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 three 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 position 6 of the benzo[b]thiophene nucleus. The screening lead to the identification of eight hits, including (E)-6-chloro-N-(pyridin-2-ylmethylene)benzo[b]thiophene-2-carboxyhydrazide (IIb), a non-cytotoxic derivative showing a minimal inhibitory concentration of 4 µg/mL on three S. aureus strains, among which were a reference classical strain and two clinically isolated strains resistant to methicillin and daptomycin, respectively.

Keywords: benzo thiophene; acylhydrazone; Staphylococcus aureus; antibacterial; MRSA

1. 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] that display a broad range of activity including antimicrobial [6,7], anticanancer [8], anti-diabetic [9], anti-depressant [10], anti-inflammatory and analgesic agents [11,12]. As 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 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 of 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.

2. Materials and Methods

2.1. Chemistry

2.1.1. General Information

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 Bruker AV300, Bruker AV400 or Bruker AV500 spectrometers, operating at 300 MHz, 400 MHz and 500 MHz, respectively, for the proton (¹H) NMR and at 75 MHz, 100 MHz and 125 MHz, respectively, for the carbon (¹³C) NMR. Chemical shifts were reported in parts per million (ppm) on a 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 doublet of doublets; m, multiplet. Coupling constants were measured in Hertz (Hz). Copies of NMR spectra of new products are available in Supplementary Materials (Figure S1).

High-resolution mass spectra (HRMS) and low-resolution mass spectra were obtained 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](https://www.molinspiration.com) (accessed on 13 January 2022), Slovensky Grob, Slovakia.

2.1.2. General Procedure for Ethyl 6-halogenobenzo[b]thiophene-2-carboxylate (4)

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) was stirred at 80 °C for 2 h and at room temperature overnight. The mix was 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. (Adapted from Fedi et al. 2007 [14])

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

Yellow crystals (3.446 g, 96% yield). ¹H NMR (300 MHz, DMSO-d₆) 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 [15]).

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

No crystallization 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-d₆) 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. [16]).

2.1.3. Procedure for Ethyl 6-(trifluoromethyl)benzo[b]thiophene-2-carboxylate (4c)

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. (Adapted from patent WO2018/122232 [15]).
Ethyl 6-(trifluoromethyl)benzo[b]thiophene-2-carboxylate (4c)
White powder (814 mg, 57% yield). \(^1\)H NMR (300 MHz, Chloroform-\(d\)) \(\delta\) 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. [17]).

2.1.4. Procedure for Ethyl 6-chlorobenzofuran-2-carboxylate (4d)
A solution of 4-chlorosalicylaldehyde (6.39 mmol, 1 eq.), ethyl bromomalonate (13.03 mmol, 2.04 eq.) and \(\text{K}_2\text{CO}_3\) (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 NaHCO\(_3\) and brine, dried over \(\text{Na}_2\text{SO}_4\) and concentrated under vacuum. The residue was then purified by chromatography (eluent: pentane/Et\(_2\)O 9:1). (Adapted from Patent US2015315198 [18]).

Ethyl 6-chlorobenzofuran-2-carboxylate (4d)
White powder (611 mg, 43% yield). \(^1\)H NMR (300 MHz, Chloroform-\(d\)) \(\delta\) 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 [19]).

2.1.5. General Procedure for Benzo[b]thiophene-2-carboxylic Acids (1)
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 H\(_2\)O (75 mL), acidified with HCl 1 N, extracted with EtOAc, dried over \(\text{Na}_2\text{SO}_4\) and concentrated under vacuum. (Adapted from Patent WO2018/122232 [15]).

6-Chlorobenzo[b]thiophene-2-carboxylic acid (1b)
White powder (2.616 g, 87% yield). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 13.57 (bs, 1H), 8.23 (d, \(J = 2.0\) Hz, 1H), 8.12 (s, 1H), 8.05 (d, \(J = 8.6\) Hz, 1H), 7.50 (dd, \(J = 8.6, 2.0\) Hz, 1H); \(^{13}\)C NMR (101 MHz, DMSO-\(d_6\)) \(\delta\) 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). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 13.46 (s, 1H), 8.12 (d, \(J = 0.8\) Hz, 1H), 8.05 (d, \(J = 5.4\) Hz, 1H), 7.98 (dd, \(J = 9.4, 2.6\) Hz, 1H), 7.35 (td, \(J = 9.0, 2.6\) Hz, 1H); \(^{13}\)C NMR (101 MHz, DMSO-\(d_6\)) \(\delta\) 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. [20]).

6-(Trifluoromethyl)benzo[b]thiophene-2-carboxylic acid (1d)
White powder (400 mg, 89% yield). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 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. [17]).

6-Chlorobenzofuran-2-carboxylic acid (1e)
White powder (378 mg, 86% yield). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 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. [21]).

2.1.6. General Procedure for Tert-butyl 2-(benzo[b]thiophene-2-carboxyl)hydrazine-1-carboxylates (2)
Under a dried and inert atmosphere (N\(_2\)), 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, filtered on Celite® and washed with DCM. The filtrate was then concentrated, and the viscous yellow oil was purified by chromatography (eluent: pentane/EtO, 1:1).

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

White powder (2.630 g, 88% yield). $^1$H NMR (300 MHz, Chloroform-d) δ 8.77 (s, 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); $^{13}$C NMR (75 MHz, Chloroform-d) δ 162.4, 156.2, 141.1, 138.9, 135.3, 126.6, 126.5, 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). $^1$H 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); $^{13}$C 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). $^1$H 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); $^{13}$C 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, 115.44 (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). $^1$H 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); $^{13}$C 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). $^1$H NMR (300 MHz, Acetone-d$_6$) δ 9.74 (s, 1H), 8.17 (s, 1H), 7.77 (d, J = 8.4 Hz, 1H), 7.60 (dd, J = 8.7, 1.7 Hz, 1H), 6.76 (s, 1H), 1.53 (s, 9H); $^{13}$C 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.

2.1.7. 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) was stirred at room temperature for 18 h. The mixture was co-evaporated with toluene to produce a white solid, which was engaged as crude material 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 performed, and the final compound crystallized from the reaction mixture. Then, the reaction mixture was cooled to 0 °C, and 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). $^1$H 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); $^{13}$C 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: calcld. for C_{17}H_{13}N_{2}O_{5}S [M + H]^{+} 325.0641, found 325.0641.

(E)-N’-(4-(Dimethylamino)benzylidene)benzo[b]thiophene-2-carbohydrazide (Lb)

Yellow solid (99 mg, 45% yield). $^1$H 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 (Le)

Intermediate 2a (938 mg) was deprotected under the reported acidic conditions (TFA) to produce, after an aqueous workup with NaHCO$_3$ 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 h 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). $^1$H 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); $^{13}$C 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, 137.6, 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: calcld. for C$_{15}$H$_{12}$N$_3$OS [M + H]^+ 282.0696, found 282.0682.

(E)-N’-(Pyridin-3-ylmethylene)benzo[b]thiophene-2-carbohydrazide (Ld)

White powder (129 mg, 67% yield). $^1$H 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); $^{13}$C 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: calcld. for C$_{15}$H$_{12}$N$_3$OS [M + H]^+ 282.0696, found 282.0694.

(E)-N’-(Pyridin-4-ylmethylene)benzo[b]thiophene-2-carbohydrazide (Le)

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). $^1$H 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 z(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 (If)

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). $^1$H 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 (Ig)

White powder (32 mg, 10% yield). $^1$H 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[θ]thiophene-2-carbohydrazide (Ih)

White powder (141 mg, 41% yield). 1H NMR (300 MHz, DMSO-d6) 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[θ]thiophene-2-carbohydrazide (Ii)

White solid (216 mg, 62% yield). 1H NMR (300 MHz, DMSO-d6) 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[θ]thiophene-2-carbohydrazide (Ij)

White solid (173 mg, 44% yield). 1H NMR (300 MHz, DMSO-d6) 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[θ]thiophene-2-carbohydrazide (Ii)

Yellowish solid (111 mg, 36% yield). 1H NMR (300 MHz, DMSO-d6) 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[θ]thiophene-2-carbohydrazide (II)

Yellowish solid (110 mg, 30% yield). 1H NMR (300 MHz, DMSO-d6) 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-d6) δ 161.98, 158.47, 147.90, 145.52, 142.41, 141.97, 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[θ]thiophene-2-carbonyl)hydrazono)methyl)benzoic acid (I.m)

White solid (88 mg, 45% yield). 1H NMR (300 MHz, DMSO-d6) 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[θ]thiophene-2-carbonyl)hydrazineylidene)methyl)benzoic acid (I.n)

White powder (188 mg, 85% yield). 1H NMR (300 MHz, DMSO-d6) 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-d6) 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 C17H15N3O3S [M + H]+ 325.0641, found 325.0626.

(E)-4-((2-(Benzo[θ]thiophene-2-carbonyl)hydrazono)methyl)benzoic acid (I.o)

White solid (92 mg, 39% yield). 1H NMR (300 MHz, DMSO-d6) 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-ylmethyl)-6-chlorobenzylidene)thiophene-2-carbohydrazide (IIa)

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). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 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, 1H), 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-\(d_6\)) \(\delta\) 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 \(C_{17}H_{12}ClN_2O_3S~[M + H]^+\) 359.0252, found 359.0254.

\(^{(E)}\)6-Chloro-N’-(pyridin-2-ylmethylene)benzo[b]thiophene-2-carbohydrazide (IIb)

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 (44 mg, 40% yield). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 12.05 (s, 0.5H), 11.93 (s, 0.5H), 8.39 (d, \(J = 8.3\) 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-\(d_6\)) \(\delta\) 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, 127.20, 126.99, 125.68, 125.58, 124.63, 122.54, 122.24, 120.51, 120.16; HRMS (ESI) \(m/z\): calcd. for \(C_{15}H_{11}ClN_3OS~[M + H]^+\) 316.0306, found 316.0315.

\(^{(E)}\)N’-(Benzo[d][1,3]dioxol-5-ylmethylene)-6-fluorobenz[b]thiophene-2-carbohydrazide (IIc)

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, 24% yield). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 12.31 (s, 1H), 8.64 (d, \(J = 6.5\) Hz, 1H), 7.59–7.14 (m, 2H); \(^13\)C NMR (101 MHz, DMSO-\(d_6\)) \(\delta\) 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 \(C_{15}H_{11}FNO_3S~[M + H]^+\) 343.0547, found 343.0550.

\(^{(E)}\)6-Fluoro-N’-(pyridin-2-ylmethylene)benzo[b]thiophene-2-carbohydrazide (IId)

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 powder (90 mg, 48% yield). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 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), 7.83–7.96 (m, 1.5H), 7.90–7.57 (m, 1.5H), 7.67–7.40 (m, 1.5H); \(^13\)C NMR (101 MHz, DMSO-\(d_6\)) \(\delta\) 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) \(m/z\): calcd. for \(C_{17}H_{12}ClN_2O_3S~[M + H]^+\) 327.0 [M + Na]\(^+\).

\(^{(E)}\)6-Chloro-N’-(furan-2-ylmethylene)benzo[b]thiophene-2-carbohydrazide (IIId)

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). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 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); $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 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 (IIIc)

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). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 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); $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 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 (IIIe)

Intermediate 2e 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 powder (63 mg, 44% yield). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 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); $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 154.59, 153.05, 149.11, 148.57, 137.00, 131.93, 126.04, 124.67, 124.27, 120.17, 117.29, 111.20; HRMS (ESI) $m/z$: calcd. for C$_{15}$H$_{11}$ClN$_3$O$_2$ [M + H]$^+$ 300.0534, found 300.0533.

(E)-N'-(Pyridin-2-ylmethylene)-6-(trifluoromethyl)benzo[b]thiophene-2-carbohydrazide (IIIf)

Intermediate 2d 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 powder (64 mg, 44% yield). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 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); $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 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, 125.81, 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 C$_{16}$H$_{11}$F$_3$N$_3$OS [M + H]$^+$ 350.0572, found 350.0572.

2.1.8. Procedure for N'-(Pyridin-2-ylmethyl)benzo[b]thiophene-2-carbohydrazide (Lp)

Under H$_2$ 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 was stirred at room temperature for 7 h. The mixture was filtered on Celite® and washed with EtOAc, and the residue was concentrated under vacuum and purified by chromatography (eluent: EtOAc/MeOH 98:2). (Adapted from Kim et al. [22]).

N'-(Pyridin-2-ylmethyl)benzo[b]thiophene-2-carbohydrazide (Lp)

White powder (30 mg, 60% yield). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 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); $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 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 C$_{15}$H$_{14}$N$_3$OS [M + H]$^+$ 284.0852, found 284.0858.
2.1.9. Procedure for Pyridine 2-aldoxime (6)

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 Na$_2$SO$_4$ and concentrated under vacuum. (Adapted from Younus Wani et al. 2011 ([23]).

Pyridine 2-aldoxime (6)

White powder (186 mg, 81%). $^1$H NMR (300 MHz, Chloroform-d) $\delta$ 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 with Zhang et al. [24]).

2.1.10. Procedure for (E)-Picolinaldehyde O-(6-chlorobenzothiophene-2-carbonyl) oxime (III.d)

Under a dried and inert atmosphere (N$_2$), 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$^\circ$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 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-chlorobenzothiophene-2-carbonyl) oxime (III.d)

Orange powder (166 mg, 64% yield). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ 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); $^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 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 C$_{15}$H$_{10}$ClN$_2$O$_2$S [M + H]$^+$ 317.0146, found 317.0136.

2.2. Biological Assays

2.2.1. 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, USA). 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 carried out in triplicate and determined after 18 h of incubation at 37 $^\circ$C. The median values were reported.

2.2.2. 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 $\times$ 10$^5$ cells/mL in DMEM GlutaMAX (Gibco) supplemented with 10% fetal bovine serum for 24 h in a humidified atmosphere at 37 $^\circ$C and 5% CO$_2$. 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 in triplicate and mean
values are displayed with standard deviation.

3. Results and Discussion

3.1. 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.](image)

The synthetic route to these compounds was achieved by the conversion of benzothio-
ephene-2-carboxylic acid 1a into tert-butyl 2-(benzothiophene-2-carbonyl)hydrazinecarboxylate
2a in 88% yield using DCC as a coupling reagent. This method was preferred to the sub-
stitution of methyl benzothiophene-2-carboxylate with hydrazine, leading to a mixture
of products difficult to purify [25]. Then, the tert-butyl carboxylate group was removed
under acidic conditions using TFA to give a crude material which was then engaged with
purification for reaction with diversely substituted aromatic or heteroaromatic aldehy-
des under acidic and refluxing conditions to form the imine bond of the acylhydrazone
(Scheme 1).

![Scheme 1. Synthesis of set I derivatives.](image)
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 such as the 2-pyridinyl derivative Lc required the 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 13C NMR signals were split in two (spectra available in Supplementary Materials, Figure S1). 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 [13,26,27]. To demonstrate the presence of rotamers in the case of the piperonyl derivative Ia, the temperature of 1H NMR experiments was gradually increased, and the expected coalescence phenomenon was observed at 330 K and at higher temperatures (Figure 2).

Figure 2. 1H NMR spectra of Ia 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, resistant to methicillin (SF8300) and daptomycin (ST20171643), respectively. 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,
inoculum, for the benzo[b]thiophene-derived acylhyrazones. The results are presented in Table 1.

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

| Name | Structure | LogP | MIC (µg/mL) |
|------|-----------|------|-------------|
|      |           |      | ATCC 29213  | SF8300  | ST20171643 |
| I.a  | ![Structure](image1) | 4.20 | 32 | 64 | 16 |
| I.b  | ![Structure](image2) | 4.41 | >256 | >256 | >256 |
| I.c  | ![Structure](image3) | 3.13 | 16 | 16 | 16 |
| I.d  | ![Structure](image4) | 3.07 | >256 | >256 | >256 |
| I.e  | ![Structure](image5) | 3.02 | >256 | >256 | >256 |
| I.f  | ![Structure](image6) | 3.80 | >256 | >256 | ND |
| I.g  | ![Structure](image7) | 3.83 | >256 | >256 | >256 |
| I.h  | ![Structure](image8) | 3.64 | >256 | >256 | >256 |
| I.i  | ![Structure](image9) | 4.98 | 128 | 128 | 128 |
| I.j  | ![Structure](image10) | 4.47 | >256 | >256 | 128 |
| I.k  | ![Structure](image11) | 4.24 | >256 | >256 | ND |
| I.l  | ![Structure](image12) | 4.26 | 64 | 64 | 32 |
was found to be important, as the other pyridinyl compounds prevent the free rotation of the pyridine ring and allowing conjugation due to the nitrogen atom in position 2 of the pyridine moiety in I.c, which 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.f, I.g and, to a lesser extent, I.h), with interesting MICs for the products, while the others 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 a 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.

![Scheme 2. Synthesis of compound I.p.](image)

(i) H₂, Pd/C, MeOH, EtOAc, r.t., 7 h

### 3.2. Synthesis and Biological Evaluation of Series II—6-Halogenobenzo[b]thiophene-2-Acylhydrazones

Based on this first set of results, we selected the two compounds I.a and I.c (bearing a 5-piperonyl and a 2-pyridinyl moiety, respectively) as hits for further structural modulation. With 6-halogenobenzo[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).

Figure 3. General structure of set II derivatives.

In order to synthesize the acylhydrazones, 6-chloro- and 6-fluorobenzothiophene-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 4b and 4c, as described in the literature [14]. 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.

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.
µ equal to 256 through the conversion of pyridine-2-carboxaldehyde into the corresponding oxime cis is better for compound chlorinated derivatives furan and 5-hydroxymethylfuran. The procedure used was the same as the one used for undertake by replacing the pyridine ring with three five-bond rings, namely imidazole, III 3.3. Synthesis and Biological Evaluation of Series I.c, for the pyridinyl compound did not change the activity. Both halogenated compounds were deprived of biological activity (II.a and II.c).

This short series allowed us to identify the chloropyridinyl compound II.b as significantly more active compared with 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 the activity. Both halogenated compounds derived from the piperonyl compound I.a were deprived of biological activity (II.a and II.c).

3.3. Synthesis and Biological Evaluation of Series III—Structural Modifications of II.b

Attempts to improve the activity of the chloropyridinyl compound II.b were then undertaken by replacing the pyridine ring with 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 (Scheme 4).

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

| Name | Structure | LogP | MIC (µg/mL) |
|------|-----------|------|-------------|
|      |           |      | ATCC 29213 | SF8300 | ST20171643 |
| II.a | ![Structure II.a](image) | 4.85 | >256 | >256 | >256 |
| II.b | ![Structure II.b](image) | 3.79 | 4 | 4 | 4 |
| II.c | ![Structure II.c](image) | 4.33 | >256 | >256 | >256 |
| II.d | ![Structure II.d](image) | 3.27 | 32 | 16 | 16 |

II.a: methicillin-sensitive S. aureus; II.b: methicillin-resistant S. aureus; II.c: daptomycin-resistant S. aureus.

Scheme 4. Synthesis of III.d.

(i) HO-NH₂.HCl, NaHCO₃, H₂O, 65°C, 3 h; (ii) 1b, DCC, DMAP, DCM (anh.), 0°C then r.t., 24 h

Compound III.d showed no anti-staphylococcal activity at concentrations lower or equal to 256 µg/mL (Table 3). Therefore, the rigidity imposed by the acylhydrazone moiety is critical to the biological activity.
As shown in Table 3, compound III.e showed a weak activity against S. aureus ATCC 29213 and a moderate one against S. aureus SF8300 and S. aureus ST20171643.

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

| Name | Structure | LogP | ATCC 29213 \(^a\) | SF8300 \(^b\) | ST20171643 \(^c\) |
|------|-----------|------|-----------------|---------------|-----------------|
| III.a | ![III.a structure](structure1.png) | 3.19 | 256 | 64 | 64 |
| III.b | ![III.b structure](structure2.png) | 4.22 | >256 | 256 | 256 |
| III.c | ![III.c structure](structure3.png) | 3.79 | >256 | 256 | 256 |
| III.d | ![III.d structure](structure4.png) | 3.89 | >256 | >256 | >256 |
| III.e | ![III.e structure](structure5.png) | 3.15 | >256 | 256 | 128 |
| III.f | ![III.f structure](structure6.png) | 4.01 | 32 | 8 | 16 |

\(^a\): methicillin-sensitive S. aureus; \(^b\): methicillin-resistant S. aureus; \(^c\): daptomycin-resistant S. aureus.

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.

![Scheme 5](scheme.png)

(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 atom by an oxygen atom decreases the overall LogP of the compound (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.

### 3.4. Cytotoxicity Assay of II.b

Due to its interesting antistaphylococcal activity, the potential mammalian cytotoxicity of the chloropyridinyl 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 \( \alpha \)-hemolysin as a 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](image_url)

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

### 4. Conclusions

In this study, benzo[b]thiophene-based acylhydrazones were shown to be interesting compounds acting as anti-staphylococcal agents. More precisely, (E)-6-chloro-N’-(pyridin-2-ylmethylene)benzo[b]thiophene-2-carboxydrazide II.b showed an equal minimum inhibitory concentration of 4 \( \mu\text{g/mL} \) for the three strains of S. aureus, including two clinically isolated strains of drug-resistant S. aureus. Moreover, II.b showing no cytotoxicity on A549 cells; this suggests that the chloropyridinyl benzothiophene acylhydrazone structure is pertinent for future work on chemical diversification and the understanding of the mode of action.
Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biom12010131/s1, Figure S1: $^1$H- and $^{13}$C-NMR spectra for new compounds.

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