65 (d, 1H), 8.50 (d, 1H), 8.15 (br s, 1H), 7.75 (s, 1H), 7.65 (br s, 2H, NH_2), 7.45 (dd, 1H), 7.40 (s, 1H), 3.95 (s, 3H). IR (neat) ν 3522, 2566, 1645, 1579, 1645, 1579. Anal. Calcd for $C_{13}H_{12}N_6OS$: C, 51.99; H, 4.03; N, 27.98; S, 10.68. Found: C, 52.69; H, 4.07; N, 26.82; S, 10.43. HRMS (ESI): calcd for $C_{13}H_{13}N_6OS$ $[M+H]^+$ 301.0872, found 301.0875.

4.1.9.7. 3-Amino-4,6-dimethyl-thieno[2,3-*b*]pyridine-2-carboxylic acid [1-(2-dimethylamino-ethyl)-1*H*-pyrrol-2-yl-methylene] hydrazide (13). The product was prepared according to the general procedure A and afforded 292 mg (38%) of a green-yellow powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.00 (br s, 1H, NH), 8.20 (br s, 1H), 7.25 (br s, 2H, NH_2), 7.00 (m, 2H), 6.60 (br s, 1H), 6.15 (t, 1H), 4.20 (t, 2H), 3.75 (s, 3H), 2.55 (t, 2H), 2.55 (s, 3H), 2.18 (s, 6H). IR (neat) ν 3484, 3309, 1621 (C=O). Anal. Calcd for $C_{19}H_{24}N_6OS$: C, 59.35; H, 6.29; N, 21.86; S, 8.34. Found: C, 58.55; H, 6.32; N, 20.61; S, 8.15. HRMS (ESI): calcd for $C_{19}H_{25}N_6OS$ $[M+H]^+$ 385.1811, found 385.1808.

4.1.9.8. 3-Amino-4,6-dimethyl-thieno[2,3-*b*]pyridine-2-carboxylic acid (1-methyl-1*H*-pyrrol-2-yl-methylene) hydrazide (14). The product was prepared according to the general procedure A and yielded 177 mg (27%) of a yellow solid; mp 230–232 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.00 (s, 1H, NH), 8.10 (br s, 1H), 7.28 (br s, 2H, NH_2), 7.00 (s, 1H), 6.95 (s, 1H), 6.58 (br s, 1H), 6.13 (t, 1H), 3.88 (s, 3H), 2.8–2.5 (2s, 6H). IR (neat) ν 3483, 3301, 1627. Anal. Calcd for $C_{16}H_{17}N_5OS$: C, 58.70; H, 5.23; N, 21.39; S, 9.79. Found: C, 58.65; H, 4.84; N, 20.80; S, 9.62. HRMS (ESI): calcd for $C_{16}H_{17}N_5OSNa$ $[M+Na]^+$ 350.1051, found 350.1039.

4.1.9.9. 3-Amino-4-methyl-6-trifluoromethyl-thieno[2,3-*b*]pyridine-2-carboxylic acid naphthalen-1-yl-methylene hydrazide (15). The product was prepared according to the general procedure A and afforded 514 mg (60%) of a yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.70 (br s, 1H, NH), 9.00 (br s, 1H), 8.40 (br s, 1H), 8.20 (m, 1H), 8.05 (m, 2H), 7.75 (s, 1H), 7.8–7.6 (m, 3H), 7.22 (br d, 2H, NH_2), 2.70 (s, 3H). IR (neat) ν 3518, 3305, 1629. Anal. Calcd for $C_{21}H_{15}F_3N_4OS$: C, 58.87; H, 3.53; N, 13.08; S, 7.48. Found C, 58.11; H, 3.78; N, 13.34; S, 6.95. HRMS (ESI): calcd for $C_{21}H_{16}F_3N_4OS$ $[M+H]^+$429.099, found 429.1008.

4.1.9.10. 3-Amino-4-methyl-6-trifluoromethyl-thieno[2,3-*b*]pyridine-2-carboxylic acid (3-methyl-3*H*-imidazol-4-yl-methylene)-hydrazide (16). The product was prepared according to the general procedure A and gave 336 mg (44%) of a yellow solid; mp >230 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.20 (s, 1H, OH), 11.45 (br s, 1H, NH), 8.15 (br s, 1H), 7.80 (s, 1H), 7.75 (s, 1H), 7.40 (s, 1H), 7.10 (br s, 2H, NH_2), 6.60 (s, 1H), 3.90 (s, 3H), 2.70 (s, 3H). IR (neat) ν 3640, 3260, 1690, 1625, 1347, 1128. Anal. Calcd for $C_{15}H_{13}F_3N_6OS$: C, 46.36; H, 3.43; N, 19.08; S, 7.28. Found: C, 46.49; H, 3.52; N, 18.78; S, 7.28. HRMS (ESI): calcd for $C_{15}H_{14}F_3N_6OS$ $[M+H]^+$ 383.0902, found 383.0914.

4.1.9.11. 3-Amino-6-(2-dimethylamino-ethylamino)-4-methyl-thieno[2,3-*b*]pyridine-2-carboxylic acid naphthalen-1-yl-methylene-hydrazide (17). The product was prepared according to the general procedure A and afforded 295 mg (33%) of a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.13 (br s, 1H, NH), 8.90 (s, 1H), 8.45 (m, 1H), 8.14 (d, 1H), 8.02 (d, 2H), 7.7–7.6 (m, 3H), 7.25 (m, 2H, NH_2), 6.95 (t, 1H, NH), 6.30 (s, 1H), 3.40 (q, 2H), 2.59 (s, 3H), 2.42 (t, 2H), 2.19 (s, 6H). IR (neat) ν 3487, 3407, 3292, 1627, 1602, 1571. Anal. Calcd for $C_{24}H_{26}N_6OS$: C, 64.55; H, 5.87; N, 18.82; S, 7.18. Found C, 64.40; H, 5.95; N, 18.24; S, 7.01. HRMS (ESI): calcd for $C_{24}H_{27}N_6OS$ $[M+H]^+$ 447.1967, found 447.1951.

4.1.9.12. 3-Amino-6-(2-dimethylamino-ethylamino)-4-methyl-thieno[2,3-*b*]pyridine-2-carboxylic acid (1-methyl-1*H*-pyrrol-2-yl-methylene)-hydrazide (18). The product was prepared according to the general procedure A and yielded 711 mg (89%) of a yellow solid; mp 185–188 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.68 (s, 1H, NH), 7.12 (br s, 1H), 7.12 (m, 2H, NH_2), 6.92 (dd, 1H), 6.89 (t, 1H, NH), 6.52 (m, 1H), 6.27 (s, 1H), 6.11 (dd, 1H), 3.85 (s, 3H), 3.38 (q, 2H), 2.57 (s, 3H), 2.41 (t, 2H), 2.18 (s, 6H). IR (neat) ν 3468, 3404, 3286, 1626, 1597. Anal. Calcd for $C_{19}H_{25}N_7OS$: C, 57.12; H, 6.31; N, 24.54; S, 8.03. Found C, 57.04; H, 6.43; N, 24.01; S, 7.92. HRMS (ESI) calcd for $C_{19}H_{26}N_7OS$ $[M+H]^+$ 402.2076, found 402.2093.

4.1.9.13. 3-Amino-6-(2-dimethylamino-ethylamino)-4-phenyl-thieno[2,3-*b*]pyridine-2-carboxylic acid naphthalen-1-yl-methylene-hydrazide hydrochloride (19). The product was prepared according to the general procedure A and led to 963 mg (95%) of a yellow solid; mp 215–217 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.25 (s, 1H, NH), 8.90 (s, 1H), 8.43 (d, 1H), 8.18 (d, 1H), 8.05 (d, 2H), 7.72–7.60 (m, 3H), 7.58 (m, 3H), 7.47 (m, 2H), 7.45 (m, 1H, NH), 6.38 (s, 1H), 6.30–6.20 (br s, 2H, NH_2), 3.62 (q, 2H), 2.90 (m, 2H), 2.55 (br s, 6H). IR (neat) ν 3480, 3300, 3300, 2459, 1610, 1589. Anal. Calcd for $C_{29}H_{28}N_6OS{\cdot}0.50HCl$: C, 63.90; H, 5.36; N, 15.42; S, 5.88; Cl, 6.50. Found: C, 65.90; H, 5.44; N, 15.75; S, 6.10; Cl, 2.91. HRMS (ESI): calcd for $C_{29}H_{29}N_6OS$ $[M+H]^+$ 509.2124, found 509.2109.

4.1.9.14. 3-Amino-6-(2-dimethylamino-ethylamino)-4-phenyl-thieno[2,3-*b*]pyridine-2-carboxylic acid (3-methyl-3*H*-imidazol-4-yl-methylene)-hydrazide hydrochloride (20). The product was prepared according to the general procedure A and gave 637 mg (71%) of a yellow powder; mp 210–214 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10 (m, 1H), 7.78 (s, 1H), 7.58 (d, 2H), 7.58 (t, 1H), 7.45 (m, 2H), 7.38 (s, 1H), 6.30 (s, 1H), 6.20 (m, 2H), 3.90 (s, 3H), 3.70 (q, 2H), 3.20 (m, 2H), 2.75 (br s, 6H). IR (neat) ν 3500, 3400, 3300, 1589, 1589, 1620, 1605. Anal. Calcd for $C_{23}H_{26}N_8OS{\cdot}0.9HCl$: C, 55.76; H, 5.47; N, 22.62; S, 6.47; Cl, 6.44. Found C, 55.75; H, 5.12; N, 22.70; S, 6.61; Cl, 6.45. HRMS (ESI): calcd for $C_{23}H_{27}N_8OS$ $[M+H]^+$ 463.2029, found 463.2045.

4.1.9.15. 3-Amino-6-(2-dimethylamino-ethylamino)-4-phenyl-thieno[2,3-*b*]pyridine-2-carboxylic acid (1-methyl-1*H*-pyrrol-2-yl-methylene)-hydrazide (21). The product was prepared according to the general procedure A and led to 591 mg (64%) of a yellow solid; mp 197–199 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.70 (s, 1H, NH), 8.04 (s, 1H), 7.54 (m, 3H), 7.43 (m, 2H), 7.14 (t, 1H, NH), 6.91 (t, 1H), 6.55 (dd, 1H), 6.32 (s, 1H), 6.11 (dd, 1H), 6.2–5.9 (br s, 2H, NH_2), 3.85 (s, 3H), 3.44 (q, 2H), 2.44 (t, 2H), 2.20 (s, 6H). IR (neat) ν 3469, 3394, 3275, 3020, 2700, 1615, 1589. Anal. Calcd for $C_{24}H_{27}N_7OS$: C, 62.45; H, 5.90; N, 21.24; S, 6.95. Found: C, 62.21; H, 5.77; N, 20.93; S, 6.66. HRMS (ESI): calcd for $C_{24}H_{28}N_7OS$ $[M+H]^+$ 462.2076, found 462.2095.

4.1.9.16. 3-Amino-4-benzyl-6-(2-dimethylamino-ethylamino)-thieno[2,3-*b*]pyridine-2-carboxylic acid (1-methyl-1*H*-pyrrol-2-yl-methylene)-hydrazide (22). The product was prepared according to the general procedure A and afforded 713 mg (75%) of a yellow powder; mp 196–201 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.70 (s, 1H, NH), 8.05 (br s, 1H), 7.35 (t, 2H), 7.28 (t, 1H), 7.25 (d, 2H), 7.10 (br s, 2H, NH_2), 7.00 (t, 1H, NH), 6.91 (t, 1H), 6.52 (m, 1H), 6.11 (t, 1H), 6.01 (s, 1H), 4.37 (s, 2H), 3.85 (s, 3H), 3.35 (q, 2H), 2.37 (t, 2H), 2.14 (s, 6H). IR (neat) ν 3540, 2430, 1612, 1580. Anal. Calcd for $C_{25}H_{29}N_7OS$: C, 63.13; H, 6.15; N, 20.62; S, 6.74. Found: C, 62.97; H, 6.11; N, 20.50; S, 6.57. HRMS (ESI): calcd for $C_{25}H_{30}N_7OS$ $[M+H]^+$ 476.2233, found 476.2244.

4.1.9.17. 3-Amino-6-(2-dimethylamino-ethylamino)-4-propyl-thieno[2,3-*b*]pyridine-2-carboxylic acid (1-methyl-1*H*-pyrrol-2-yl-methylene)-hydrazide (23). The product was prepared according to the general procedure A and yielded 522 mg (61%) of a yellow powder; mp 172–175 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.50 (s, 1H, NH), 8.09 (s, 1H), 7.01 (br s, 2H, NH_2), 6.89 (t, 1H), 6.74 (t, 1H, NH), 6.50 (m, 1H), 6.30 (s, 1H), 6.10 (dd, 1H), 3.86 (s, 3H), 3.39 (q, 2H), 2.89 (dd, 2H), 2.44 (t, 2H), 2.20 (s, 6H), 1.69 (m, 2H), 0.99 (t, 3H). IR (neat) ν 3492, 3315, 3239, 3172, 2647, 1617, 1604, 1581, 1523. Anal. Calcd for $C_{21}H_{29}N_7OS$: C, 58.99; H, 6.84; N, 22.93; S, 7.50. Found: C, 58.90; H, 6.82; N, 22.88; S, 7.12. HRMS (ESI): calcd for $C_{21}H_{30}N_7OS$ $[M+H]^+$ 428.2233, found 428.2250.

4.1.9.18. 3-Amino-6-(2-dimethylamino-ethylamino)-5-phenyl-thieno[2,3-*b*]pyridine-2-carboxylic acid (1-methyl-1*H*-pyrrol-2-yl-methylene) hydrazide (24). The product was prepared according to general procedure B and gave 2.72 g (59%) of a yellow solid, mp = 220 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.80 (m, 1H, NH), 8.05 (m, 1H), 8.00 (s, 1H), 7.6–7.4 (m, 2H, NH_2), 7.6–7.4 (m, 5H), 6.95 (br s, 1H), 6.55 (br s, 1H), 6.15 (t, 1H), 6.05 (t, 1H, NH), 3.85 (s, 3H), 3.45 (q, 2H), 2.40 (m, 2H), 2.15 (s, 6H). IR (neat) ν 3460 (NH_2), 3150, 1616, 1600. Anal. Calcd for $C_{24}H_{27}N_7OS$: C, 62.45; H, 5.90; N, 21.24; S, 6.95. Found: C, 61.92; H, 5.78; N, 20.76; S, 6.58. HRMS (ESI): calcd for $C_{24}H_{28}N_7OS$ $[M+H]^+$ 462.2076, found 462.2077.

4.1.9.19. 3-Amino-5-benzyl-6-(2-dimethylamino-ethylamino)-thieno[2,3-*b*]pyridine-2-carboxylic acid (1-methyl-1*H*-pyrrol-2-yl-methylene) hydrazide (25). The product was prepared according to general procedure A and gave 751 mg (79%) of a yellow powder, mp = 192 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.70 (m, 1H, NH), 8.05 (m, 1H), 7.95 (s, 1H), 7.40 (m, 2H, NH_2), 7.35–7.26 (m, 5H), 6.90 (m, 1H), 6.55 (m, 1H), 6.25 (t, 1H, NH), 6.10 (m, 1H), 3.90 (s, 2H), 3.85 (s, 3H), 3.40 (q, 2H), 2.40 (t, 2H), 2.10 (s, 6H). IR (neat) ν 3340 (NH), 3150, 1614 (C=O), 1596 (C=N). Anal. Calcd for $C_{25}H_{29}N_7OS$: C, 63.13; H, 6.15; N, 20.62; S, 6.74. Found: C, 63.03; H, 6.09; N, 20.04; S, 6.92. HRMS (ESI): calcd for $C_{25}H_{30}N_7OS$ $[M+H]^+$ 476.2233 found 476.2240.

4.1.9.20. 3-Amino-6-(2-dimethylamino-ethylamino)-5-propyl-thieno[2,3-*b*]pyridine-2-carboxylic acid (1-methyl-1*H*-pyrrol-2-yl-methylene)-hydrazide (26). The product was isolated according to the general procedure B and yielded 2.01 g (47%) of a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.70 (m, 1H, NH), 8.05 (m, 1H), 7.85 (s, 1H), 7.40 (m, 2H, NH_2), 6.90 (t, 1H), 6.50 (m, 1H), 6.35 (t, 1H, NH), 6.10 (m, 1H), 3.85 (s, 3H), 3.50 (q, 2H), 2.45 (m, 4H), 2.20 (s, 6H), 1.60 (m, 2H), 0.95 (t, 3H). IR (neat) ν 3490, 3170, 1610, 1593. HRMS (ESI): calcd for $C_{21}H_{29}N_7OS$ [M+H$^+$] 428.2233 found 428.2240.

4.2. Molecular modeling

Modeling and docking studies were performed using the available c-Src crystal structure (PDB code 2SRC). This inactive form crystal structure of c-Src was the only one available at that time. Hydrogen atoms were added and their position optimized by energy minimization.

Inhibitors were build using SYBYL 6.5 version from Tripos,[14] no charges being computed, 3D structures were obtained using the CONCORD program within SYBYL.

The compounds were then docked using the GOLD[15] program that automatically positioned molecules in the ATP binding pocket (the GOLD protocol attributes formal charges both to the enzyme and to the inhibitors). 30 solutions (genetic algorithm parameters set to 7–8) were calculated but only the 10 best ones, based on the build in fitness function, were retained. Known structure activity data were then used to visually compare and filter this chosen solutions. In this manner the two orientations depicted in Figure 3A and B were identified. Molecular surfaces (Connolly) were computed using the MOLCAD program within SYBYL.

4.3. Biological evaluation

4.3.1. High throughput screening (HTS)

Src activity was measured using the time-resolved fluorescence resonance energy transfer assay (TR-FRET, LANCE®, Perkin Elmer) adapted from Sadler and al.[16]

Briefly, Src activation results in the tyrosine phosphorylation of a model peptide (N-terminus biotynilated peptide, whose biotin moiety is recognized by a streptavidine-allophytocyanin compound).

The phosphorylated peptide was recognised by an Europium-labelled antibody PY20 (AD0067, Perkin–Elmer). The close proximity of this labelled antibody and of the labelled streptovidine gives rise to a 665 nm fluorescence, easily detectable from the experiment background. Compounds were tested twice independently at 10 μM, final concentration (staurosporine was used as positive control in all individual assay plates, 384-well format). The final results of the screen were analysed and compounds showing significant activities were again tested using a concentration range from 0.1 nM to 100 μM. Obtained IC_{50} (n = 2–3) were used to make a pre-selection of the most interesting compounds (more than 90,000 compounds tested).

4.3.2. Inhibition of c-Src enzyme

The inhibitors were diluted using a robotic Tecan Evo 150 platform and the kinase assay was performed with 4 μl of a 10% DMSO diluted inhibitor, assay buffer concentrated four times (80 mM $MgCl_2$, 0.4 mM EDTA, 2 mM dTT), 10 μl peptide substrate (KVEKIGEGYYGVVYK, 370 μM) and 6 μl Src kinase (stock GTP purified diluted with kinase assay buffer to 200 nM).

10 μl co-substrate (40 μM ATP with 0.2 μCiP^{33}-γ-ATP) was then added using a robotic Precision 2000 Biotek) platform. The assay was incubated 20 min at 30 °C then stopped by adding 200 μl of a 0.85% orthophosphoric acid followed by transfer to a phosphocellulose filter microplate (Whatman-P81). After washing three times with 200 μl, 0.85% orthophosphoric acid the fitter plate was dried with 200 μl acetone. The residual activity was measured on a top count using 25 μl scintillation solution (Packard Ultimagold).

4.3.3. Inhibition of c-Src in culture cells

Although our main goal was to explore new chemical avenues for c-Src inhibitors, we investigated the ability of these molecules to inhibit c-Src in cells. Indeed in a complex cellular context the higher ATP concentration and the interaction with other signaling pathways may limit the effect of ATP competitors on the kinase. Furthermore, we considered relevant to test the effect of our inhibitors on the phosphorylation of an endogenous and physiologically relevant substrate like FAK.

4.3.3.1. Phosphor-FAK Western blot assays. HCT116 human colon carcinoma cells (ATTC) were seeded in 6-well plates overnight and serum starved (0% serum). After 5 h incubation in presence of inhibitors, cell lysates were prepared in 200 μl of 2X Laemmli buffer (Sigma). After boiling and sonication, protein extracts were separated in 4–20% gradient polyacrylamide gels and transferred to PVDF membranes by liquid transfer. Detection of phosphor-FAK was done using a rabbit anti-pTyr861 antibody from Biosource. Total FAK was detected using a rabbit anti-FAK antibody from Upstate, and measured in a separate blot.

The ratio of phospho-FAK/total FAK was calculated by densitometric measurements of X-ray films.

DMSO was used as negative control (100% phosphorylation). The 4-anilinoquinoline-3-carbonitrile[3b] (Fig. 1) was used as positive control.

Inhibition of pFAK by compounds was tested at 3 μM and 10 μM final concentration. If no significant inhibition was observed at 10 μM it was then assumed that the 50% inhibition would be seen above 10 μM. Given the semi-quantitative nature of the assay no precise IC_{50} determination was done.

4.3.3.2. Phospho-STAT3. Western blot assays. A431 epidermoid carcinoma cells were starved overnight and stimulated 10 min with EGF (100 ng/ml) followed by cell lysis as described above. Phosphor-STAT3 was detected using a phospho-Tyr705 antibody from Santa Cruz.

Total STAT3 was measured in a separate blot and the ratio of phosphor STAT3/total STAT3 was calculated by densitometric measurement of X-ray films.

DMSO was used as negative control in the presence of EGF (100% phosphorylation).

The 4-anilinoquinoline-3-carbonitrile[3b] (Fig. 1) was used as positive control.

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