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Synthesis and biological evaluation of benzo[*b*]thiophene acylhydrazones as antimicrobial agents against multidrug-resistant *Staphylococcus aureus*

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Abstract – The benzo[*b*]thiophene nucleus and the acylhydrazone functional group were combined to prepare 3 new series of compounds for screening against *Staphylococcus aureus*. The reaction of substituted benzo[*b*]thiophene-2-carboxylic hydrazide and various aromatic or heteroaromatic aldehydes led to a collection of 26 final products with extensive structural diversification on the aromatic ring and on the position 6 of the benzo[*b*]thiophene. The screening lead to the identification of 8 hits including (*E*)-6-chloro-*N'*-(pyridin-2-ylmethylene)benzo[*b*]thiophene-2-carbohydrazide (**II.b**), a non-cytotoxic derivative showing a minimal inhibitory concentration of 4 µg/mL on three *S. aureus* strains, among which a reference classical strain and two clinically isolated strains resistant to methicillin and daptomycin, respectively.

Key words: Benzothiophene, acylhydrazone, *Staphylococcus aureus*, Antibacterial, MRSA

Introduction

Antimicrobial resistance (AMR) is a public health issue that will continue to worsen in the years to come. At least 700,000 deaths are attributed each year to drug-resistant pathogens, but this could rise to 10,000,000 deaths per year by 2050 [1-3]. Among those pathogens, some are widely spread, such as vancomycin-resistant *Enterococcus faecium* or methicillin-resistant *Staphylococcus aureus* (MRSA) [4]. The development of new antibiotics targeting antibioresistant bacteria is therefore critical. Substituted benzo[*b*]thiophenes are interesting compounds in medicinal chemistry [5] by displaying a broad range of activity including antimicrobial [6, 7], anticancer [8], anti-diabetic [9], anti-depressant [10], anti-inflammatory and analgesic agents [11, 12]. As a part of an ongoing research program aiming at the discovery of new potential antibiotics targeting multidrug-resistant *Staphylococcus aureus* strains, we combined the benzo[*b*]thiophene nucleus with the acylhydrazone functional group which is also relevant in bioactive molecules design [13]. We focused our efforts on the synthesis and biological evaluation of a

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collection of acylhydrazones built from various aromatic or heteroaromatic aldehydes and benzo[*b*]thiophene-2-carboxylic hydrazide, readily accessible from benzo[*b*]thiophene-2-carboxylic acid. This sequence allowed an easy structural diversification on both the heteroaromatic benzo[*b*]thiophene nucleus and the aromatic system. The purpose was then to screen the collection of benzo[*b*]thiophene-2-acylhydrazones for identifying new hits against drug-resistant *S. aureus* strains.

Results and discussion

Synthesis and biological evaluation of series I – benzo[*b*]thiophene-2-acylhydrazones

Benzo[*b*]thiophene-2-acylhydrazones **I** derived from various aldehydes were first investigated (Figure 1).

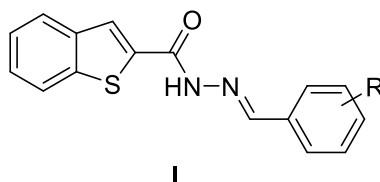
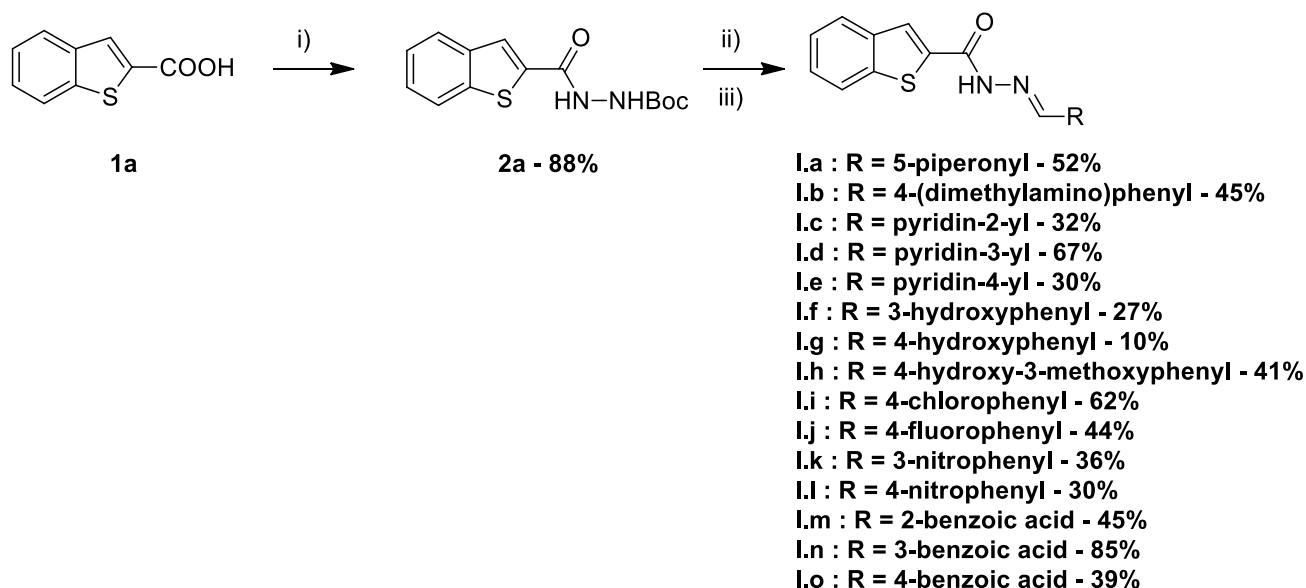


Figure 1 : General structure of set **I** derivatives

The synthetic route to these compounds was achieved by conversion of benzothiophene-2-carboxylic acid **1a** into *tert*-butyl 2-(benzothiophene-2-carbonyl)hydrazinecarboxylate **2a** in 88% yield using DCC as coupling reagent. This method was preferred from the substitution of methyl benzothiophene-2-carboxylate with hydrazine leading to a mixture of products difficult to purify [14]. Then, the *tert*-butyl carboxylate group was removed under acidic conditions using TFA to give a crude material which was then engaged without purification in the presence of diversely substituted aromatic or heteroaromatic aldehydes under acidic and refluxing conditions to form the imine bond of the acylhydrazone (

Scheme **1**).

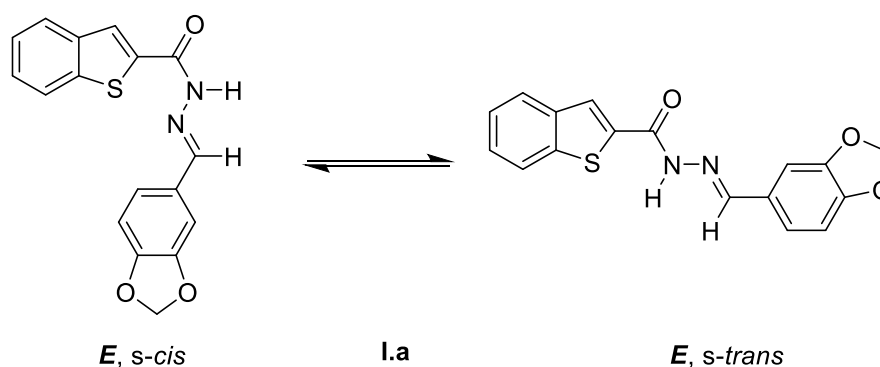


i) Boc-hydrazide, DCC, DMAP, DCM (anh.), r.t., 24 h; ii) TFA, DCM, r.t., 18 h;
 iii) Aldehyde, MeOH (anh.), 65 °C, 2 h;

Scheme 1 : Synthesis of set I derivatives

All the synthesized derivatives **I** were conveniently obtained in satisfactory yields by spontaneous precipitation in pure form in the reaction medium after two hours of reflux. Compounds like the 2-pyridinyl derivative **I.c** required careful removal of all traces of acid after TFA deprotection, by a basic aqueous work-up and extraction with ethyl acetate before reaction with 2-pyridinecarboxaldehyde.

Structural analysis showed the presence of mixtures of geometric isomers for each final compound, as ^1H and ^{13}C NMR signals were split in two. Theoretically, each N-acylhydrazone derivative could present four different isomers due to (*E*)/(*Z*) and *s-cis*/*s-trans* isomerism. Previous studies have shown that only the more stable (*E*)-isomer is generated due to the steric hindrance and under such thermodynamic conditions, and that *s-cis*- and *s-trans*-isomers (rotamers) were identified by ^1H NMR spectroscopy [13, 15, 16]. To demonstrate the presence of rotamers in our case for the piperonyl derivative **I.a**, the temperature of ^1H NMR experiments was gradually increased, and the expected coalescence phenomenon was observed at 330 K and at higher temperature (Figure 2).



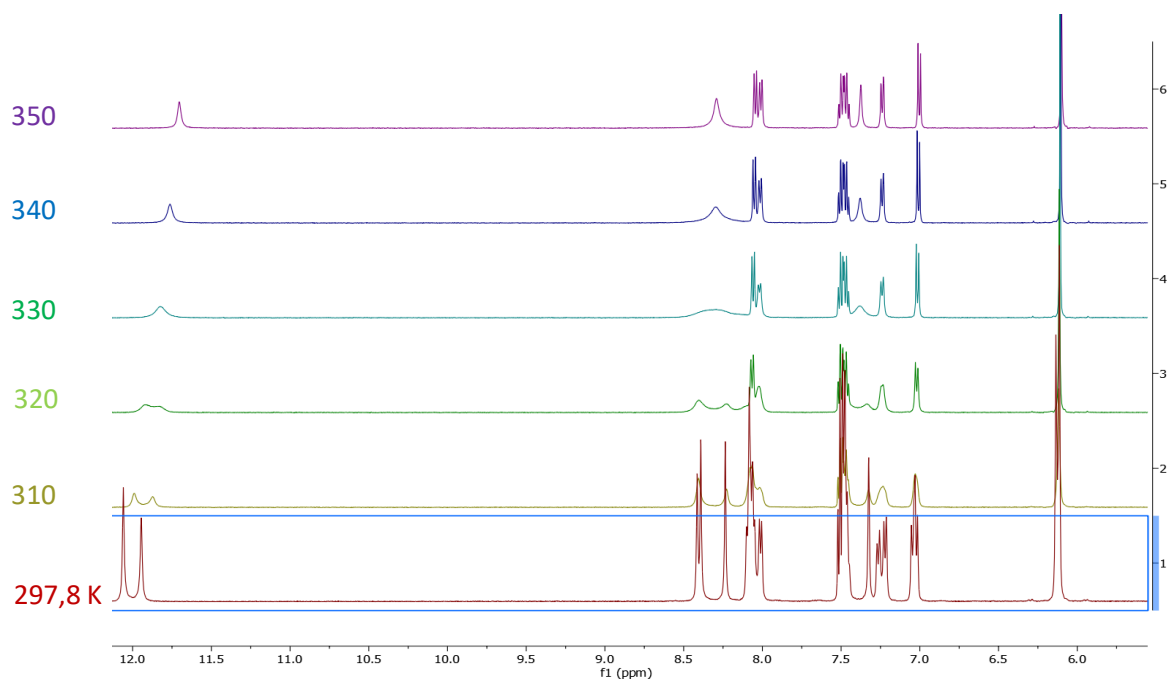
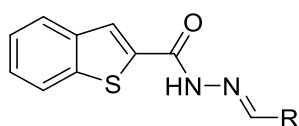




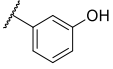
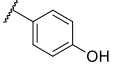
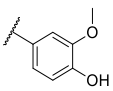


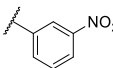
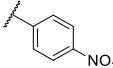

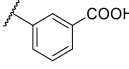
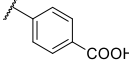

Figure 2 : ^1H NMR spectra of **I.a** at different temperatures

This first set of compounds was then tested on three strains of *Staphylococcus aureus*, including a reference (ATCC29213) and two clinically isolated strains respectively resistant to methicillin (SF8300) and daptomycin (ST20171643). The purpose of this experiment was to establish the minimum inhibitory concentration (MIC), which is the minimum concentration of compound needed to prevent the growth of a standardized bacterial inoculum, for the benzo[*b*]thiophene derived acylhydrazones. The results are presented in Table 1.

Table 1 : Minimum inhibitory concentrations of set **I** molecules against *S. aureus* strains



Name	R	LogP	MIC ($\mu\text{g/mL}$)		
			ATCC 29213 ^a	SF8300 ^b	ST20171643 ^c
I.a		4.20	32	64	16
I.b		4.41	> 256	> 256	> 256
I.c		3.13	16	16	16

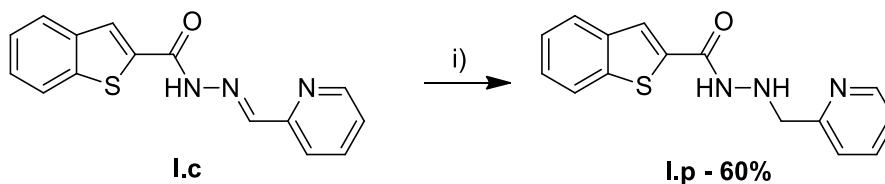
I.d		3.07	> 256	> 256	> 256
I.e		3.02	> 256	> 256	> 256
I.f		3.80	> 256	> 256	ND
I.g		3.83	> 256	> 256	> 256
I.h		3.64	> 256	> 256	> 256
I.i		4.98	128	128	128
I.j		4.47	> 256	> 256	128
I.k		4.24	> 256	> 256	ND
I.l		4.26	64	64	32
I.m		3.83	256	> 256	> 256
I.n		4.19	> 256	> 256	256
I.o		4.22	> 256	> 256	256
I.p		2.33	64	64	64

^a : Methicillin-sensitive *S. aureus* ; ^b : Methicillin-resistant *S. aureus* ; ^c : Daptomycin-resistant *S. aureus*

In this series, the two most active compounds are the piperonyl derivative **I.a** which shows a MIC of 32 µg/mL for ATCC29213 and a range from 16 to 64 µg/mL for the two strains of antibiotic-resistant *S. aureus*, and the pyridinyl compound **I.c**, which shows a MIC of 16 µg/mL for every strain tested, making it the most potent molecule in this series. Interestingly, the position of the nitrogen atom in position 2 of the pyridine moiety in **I.c** was found to be important, as the other pyridinyl compounds **I.d** and **I.e**, in which the nitrogen is respectively in position 3 and 4, were found to be inactive. In this set, another position of interest in the phenyl ring is position 4 (**I.i**, **I.l** and, to a lesser extent **I.a**), with interesting MICs for the products, while the other ones had no biological activity.

To examine the effect of the reduction of the imine bond and its consequences on free rotation and conjugation, the pyridinyl compound **I.c** was reduced using palladium over carbon under H₂ atmosphere with a 60% yield (Scheme 2). Reduced compound **I.p** exhibited a MIC four times higher than the one of **I.c** (Table 1). It is therefore suggested that preventing the free rotation of the pyridine ring and allowing

conjugation due to the tautomeric form of the amide bond is important for the activity of **I.c**. An effect of hydrophobicity is also observed, decreasing the LogP from 3.13 for **I.c** to 2.33 for **I.p**.



i) H₂, Pd/C, MeOH, EtOAc, r.t., 7 h

Scheme 2 : Synthesis of compound I.p

Synthesis and biological evaluation of series II – 6-halogenbenzo[b]thiophene-2-acylhydrazones

Based on this first set of results, we selected the two compounds **I.a** and **I.c** (respectively bearing a 5-piperonyl and a 2-pyridinyl moiety) as hits to improve their activity. 6-halogenbenzo[b]thiophene rings being easily accessible using 2-fluoro-4-halogenobenzaldehyde as precursor, we prepared a series of four compounds, namely two piperonyl or pyridinyl compounds bearing a fluorine or chlorine atom respectively.

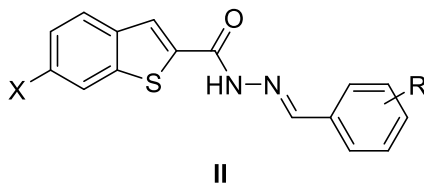
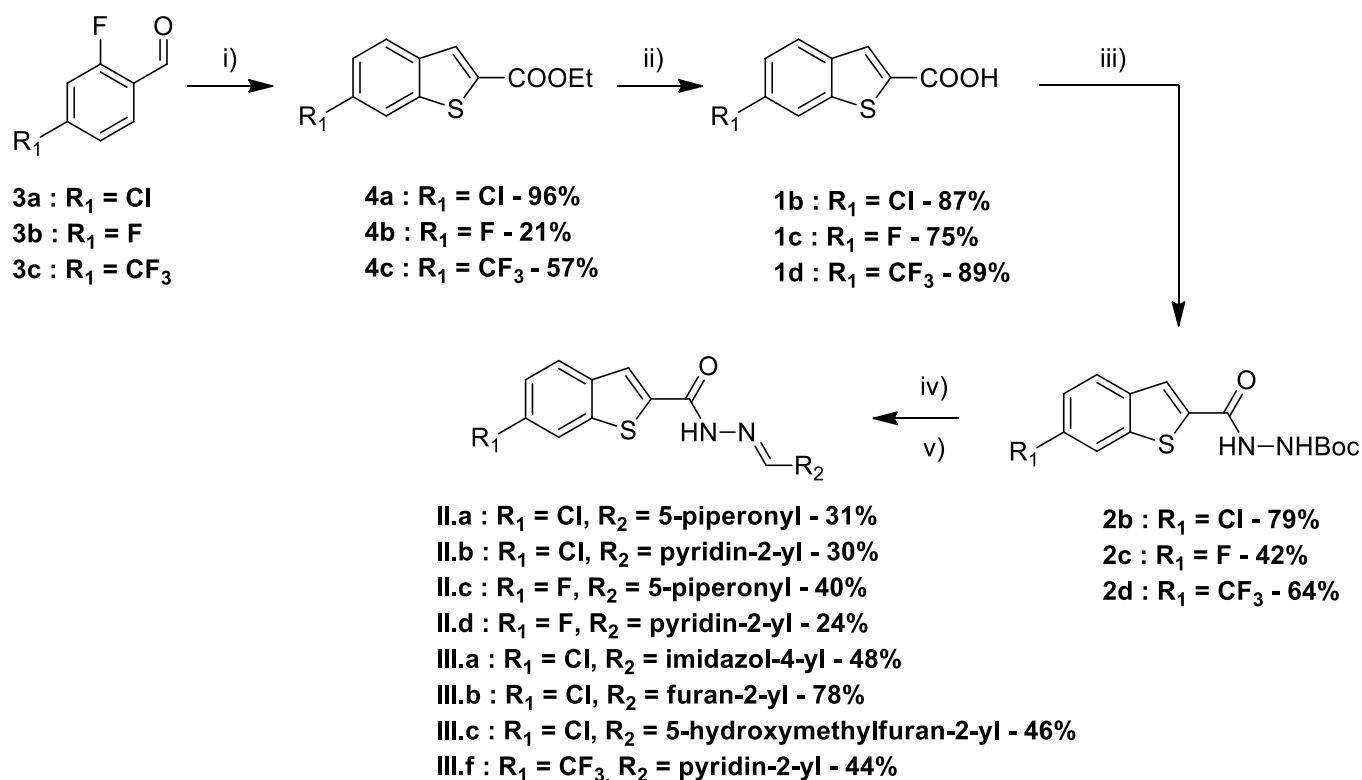


Figure 3 : General structure of set II derivatives

In order to synthesize the acylhydrazones, 6-chloro- and 6-fluorobenzo[b]thiophene-2-carboxylic acids **1b** and **1c** were thus prepared. First, 2-fluoro-4-halogenobenzaldehyde reacted with ethyl thioglycolate by nucleophilic substitution followed by an intramolecular cyclization to obtain compounds **4a** and **4b**, as described in the literature [17]. These esters were then saponified with sodium hydroxide to give the desired carboxylic acids **1b** and **1c**. The general procedure used for the first set was applied to the latter to obtain compounds **II.a-d** with similar yields, as shown in Scheme 3.

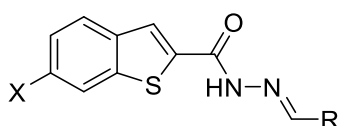


i) Ethyl thioglycolate, Et_3N , DMSO (anh.), 80 °C, 2 h then r.t., 18 h; ii) NaOH, H_2O , EtOH, r.t., 18 h; iii) Boc-hydrazide, DCC, DMAP, DCM (anh.), r.t., 24 h; iv) TFA, DCM (anh.), r.t., 18 h; v) Aldehyde, MeOH (anh.), 65 °C, 2 h;

Scheme 3 : Synthesis of set **II** and **III** derivatives

These compounds were also tested to establish their MIC against *S. aureus* with the same method used for set **I**, and the results are presented in Table 2.

Table 2: Minimum inhibitory concentrations of set **II** molecules against *S. aureus* strains



Name	X	R	LogP	MIC ($\mu\text{g/mL}$)		
				ATCC 29213 ^a	SF8300 ^b	ST20171643 ^c
II.a	Cl		4.85	> 256	> 256	> 256
II.b	Cl		3.79	4	4	4
II.c	F		4.33	> 256	> 256	> 256
II.d	F		3.27	32	16	16

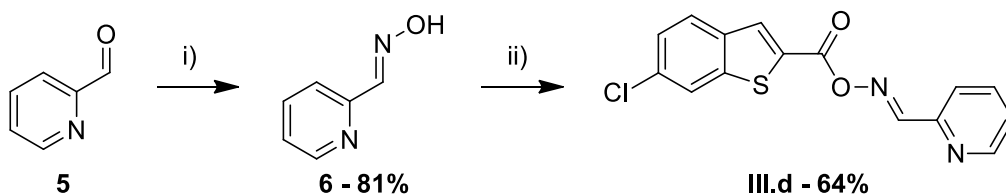
^a : Methicillin-sensitive *S. aureus* ; ^b : Methicillin-resistant *S. aureus* ; ^c : Daptomycin-resistant *S. aureus*

This short series allowed to identify the chloro pyridinyl compound **II.b** as significantly more active compared to its non-halogenated counterpart **I.c**, reaching 4 µg/mL for the three strains. The presence of a fluorine atom in position 6 of the benzothiophene for the pyridinyl compound did not change its activity. Both halogenated compounds derived from the piperonyl compound **I.a** lost their biological activity (**II.a** and **II.c**).

Synthesis and biological evaluation of series III – structural modifications of II.b

Attempts for improving the activity of the chloro pyridinyl compound **II.b** was then undertaken by replacing the pyridine ring by three five-bond rings, namely imidazole, furan and 5-hydroxymethylfuran. The procedure used was the same as the one used for chlorinated derivatives **II.a** and **II.b** (Scheme 3). As shown in Table 3, the activity remains better for compound **II.b**, confirming the importance of the 2-pyridinyl group.

Then the acylhydrazone was replaced by an acyloxime moiety, leading to a more flexible molecule and losing the *s-cis/s-trans* isomerism. Compound **III.d** was obtained through the conversion of pyridine-2-carboxaldehyde into the corresponding oxime **6**, followed by the acylation from 6-chlorobenzo[*b*]thiophene-2-carboxylic acid **1b** (Erreur ! Source du renvoi introuvable.).

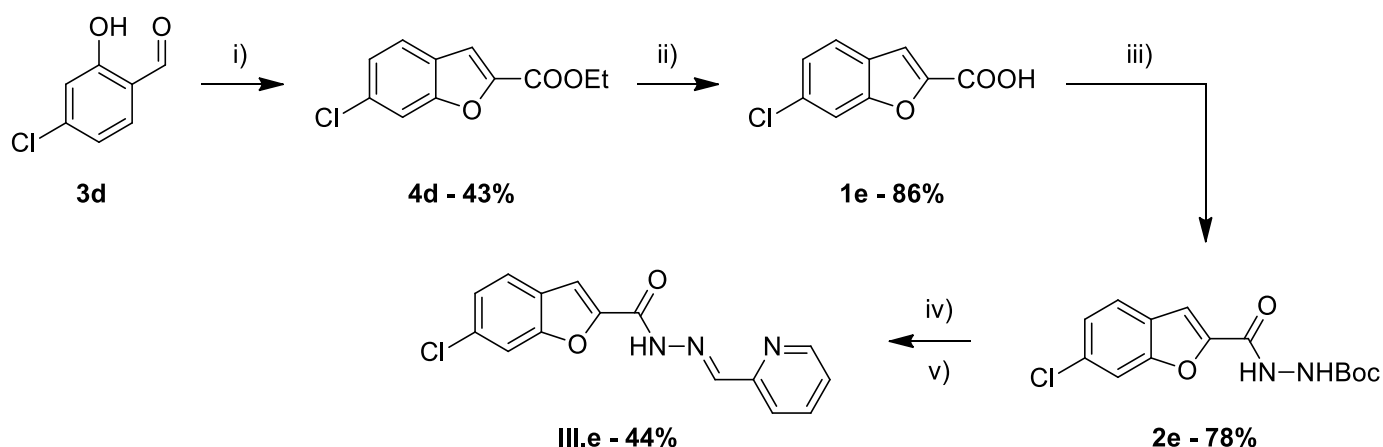


i) $\text{HO-NH}_2\cdot\text{HCl}$, NaHCO_3 , H_2O , 65°C , 3 h; ii) **1b**, DCC, DMAP, DCM (anh.), 0°C then r.t., 24 h

Scheme 4 : Synthesis of III.d

Compound **III.d** showed no anti-staphylococcal activity at concentration lower or equal to 256 µg/mL (Table 3). Therefore, the rigidity imposed by the acylhydrazone moiety is critical to the biological activity.

Then, the effect of the hydrophobicity of the compounds on the antibacterial activity was investigated using the benzo[*b*]furan counterpart of **II.b** (Scheme 5). First, 4-chloro-2-hydroxybenzaldehyde **3d** was converted into the ester **4d** using diethyl 2-bromomalonate as described [18]. Then, the synthetic route was the same as described before for benzo[*b*]thiophene acylhydrazone derivatives.



i) Diethyl 2-bromomalonate, K_2CO_3 , butan-2-one, 90 °C, 7 h then r.t., 18 h; ii) NaOH, H_2O , EtOH, r.t., 2 h; iii) Boc-hydrazide, DCC, DMAP, DCM (anh.), r.t., 24 h; iv) TFA, DCM, r.t., 18 h; v) Pyridine-2-carboxaldehyde, MeOH (anh.), 65 °C, 2 h;

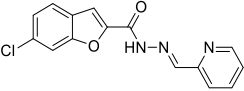
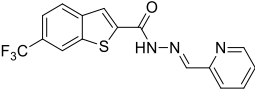
Scheme 5 : Synthesis of III.e

As shown in Table 3, compound **III.e** showed a weak activity against *S. aureus* with MIC values $\geq 128 \mu\text{g/mL}$. Therefore, the hydrophobicity of the benzo[*b*]thiophene ring is critical for the biological activity. As expected, the replacement of the sulfur by an oxygen atom decreases the overall LogP of the compound (respectively 3.79 for **II.b** and 3.15 for **III.e**).

Finally, the chlorine atom was replaced by a bioisostere, a trifluoromethyl group, using the same synthetic strategy (Scheme 3), leading to compound **III.f**. Even if the MICs are interesting (Table 3), the chlorinated derivative **II.b** remains the most active compound.

Table 3 : Minimum inhibitory concentrations of set III molecules against *S. aureus* strains

Name	Structure	LogP	MIC ($\mu\text{g/mL}$)		
			ATCC 29213 ^a	SF8300 ^b	ST20171643 ^c
III.a		3.19	256	64	64
III.b		4.22	> 256	256	256
III.c		3.79	> 256	256	256
III.d		3.89	> 256	> 256	> 256

III.e		3.15	> 256	256	128
III.f		4.01	32	8	16

^a : Methicillin-sensitive *S. aureus* ; ^b : Methicillin-resistant *S. aureus* ; ^c : Daptomycin-resistant *S. aureus*

Cytotoxicity assay of II.b

Due to its interesting antistaphylococcal activity, the potential mammalian cytotoxicity of the chloro pyridinyl derivative **II.b** was examined. Therefore, a 7 h propidium iodide (PI)-based assay was used on adenocarcinomic human alveolar basal epithelial cells (A549), with recombinant *S. aureus* α -hemolysin as positive control. As depicted in Figure 4, no cytotoxicity was found for **II.b** after 7 h at a concentration of 128 $\mu\text{g/mL}$ (32 times the MIC).

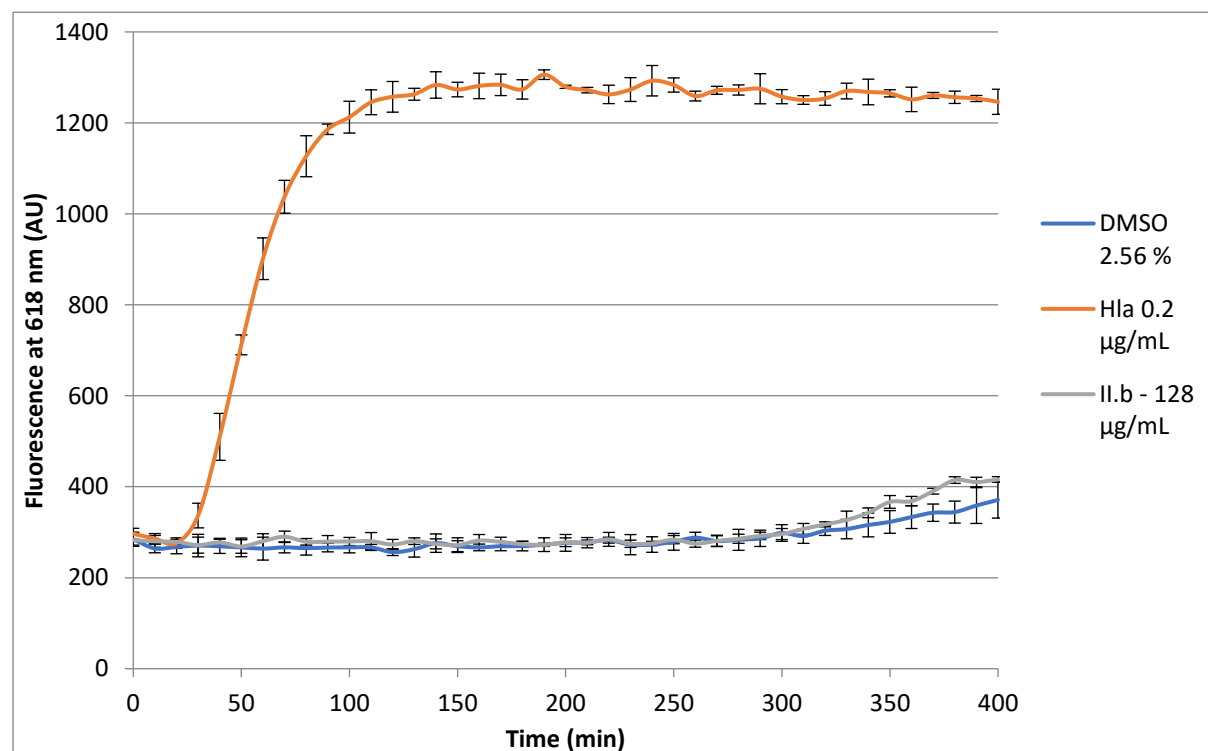


Figure 4 : Effect of **II.b** on the growth of A549 cells

Conclusion

In this study, benzo[*b*]thiophene-based acylhydrazones are shown to be interesting compounds acting as anti-staphylococcal agents. More precisely, (*E*)-6-chloro-*N'*-(pyridin-2-ylmethylene)benzo[*b*]thiophene-2-carbohydrazide **II.b** showed an equal minimum inhibitory concentration of 4 µg/mL for the three strains of *S. aureus*, including two clinical isolated strains of drug resistant *S. aureus*. Moreover, **II.b** showing no cytotoxicity on A549 cells, this suggests that the chloro-pyridinyl benzothiophene acylhydrazone structure is pertinent for future work on chemical diversification and the understanding of the mode of action.

Experimental

All commercial materials were used as received without further purification. Flash chromatography was carried out using Macherey-Nagel Kieselgel 60 M silica. Analytical thin layer chromatography was realized using aluminum-backed plates coated with Macherey-Nagel Kieselgel 60 XtraSIL G/UV254 and were visualized under UV light (at 254 nm or 365 nm) or stained using ninhydrin. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVL300 or a Bruker AV400 or a Bruker AV500 spectrometer, operating respectively at 300 MHz, 400 MHz and 500 MHz for the proton (¹H) NMR. Carbon (¹³C) NMR spectra were recorded on a Bruker AVL300 or a Bruker AV400 or a Bruker AV500 spectrophotometer, operating respectively at 75 MHz, 100 MHz and 101 MHz. Chemical shifts were reported in parts per million (ppm) in the scale relative to residual solvent signals. Multiplicities are abbreviated as: s, singlet; d, doublet; t, triplet; q, quadruplets; dd, doublet of doublets; dt, doublet of triplets; td, triplet of doublets; ddd, doublet of doublets; m, multiplet. Coupling constants are measured in Hertz (Hz). High-resolution mass spectra (HRMS) and low-resolution mass spectra were performed by the Centre Commun de Spectrométrie de Masse (CCSM), University of Lyon 1, Lyon, France. LogP calculations were performed using Molinspiration Cheminformatics free web services, <https://www.molinspiration.com>, Slovensky Grob, Slovakia.

General procedure for ethyl 6-halogenobenzo[*b*]thiophene-2-carboxylate (4) (adapted from Fedi *et al.* 2007 [17])

Under a dried and inert atmosphere (N₂), a solution of 4-chloro-2-fluorobenzaldehyde (14.9 mmol, 1 eq.), ethyl thioglycolate (16.5 mmol, 1.1 eq.) and triethylamine (45 mmol, 3 eq.) in anhydrous DMSO (20 mL) is stirred at 80 °C for 2 h and at room temperature overnight. The mix is then poured into 800 mL of ice/water and stirred vigorously. After 1 h of digestion the formed solid was filtered, washed with water, and dried by suction.

Ethyl 6-chlorobenzo[*b*]thiophene-2-carboxylate (4a)

Yellow crystals (3.446 g, 96% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.25 (d, *J* = 2.0 Hz, 1H), 8.21 (s, 1H), 8.04 (d, *J* = 8.6 Hz, 1H), 7.52 (dd, *J* = 8.6, 2.0 Hz, 1H), 4.35 (q, *J* = 7.1 Hz, 2H), 1.33 (t, *J* = 7.1 Hz, 3H). (Spectrum in accordance with patent WO2018/122232 [19])

Ethyl 6-fluorobenzo[*b*]thiophene-2-carboxylate (4b)

No crystallisation occurred in water, and the residue was purified by chromatography (eluent: pentane / Et₂O 10:1). White powder (710 mg, 21% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.20 (s, 1H), 8.07 (dd, *J* = 9.1, 5.4 Hz, 1H), 7.99 (dd, *J* = 9.1, 2.4 Hz, 1H), 7.37 (td, *J* = 9.1, 2.4 Hz, 1H), 4.35 (q, *J* = 7.1 Hz, 2H), 1.33 (t, *J* = 7.1 Hz, 3H).

(Spectrum in accordance with Cheng *et al.* [20])

Procedure for ethyl 6-(trifluoromethyl)benzo[*b*]thiophene-2-carboxylate (4c) (adapted from patent WO2018/122232 [19])

Under a dried and inert atmosphere (N₂), a solution of 2-fluoro-4-(trifluoromethyl)benzaldehyde (5.20 mmol, 1 eq.), ethyl thioglycolate (6.20 mmol, 1.2 eq.) and K₂CO₃ (5.70 mmol, 1.1 eq.) in anhydrous DMF (10 mL) was stirred at 60 °C for 2 h. The mix was then diluted with water (20 mL), extracted with Et₂O, dried over Na₂SO₄ and concentrated under vacuum. The residue was then recrystallized in MeOH and filtered.

Ethyl 6-(trifluoromethyl)benzo[*b*]thiophene-2-carboxylate (4c)

White powder (814 mg, 57% yield). ¹H NMR (300 MHz, Chloroform-*d*) δ 8.16 (q, *J* = 0.8 Hz, 1H), 8.10 (d, *J* = 0.8 Hz, 1H), 7.98 (dt, *J* = 8.4, 0.8 Hz, 1H), 7.63 (dd, *J* = 8.4, 1.6 Hz, 1H), 4.43 (q, *J* = 7.1 Hz, 2H), 1.43 (t, *J* = 7.1 Hz, 3H).

(Spectrum in accordance with Tan *et al.* [21])

Procedure for ethyl 6-chlorobenzofuran-2-carboxylate (4d) [adapted from patent US2015315198 [18]]

A solution of 4-chlorosalicylaldehyde (6.39 mmol, 1 eq.), ethyl bromomalonate (13.03 mmol, 2.04 eq.) and K₂CO₃ (19.74 mmol, 3.09 eq.) in 2-butanone (6 mL) was stirred at 90 °C for 7 h then at room temperature for 18 h. The mixture was then diluted with EtOAc, neutralized with HCl 1 N and extracted. The organic phase was washed with saturated NaHCO₃ and brine, dried over Na₂SO₄ and concentrated under vacuum. The residue was then purified by chromatography (eluent: pentane / Et₂O 9:1).

Ethyl 6-chlorobenzofuran-2-carboxylate (4d)

White powder (611 mg, 43% yield). ¹H NMR (300 MHz, Chloroform-*d*) δ 7.62 – 7.57 (m, 2H), 7.49 (d, *J* = 1.3 Hz, 1H), 7.29 (dd, *J* = 8.5, 1.3 Hz, 1H), 4.44 (q, *J* = 7.1 Hz, 2H), 1.43 (t, *J* = 7.1 Hz, 3H).

(Spectrum in accordance with Chen *et al.* 2017 [22])

General procedure for benzo[*b*]thiophene-2-carboxylic acids (1) (adapted from patent WO2018/122232 [19])

To a solution of ethyl 6-chlorobenzo[*b*]thiophene-2-carboxylate (14.1 mmol, 1 eq.) in EtOH (15 mL) was added a solution of NaOH 3N (28.2 mmol, 2 eq.). The solution was stirred at room temperature overnight, concentrated under vacuum, diluted with H₂O (75 mL), acidified with HCl 1 N, extracted with EtOAc, dried over Na₂SO₄ and concentrated under vacuum.

6-chlorobenzo[*b*]thiophene-2-carboxylic acid (1b)

White powder (2.616 g, 87% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.57 (bs, 1H), 8.23 (d, *J* = 2.0 Hz, 1H), 8.12 (s, 1H), 8.02 (d, *J* = 8.6 Hz, 1H), 7.50 (dd, *J* = 8.6, 2.0 Hz, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 163.48, 142.6, 137.59, 135.82, 132.07, 129.89, 127.20, 125.84, 122.60.

6-fluorobenzo[*b*]thiophene-2-carboxylic acid (1c)

Yellowish powder (295 mg, 75% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.46 (s, 1H), 8.12 (d, *J* = 0.8 Hz, 1H), 8.05 (dd, *J* = 9.0, 5.4 Hz, 1H), 7.98 (dd, *J* = 9.4, 2.6 Hz, 1H), 7.35 (td, *J* = 9.0, 2.6 Hz, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 163.48 161.43 (d, *J* = 245.2 Hz), 142.76 (d, *J* = 11.3 Hz), 135.75, 134.96 (d, *J* = 3.9 Hz), 129.99, 127.70 (d, *J* = 9.6 Hz), 114.48 (d, *J* = 24.8 Hz), 109.13 (d, *J* = 26.2 Hz).

(Spectrum in accordance with Cai *et al.* [23])

6-(trifluoromethyl)benzo[*b*]thiophene-2-carboxylic acid (1d)

White powder (400 mg, 89% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.59 (q, *J* = 0.8 Hz, 1H), 8.10 (d, *J* = 0.8 Hz, 1H), 7.98 (dt, *J* = 8.5, 0.8 Hz, 1H), 7.75 (dd, *J* = 8.5, 1.3 Hz, 1H).

(Spectrum in accordance with Tan *et al.* [21])

6-chlorobenzofuran-2-carboxylic acid (1e)

White powder (378 mg, 86% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.90 (d, *J* = 1.9 Hz, 1H), 7.80 (d, *J* = 8.4 Hz, 1H), 7.68 (d, *J* = 1.0 Hz, 1H), 7.40 (dd, *J* = 8.4, 1.9 Hz, 1H).

(Spectrum in accordance with Gensini *et al.* [24])

General procedure for *tert*-butyl 2-(benzo[*b*]thiophene-2-carbonyl)hydrazine-1-carboxylates (2)

Under a dried and inert atmosphere (N₂), a solution of benzothiophene-2-carboxylic acid (11 mmol, 1.1 eq.) and *tert*-butyl carbazate (10 mmol, 1.0 eq.) in anhydrous DCM (40 mL) was cooled to 0 °C. Then, DMAP (1.3 mmol, 0.13 eq.) was added to the mixture. DCC (12 mmol, 1.2 eq.) in anhydrous DCM (10 mL) was added dropwise. The mixture was then stirred at room temperature for 24 h and then filtered on Celite® and washed with DCM. The filtrate was then concentrated, and the viscous yellow oil was purified by chromatography (eluent: pentane / Et₂O, 1:1).

***tert*-butyl 2-(benzo[*b*]thiophene-2-carbonyl)hydrazine-1-carboxylate (2a)**

White powder (2.630 g, 88% yield). ¹H NMR (300 MHz, Chloroform-*d*) δ 8.77 (sz, 1H), 7.83 (s, 1H), 7.75 (t, *J* = 6.9 Hz, 2H), 7.47 – 7.31 (m, 2H), 6.82 (s, 1H), 1.50 (s, 9H); ¹³C NMR (75 MHz, Chloroform-*d*) δ 162.4, 156.2, 141.1, 138.9, 135.3, 126.6, 126.5, 125.4, 124.8, 122.4, 82.3, 28.1.

***tert*-butyl 2-(6-chlorobenzo[*b*]thiophene-2-carbonyl)hydrazine-1-carboxylate (2b)**

Acid **1b** was used. White powder (1.435 g, 79% yield). ¹H NMR (300 MHz, Chloroform-*d*) δ 8.73 (s, 1H), 7.75 (s, 1H), 7.71 (s, 1H), 7.67 (d, *J* = 8.6 Hz, 1H), 7.33 (dd, *J* = 8.6, 1.9 Hz, 1H), 6.72 (s, 1H), 1.51 (s, 9H); ¹³C NMR (101 MHz, Acetone-*d*₆) δ 162.42, 156.46, 142.96, 139.20, 138.93, 132.97, 127.41, 126.55, 125.83, 123.03, 80.78, 28.39.

***tert*-butyl 2-(6-fluorobenzo[*b*]thiophene-2-carbonyl)hydrazine-1-carboxylate (2c)**

Acid **1c** was used. White powder (305 mg, 42% yield). ¹H NMR (300 MHz, Chloroform-*d*) δ 8.41 (s, 1H), 7.79 (s, 1H), 7.75 (dd, *J* = 8.9, 5.2 Hz, 1H), 7.45 (d, *J* = 8.9 Hz, 1H), 7.14 (td, *J* = 8.9, 2.4 Hz, 1H), 6.67 (s, 1H), 1.51 (s, 9H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 162.19, 160.52 (d, *J* = 153.3 Hz), 155.39, 141.54 (d, *J* = 10.8 Hz), 137.52 (d, *J* = 3.0 Hz), 135.91, 127.24 (d, *J* = 9.4 Hz), 125.14, 114.27 (d, *J* = 24.4 Hz), 108.98 (d, *J* = 26.2 Hz), 79.49, 28.08.

***tert*-butyl 2-(6-(trifluoromethyl)benzo[*b*]thiophene-2-carbonyl)hydrazine-1-carboxylate (**2d**)**

Acid **1d** was used. White powder (213 mg, 64% yield). ¹H NMR (300 MHz, Chloroform-*d*) δ 9.11 (s, 1H), 7.94 (s, 1H), 7.82 (d, *J* = 8.7 Hz, 1H), 7.80 (s, 1H), 7.56 (dd, *J* = 8.7, 1.7 Hz, 1H), 6.76 (s, 1H), 1.53 (s, 9H); ¹³C NMR (75 MHz, Acetone-*d*₆) δ 162.23, 156.45, 142.86, 142.07, 141.57, 128.48 (q, *J* = 32.3 Hz), 127.07, 125.77, 125.4 (q, *J* = 271.5 Hz), 122.16 (q, *J* = 3.5 Hz), 121.22 (q, *J* = 4.5 Hz), 80.88, 28.40.

***tert*-butyl 2-(6-chlorobenzofuran-2-carbonyl)hydrazine-1-carboxylate (**2e**)**

Acid **1e** was used. White powder (281 mg, 78% yield). ¹H NMR (300 MHz, Acetone-*d*₆) δ 9.74 (s, 1H), 8.17 (s, 1H), 7.77 (d, *J* = 8.4 Hz, 1H), 7.65 – 7.53 (m, 2H), 7.35 (dd, *J* = 8.4, 1.8 Hz, 1H), 1.45 (s, 9H); ¹³C NMR (75 MHz, Acetone-*d*₆) δ 158.77, 156.30, 155.75, 149.81, 133.17, 127.08, 125.38, 124.68, 112.96, 111.27, 80.77, 28.39.

General procedure for N-acylhydrazones derivatives (I, II and III)

Step 1: A solution of *tert*-butyl 2-(benzo[*b*]thiophene-2-carbonyl)hydrazine-1-carboxylate (1 mmol, 1 eq.) and TFA (20 mmol, 20 eq.) in anhydrous DCM (3 mL) is stirred at room temperature for 18 h. The mixture was co-evaporated with toluene to afford a white solid which was engaged as crude in the next step.

Step 2: The crude material was diluted in MeOH (10 mL) before the addition of the corresponding substituted benzaldehyde (2 mmol, 2 eq.) at room temperature. Reflux for 2 h was done, and the final compound crystallized from the reaction mixture. Then, the reaction mixture was cooled to 0 °C, the solid was filtered off and washed with cold MeOH.

(*E*)-N'-(benzo[*d*][1,3]dioxol-5-ylmethylene)benzo[*b*]thiophene-2-carbohydrazide (I.a**)**

Light-yellow solid (127 mg, 52% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.05 (s, 0.5H), 11.94 (s, 0.5H), 8.40 (d, *J* = 7.6 Hz, 1H), 8.23 (s, 0.5H), 8.16 – 7.97 (m, 2.5H), 7.69 – 7.37 (m, 2.5H), 7.32 (s, 0.5H), 7.28 – 7.18 (m, 1H), 7.03 (t, *J* = 7.1 Hz, 1H), 6.13 (s, 1H), 6.11 (s, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 161.9, 158.5, 149.7, 149.6, 148.5, 148.4, 145.0, 143.5, 140.8, 139.5, 138.9, 137.7, 133.7, 132.3, 129.0, 128.8, 127.2, 127.0, 126.0, 125.8, 125.5, 125.2, 124.2, 124.0, 123.3, 123.1, 109.1, 109.0, 106.0, 105.7, 102.1; HRMS (ESI) *m/z*: calcd. for C₁₇H₁₃N₂O₃S [M+H]⁺ 325.0641, found 325.0641.

(*E*)-N'-(4-(dimethylamino)benzylidene)benzo[*b*]thiophene-2-carbohydrazide (I.b**)**

Yellow solid (99 mg, 45% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.85 (s, 0.5H), 11.78 (s, 0.5H), 8.40 (s, 0.5H), 8.32 (s, 0.5H), 8.20 (s, 0.5H), 8.13 – 7.94 (m, 2.5H), 7.68 (d, *J* = 8.7 Hz, 1H), 7.57 (d, *J* = 8.7 Hz, 1H), 7.52 – 7.41 (m, 2H), 6.82 (d, *J* = 8.7 Hz, 1H), 6.77 (d, *J* = 8.7 Hz, 1H), 3.00 (s, 3H), 2.99 (s, 3H); MS (ESI) *m/z* = 324.1 [M+H]⁺, 346.1 [M+Na]⁺.

(*E*)-N'-(pyridin-2-ylmethylene)benzo[*b*]thiophene-2-carbohydrazide (I.c**)**

Intermediate **2a** (938 mg) was deprotected under the reported acidic conditions (TFA) to afford, after an aqueous workup with NaHCO₃ and extractions with EtOAc, a white solid (615 mg) which was directly engaged in the presence of 2-pyridinecarboxaldehyde (6.40 mmol, 2.0 eq.) in MeOH (20 mL). After 2 hours reflux, the reaction mixture was concentrated *in vacuo* to give a brown viscous oil which was purified by recrystallization in EtOAc to give product. White powder (292 mg, 32% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.34 (s, 1H, 0.5H), 12.26 (s, 0.5H), 8.65 (d, *J* = 4.5 Hz, 1H), 8.49 (d, *J* = 10.6 Hz, 1H), 8.29 (s, 0.5H), 8.23 (s, 1H), 8.16 – 7.85 (m, 3.5H), 7.61 – 7.37 (m, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 162.3, 158.9, 153.5, 153.4, 150.1, 148.7, 145.6, 143.4, 140.9, 139.5, 138.4, 137.8, 137.6, 137.4, 133.5, 132.6, 127.3, 127.2, 126.6, 126.1, 126.0, 125.6, 125.3, 125.0, 123.3, 123.1, 120.8, 120.5; HRMS (ESI) *m/z*: calcd. for C₁₅H₁₂N₃OS [M+H]⁺ 282.0696, found 282.0682.

(*E*)-N'-(pyridin-3-ylmethylene)benzo[*b*]thiophene-2-carbohydrazide (I.d)

White powder (129 mg, 67% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.32 (s, 0.5H), 12.21 (s, 0.5H), 8.99 (s, 0.5H), 8.89 (s, 0.5H), 8.74 – 8.60 (m, 1H), 8.52 (s, 0.5H), 8.44 (s, 0.5H), 8.27 (s, 1H), 8.24 – 8.14 (m, 1H), 8.13 – 7.98 (m, 2H), 7.59 – 7.39 (m, 3H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 161.86, 161.44, 158.42, 150.89, 150.69, 148.91, 145.43, 143.10, 142.06, 140.49, 140.39, 139.08, 138.09, 137.36, 137.10, 134.08, 133.62, 132.15, 130.14, 130.06, 126.87, 126.73, 126.11, 126.03, 125.69, 125.55, 125.20, 124.90, 124.11, 122.92, 122.67; HRMS (ESI) *m/z*: calcd. for C₁₅H₁₂N₃OS [M+H]⁺ 282.0696, found 282.0694.

(*E*)-N'-(pyridin-4-ylmethylene)benzo[*b*]thiophene-2-carbohydrazide (I.e)

Crystallized very slowly in the reaction mixture after the reflux. The mixture was concentrated and then engaged in recrystallization with MeOH and EtOAc. Light-yellow powder (92 mg, 30% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.41 (s, 0.5H), 12.32 (s, 0.5H), 8.70 (s, 2H), 8.46 (s, 1H), 8.29 (s, 0.5H), 8.20 – 7.98 (m, 2.5H), 7.80 (s, 1H), 7.71 (s, 1H), 7.58 – 7.43 (m, 2H); MS (ESI) *m/z* = 282.0 [M+H]⁺, 304.0 [M+Na]⁺.

(*E*)-N'-(3-hydroxybenzylidene)benzo[*b*]thiophene-2-carbohydrazide (I.f)

Did not crystallize in the reaction mixture after the reflux. The mixture was concentrated and then engaged in recrystallization with MeOH and EtOAc (8:2). Light-brown powder (47 mg, 27% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.10 (s, 0.5H), 11.99 (s, 0.5H), 9.75 (s, 0.5H), 9.67 (s, 0.5H), 8.41 (d, *J* = 11.2 Hz, 1H), 8.25 (s, 0.5H), 8.14 – 7.95 (m, 2.5H), 7.57 – 7.44 (m, 2H), 7.38 – 7.21 (m, 2.5H), 7.14 (d, *J* = 7.7 Hz, 0.5H), 6.88 (s, 1H); MS (ESI) *m/z* = 297.1 [M+H]⁺, 319.0 [M+Na]⁺.

(*E*)-N'-(4-hydroxybenzylidene)benzo[*b*]thiophene-2-carbohydrazide (I.g)

White powder (32 mg, 10% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.95 (s, 0.5H), 11.85 (s, 0.5H), 10.00 (s, 0.5H), 9.97 (s, 0.5H), 8.41 (s, 0.5H), 8.36 (s, 0.5H), 8.22 (s, 0.5H), 8.11 – 7.96 (m, 2.5H), 7.69 (d, *J* = 8.3 Hz, 1H), 7.59 (d, *J* = 8.3 Hz, 1H), 7.55 – 7.39 (m, 2H), 6.90 (d, *J* = 8.3 Hz, 1H), 6.85 (d, *J* = 8.3 Hz, 1H); MS (ESI) *m/z* = 297.1 [M+H]⁺, 319.0 [M+Na]⁺.

(*E*)-N'-(4-hydroxy-3-methoxybenzylidene)benzo[*b*]thiophene-2-carbohydrazide (I.h)

White powder (141 mg, 41% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.97 (s, 0.5H), 11.93 (s, 0.5H), 9.62 (s, 0.5H), 9.60 (s, 0.5H), 8.42 (s, 0.5H), 8.36 (s, 0.5H), 8.22 (s, 0.5H), 8.10 – 7.96 (m, 2.5H), 7.58 – 7.41 (m, 2.5H), 7.33 (s, 0.5H), 7.20 (d, *J* = 8.2 Hz, 0.5H), 7.13 (d, *J* = 8.2 Hz, 0.5H), 6.87 (t, *J* = 7.2 Hz, 1H), 3.92 (s, 1.5H), 3.84 (s, 1.5H); MS (ESI) *m/z* = 327.1 [M+H]⁺, 349.0 [M+Na]⁺.

(E)-N'-(4-chlorobenzylidene)benzo[*b*]thiophene-2-carbohydrazide (I.i)

White solid (216 mg, 62% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.22 (s, 0.5H), 12.10 (s, 0.5H), 8.44 (d, *J* = 11.4 Hz, 1H), 8.26 (s, 0.5H), 8.16 (s, 0.5H), 8.10 – 7.97 (m, 2H), 7.88 (d, *J* = 8.0 Hz, 1H), 7.79 (d, *J* = 8.0 Hz, 1H), 7.63 – 7.39 (m, 4H); MS (ESI) *m/z* = 315.0 [M+H]⁺, 337.0 [M+Na]⁺.

(E)-N'-(4-fluorobenzylidene)benzo[*b*]thiophene-2-carbohydrazide (I.j)

White solid (173 mg, 44% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.17 (s, 0.5H), 12.06 (s, 0.5H), 8.45 (d, *J* = 14.0 Hz, 1H), 8.25 (s, 0.5H), 8.17 (s, 0.5H), 8.13 – 7.99 (m, 2H), 7.92 (t, *J* = 6.8 Hz, 1H), 7.83 (t, *J* = 6.8 Hz, 1H), 7.56 – 7.42 (m, 2H), 7.40 – 7.27 (m, 2H); MS (ESI) *m/z* = 299.1 [M+H]⁺, 321.0 [M+Na]⁺.

(E)-N'-(3-nitrobenzylidene)benzo[*b*]thiophene-2-carbohydrazide (I.k)

Yellowish solid (111 mg, 36% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.41 (s, 0.5H), 12.30 (s, 0.5H), 8.68 (s, 0.5H), 8.58 (s, 1H), 8.44 (s, 0.5H), 8.36 – 8.16 (m, 3H), 8.05 (s, 2H), 7.87 – 7.68 (m, 1H), 7.58 – 7.41 (m, 2H); MS (ESI) *m/z* = 326.1 [M+H]⁺, 348.0 [M+Na]⁺.

(E)-N'-(4-nitrobenzylidene)benzo[*b*]thiophene-2-carbohydrazide (I.l)

Yellowish solid (110 mg, 30% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.43 (s, 0.5H), 12.35 (s, 0.5H), 8.56 (s, 0.5H), 8.44 (s, 0.5H), 8.33 (d, *J* = 8.4 Hz, 3H), 8.18 – 7.94 (m, 4H), 7.59 – 7.39 (m, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 161.98, 158.47, 147.90, 145.52, 142.41, 140.44, 138.97, 137.85, 137.34, 132.22, 128.35, 128.14, 126.25, 125.58, 125.18, 124.91, 124.12, 122.89, 122.71; HRMS (ESI) *m/z*: calcd. for C₁₆H₁₀N₃O₃S [M-H]⁻ 324.0448, found 324.0449.

(E)-2-((2-(benzo[*b*]thiophene-2-carbonyl)hydrazono)methyl)benzoic acid (I.m)

White solid (88 mg, 45% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.40 (s, 1H), 12.37 (s, 0.5H), 12.18 (s, 0.5H), 9.23 (s, 0.5H), 8.94 (s, 0.5H), 8.42 (s, 0.5H), 8.32 (s, 0.5H), 8.28 – 7.97 (m, 3H), 7.93 (dd, *J* = 7.7, 1.4 Hz, 1H), 7.81 – 7.61 (m, 1H), 7.61 – 7.42 (m, 3H); MS (ESI) *m/z* = 325.1 [M+H]⁺, 347.0 [M+Na]⁺.

(E)-3-((2-(benzo[*b*]thiophene-2-carbonyl)hydrazineylidene)methyl)benzoic acid (I.n)

White powder (188 mg, 85% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.29 (s, 0.5H), 12.16 (s, 0.5H), 8.52 (s, 0.5H), 8.49 – 8.30 (m, 1.5H), 8.32 – 7.91 (m, 5H), 7.71 – 7.57 (m, 1H), 7.57 – 7.41 (m, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 166.97, 161.82, 161.41, 158.41, 147.15, 143.87, 143.00, 140.46, 139.08, 138.17, 137.40, 134.68, 132.01, 131.65, 131.51, 131.11, 130.78, 129.36, 128.54, 127.54, 126.91, 126.72, 125.97, 125.56, 125.20, 124.96, 122.93, 122.49; HRMS (ESI) *m/z*: calcd. for C₁₇H₁₃N₂O₃S [M+H]⁺ 325.0641, found 325.0626

(E)-4-((2-(benzo[*b*]thiophene-2-carbonyl)hydrazono)methyl)benzoic acid (I.o)

White solid (92 mg, 39% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.14 (s, 1H), 12.30 (s, 0.5H), 12.19 (s, 0.5H), 8.53 (s, 0.5H), 8.44 (s, 0.5H), 8.28 (s, 0.5H), 8.23 (s, 0.5H), 8.13 – 7.81 (m, 6H), 7.57 – 7.40 (m, 2H); MS (ESI) *m/z* = 325.1 [M+H]⁺, 347.0 [M+Na]⁺.

(E)-N'-(benzo[*d*][1,3]dioxol-5-ylmethylene)-6-chlorobenzo[*b*]thiophene-2-carbohydrazide (II.a)

Intermediate **2b** was deprotected under the reported acidic conditions (TFA) to afford a white solid which was engaged without further purification for the next step. White powder (75 mg, 31% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.08 (s, 0.5H), 11.97 (s, 0.5H), 8.39 (d, *J* = 8.3 Hz, 1H), 8.33 – 7.97 (m, 3H), 7.48 (d, *J* = 9.3

Hz, 1.5H), 7.31 (s, 0.5H), 7.23 (t, $J = 9.9$ Hz, 1H), 7.10 – 6.97 (m, 1H), 6.13 (s, 1H), 6.11 (s, 1H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 161.18, 157.85, 149.26, 148.16, 144.90, 144.40, 141.58, 139.44, 137.86, 136.02, 134.19, 131.37, 128.52, 128.28, 127.01, 126.83, 125.77, 125.48, 125.07, 123.96, 123.67, 122.48, 122.24, 108.65, 105.64, 105.27, 101.68; HRMS (ESI) m/z : calcd. for $\text{C}_{17}\text{H}_{12}\text{ClN}_2\text{O}_3\text{S}$ $[\text{M}+\text{H}]^+$ 359.0252, found 359.0254.

(E)-6-chloro-N'-(pyridin-2-ylmethylene)benzo[*b*]thiophene-2-carbohydrazide (II.b)

Intermediate **2b** was deprotected under the reported acidic conditions (TFA) to afford, after an aqueous workup with NaHCO_3 and extractions with EtOAc, a white solid which was engaged without further purification for the next step. White solid (52 mg, 30% yield). ^1H NMR (300 MHz, DMSO- d_6) δ 12.08 (s, 0.5H), 11.97 (s, 0.5H), 8.39 (d, $J = 8.3$ Hz, 1H), 8.33 – 7.97 (m, 3H), 7.48 (d, $J = 9.3$ Hz, 1.5H), 7.31 (s, 0.5H), 7.23 (t, $J = 9.9$ Hz, 1H), 7.10 – 6.97 (m, 1H), 6.13 (s, 1H), 6.11 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6) δ 161.52, 158.24, 153.04, 152.82, 149.69, 148.52, 145.41, 144.33, 141.70, 138.90, 137.81, 137.07, 136.13, 133.96, 131.69, 127.20, 126.99, 125.88, 125.68, 125.58, 124.63, 122.54, 122.24, 120.51, 120.16; HRMS (ESI) m/z : calcd. for $\text{C}_{15}\text{H}_{11}\text{ClN}_3\text{OS}$ $[\text{M}+\text{H}]^+$ 316.0306, found 316.0315

(E)-N'-(benzo[*d*][1,3]dioxol-5-ylmethylene)-6-fluorobenzo[*b*]thiophene-2-carbohydrazide (II.c)

Intermediate **2c** was deprotected under the reported acidic conditions (TFA) to afford a white solid which was engaged without further purification for the next step. White powder (44 mg, 40% yield). ^1H NMR (300 MHz, DMSO- d_6) δ 12.05 (s, 0.5H), 11.93 (s, 0.5H), 8.38 (d, $J = 8.7$ Hz, 1H), 8.21 (s, 0.5H), 8.16 – 7.89 (m, 2.5H), 7.46 (s, 0.5H), 7.41 – 7.28 (m, 1.5H), 7.23 (t, $J = 8.9$ Hz, 1H), 7.11 – 6.92 (m, 1H), 6.13 (s, 1H), 6.11 (s, 1H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 157.89, 149.33, 149.24, 148.14, 148.08, 148.06, 148.01, 144.80, 138.58, 138.55, 135.97, 134.17, 133.40, 131.45, 128.56, 128.31, 127.48, 125.09, 123.92, 123.62, 114.20, 109.15, 108.73, 108.63, 108.56, 108.49, 105.57, 105.26, 101.67; HRMS (ESI) m/z : calcd. for $\text{C}_{17}\text{H}_{12}\text{FN}_2\text{O}_3\text{S}$ $[\text{M}+\text{H}]^+$ 343.0547, found 343.0550

(E)-6-fluoro-N'-(pyridin-2-ylmethylene)benzo[*b*]thiophene-2-carbohydrazide (II.d)

Intermediate **2c** was deprotected under the reported acidic conditions (TFA) to afford, after an aqueous workup with NaHCO_3 and extractions with EtOAc, a white solid which was engaged without further purification for the next step. White solid (23 mg, 24% yield). ^1H NMR (300 MHz, DMSO- d_6) δ 12.31 (s, 1H), 8.64 (s, 0.5H), 8.54 – 7.71 (m, 6.5H), 7.59 – 7.14 (m, 2H); ^{13}C NMR (126 MHz, DMSO- d_6) δ 162.02, 160.08, 158.80, 150.13, 145.75, 144.71, 144.62, 141.43, 141.34, 140.47, 137.50, 137.04, 136.76, 136.33, 135.04, 132.22, 129.70, 127.83, 127.28, 127.20, 127.15, 127.07, 126.16, 125.03, 123.37, 120.85, 120.60, 118.72, 114.78, 114.43, 114.24, 114.01, 113.82, 109.37, 109.16, 108.79, 108.59; HRMS (ESI) m/z : calcd. for $\text{C}_{15}\text{H}_{11}\text{FN}_3\text{OS}$ $[\text{M}+\text{H}]^+$ 300.0601, found 300.0603.

(E)-N'-((1H-imidazol-4-yl)methylene)-6-chlorobenzo[*b*]thiophene-2-carbohydrazide (III.a)

Intermediate **2b** was deprotected under the reported acidic conditions (TFA) to afford, after an aqueous workup with NaHCO_3 and extractions with EtOAc, a white solid which was engaged without further purification for the next step. Beige powder (90 mg, 48% yield). ^1H NMR (300 MHz, DMSO- d_6) δ 12.92 (s, 0.5H), 12.52 (s, 0.5H), 11.92 (s, 0.5H), 11.85 (s, 0.5H), 8.53 – 8.35 (m, 1H), 8.31 – 8.13 (m, 1.5H), 8.13 – 7.96 (m, 1.5H), 7.90 – 7.67 (m, 1.5H), 7.67 – 7.40 (m, 1.5H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 157.66, 144.16, 141.51, 139.58, 137.86, 136.71, 131.38, 130.92, 126.77, 125.75, 125.34, 124.90, 122.48, 122.08; MS (ESI) $m/z = 305.1$ $[\text{M}+\text{H}]^+$, 327.0 $[\text{M}+\text{Na}]^+$.

(E)-6-chloro-N'-(furan-2-ylmethylene)benzo[b]thiophene-2-carbohydrazide (III.b)

Intermediate **2b** was deprotected under the reported acidic conditions (TFA) to afford a white solid which was engaged without further purification for the next step. Beige powder (145 mg, 78% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.12 (s, 0.5H), 12.04 (s, 0.5H), 8.43 (s, 0.5H), 8.34 (s, 0.5H), 8.30 – 8.12 (m, 1.5H), 8.12 – 7.96 (m, 1.5H), 7.88 (s, 1H), 7.57 – 7.41 (m, 1H), 7.06 (s, 0.5H), 6.99 (s, 0.5H), 6.67 (s, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 158.34, 149.66, 145.94, 142.04, 139.68, 138.42, 138.25, 135.33, 134.70, 131.97, 131.65, 127.49, 127.30, 126.24, 125.90, 125.64, 122.96, 122.56, 114.70, 114.23, 112.79; MS (ESI) *m/z* = 305.0 [M+H]⁺, 327.0 [M+Na]⁺.

(E)-6-chloro-N'-((5-(hydroxymethyl)furan-2-yl)methylene)benzo[b]thiophene-2-carbohydrazide (III.c)

Intermediate **2b** was deprotected under the reported acidic conditions (TFA) to afford a white solid which was engaged without further purification for the next step. Beige powder (94 mg, 46% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.09 (s, 0.5H), 12.03 (s, 0.5H), 8.44 (s, 0.5H), 8.36 – 8.15 (m, 2H), 8.12 – 7.94 (m, 1.5H), 7.50 (d, *J* = 8.4 Hz, 1H), 7.15 – 6.85 (m, 1H), 6.47 (s, 1H), 5.44 (t, *J* = 6.3 Hz, 1H), 4.63 – 4.29 (m, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 158.22, 157.89, 148.44, 141.61, 139.28, 137.89, 136.46, 135.18, 134.30, 131.54, 131.16, 127.07, 126.86, 125.82, 125.45, 125.19, 122.51, 122.11, 115.54, 114.25, 109.38, 55.82; MS (ESI) *m/z* = 335.0 [M+H]⁺, 357.0 [M+Na]⁺.

(E)-6-chloro-N'-(pyridin-2-ylmethylene)benzofuran-2-carbohydrazide (III.e)

Intermediate **2e** was deprotected under the reported acidic conditions (TFA) to afford, after an aqueous workup with NaHCO₃ and extractions with EtOAc, a white solid which was engaged without further purification for the next step. White powder (63 mg, 44% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.45 (s, 1H), 8.63 (dd, *J* = 4.8, 1.5 Hz, 1H), 8.54 (s, 1H), 8.03 – 7.73 (m, 5H), 7.49 – 7.41 (m, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 154.59, 153.05, 149.11, 148.57, 137.00, 131.93, 126.04, 124.67, 124.27, 120.17, 112.29, 111.20; HRMS (ESI) *m/z*: calcd. for C₁₅H₁₁ClN₃O₂ [M+H]⁺ 300.0534, found 300.0533.

(E)-N'-(pyridin-2-ylmethylene)-6-(trifluoromethyl)benzo[b]thiophene-2-carbohydrazide (III.f)

Intermediate **2d** was deprotected under the reported acidic conditions (TFA) to afford, after an aqueous workup with NaHCO₃ and extractions with EtOAc, a white solid which was engaged without further purification for the next step. White powder (64 mg, 44% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.42 (s, 1H), 8.70 – 8.59 (m, 2H), 8.52 (d, *J* = 13.4 Hz, 1H), 8.39 – 8.18 (m, 3H), 8.07 – 7.85 (m, 2H), 7.77 (d, *J* = 7.9 Hz, 1H), 7.46 (d, *J* = 6.5 Hz, 1H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 161.37, 158.04, 152.99, 152.78, 149.68, 148.84, 145.64, 142.91, 141.86, 141.72, 140.29, 139.92, 137.13, 137.02, 136.72, 131.50, 126.66, 126.51, 125.55, 124.69, 124.61, 121.45, 121.09, 120.88, 120.83, 120.77, 120.71, 120.68, 120.59, 120.19; HRMS (ESI) *m/z*: calcd. for C₁₆H₁₁F₃N₃OS [M+H]⁺ 350.0569, found 350.0572.

Procedure for N'-(pyridin-2-ylmethyl)benzo[b]thiophene-2-carbohydrazide (I.p) (adapted from Kim *et al.* [25])

Under H₂ atmosphere, a solution of (E)-N'-(pyridin-2-ylmethylene)benzo[b]thiophene-2-carbohydrazide (0.12 mmol, 1 eq.) and palladium on carbon 10% (0.019 mmol, 15 mol%) in 5 mL of EtOAc and 1 mL of

MeOH is stirred at room temperature for 7 h. The mixture is filtered on Celite[®], washed with EtOAc and the residue is concentrated under vacuum and purified by chromatography (eluent: EtOAc / MeOH 98:2).

***N'*-(pyridin-2-ylmethyl)benzo[*b*]thiophene-2-carbohydrazide (I.p)**

White powder (30 mg, 60% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.34 (d, *J* = 5.1 Hz, 1H), 8.63 – 8.40 (m, 1H), 8.04 – 7.98 (m, 2H), 7.95 – 7.88 (m, 1H), 7.78 (td, *J* = 7.7, 1.8 Hz, 1H), 7.54 (d, *J* = 7.7 Hz, 1H), 7.49 – 7.38 (m, 2H), 7.32 – 7.21 (m, 1H), 5.76 (q, *J* = 5.1 Hz, 1H), 4.12 (d, *J* = 5.1 Hz, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 161.02, 158.23, 148.90, 140.04, 139.12, 138.16, 136.59, 126.25, 125.20, 125.00, 124.69, 122.82, 122.36, 122.32, 56.21; HRMS (ESI) *m/z*: calcd. for C₁₅H₁₄N₃OS [M+H]⁺ 284.0852, found 284.0858

Procedure for pyridine 2-aldoxime (6) (adapted from Younus Wani *et al.* 2011 [26])

To a solution of pyridine-2-carboxaldehyde (1.87 mmol, 1 eq.) and hydroxylamine hydrochloride (2.33 mmol, 1.25 eq.) in water (5 mL) was added dropwise a solution of sodium bicarbonate (2.33 mmol, 1.25 eq.) in water (10 mL) and the mixture was stirred for 3 h at room temperature. The solution was then extracted with EtOAc and the organic layer dried over Na₂SO₄ and concentrated under vacuum.

Pyridine 2-aldoxime (6)

White powder (186 mg, 81%). ¹H NMR (300 MHz, Chloroform-*d*) δ 8.99 (s, 1H), 8.63 (ddd, *J* = 4.9, 1.8, 1.0 Hz, 1H), 8.31 (s, 1H), 7.82 (dt, *J* = 8.1, 1.0 Hz, 1H), 7.72 (td, *J* = 8.1, 1.8 Hz, 1H), 7.29 (ddd, *J* = 8.1, 4.9, 1.0 Hz, 1H).
(Spectrum in accordance to Zhang *et al.* [27]).

Procedure for (*E*)-picolinaldehyde O-(6-chlorobenzo[*b*]thiophene-2-carbonyl) oxime (III.d)

Under a dried and inert atmosphere (N₂), a solution of 6-chlorobenzothiophene-2-carboxylic acid (1.06 mmol, 1.3 eq.) and pyridine 2-aldoxime (0.82 mmol, 1.0 eq.) in anhydrous DCM (3 mL) was cooled to 0 °C. Then, DMAP (0.12 mmol, 0.15 eq.) and DCC (1.06 mmol, 1.3 eq.) were gradually added to the mixture. The mixture was then stirred at room temperature overnight and then filtered on Celite[®] and washed with DCM. The filtrate was then concentrated, and the residue was purified by column chromatography (eluent: pentane / EtOAc 2:1).

(*E*)-picolinaldehyde O-(6-chlorobenzo[*b*]thiophene-2-carbonyl) oxime (III.d)

Orange powder (166 mg, 64% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.85 (s, 1H), 8.77 – 8.73 (m, 1H), 8.43 (d, *J* = 0.7 Hz, 1H), 8.33 (dt, *J* = 1.9, 0.7 Hz, 1H), 8.10 (dd, *J* = 8.5, 0.7 Hz, 1H), 8.04 – 7.95 (m, 2H), 7.62 – 7.54 (m, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 159.04, 158.37, 150.12, 148.94, 142.67, 137.31, 137.04, 132.61, 131.55, 131.02, 127.37 (2C), 126.05, 122.57, 122.49; HRMS (ESI) *m/z*: calcd. for C₁₅H₁₀ClN₂O₂S [M+H]⁺ 317.0146, found 317.0136.

Minimum inhibitory concentration (MIC) evaluation

MICs were evaluated in CaMHB (cation-adjusted Mueller-Hinton broth) by the method of microdilution in liquid medium which follows the CLSI recommendations (Clinical and Laboratory Standards Institute (CLSI), methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard seventh edition. Clinical and Laboratory Standards Institute, Wayne, PA). Evaluated compounds were diluted in DMSO with a concentration of 5 mg/mL and then further diluted in CaMHB. The 0.5 MacFarland bacterial suspensions were made from colonies previously grown on blood agar plate (COS, Biomérieux) in a saline solution (0.45% NaCl), they were diluted in CaMHB (1/100) before addition into 96-well microplates. MICs were done in triplicate and determined after 18 h of incubation at 37 °C. The median values were reported.

Cytotoxicity assay

Cytotoxicity was evaluated using a propidium iodide assay. Briefly, human lung adenocarcinomic A549 cells were plated on black flat 96-well plates (Greiner) at a concentration of 0.5×10^6 cells / mL in DMEM GlutaMAX (Gibco) supplemented with 10% fetal bovine serum for 24 h in a humidified atmosphere at 37 °C and 5% CO₂. The medium was then removed and fresh medium with 2.5 µg/mL of propidium iodide and tested compounds (128 µg/mL of II.b and 0.2 µg/mL for recombinant Hla) was added. Absorbance at 618 nm was measured every 10 min over 7 h. Each concentration was made as a triplicate and mean values are displayed with standard deviation.

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Author Contributions

Methodology T.B., A.B., J.M., C.B., F.D., A.B.; Conceptualization Y.Q., O.D., G.L. L.S.; all authors contributed to the writing of the manuscript. All authors have read and agreed to the submitted version of the manuscript.

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Data Availability Statement

Data can be found in the manuscript.

Conflicts of Interest

623 The authors declare no conflict of interest.

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