Synthesis and Antimicrobial Evaluation of Novel Chiral 2-Amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine Derivatives

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New *N*-substituted-2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine derivatives were synthesized employing a convenient one-pot three-component method and their structures were characterized by ^{1}H-NMR and single crystal X-ray diffraction analysis. All the synthesized compounds were *in vitro* screened for antimicrobial activity against Gram-positive (*Sarcina lutea*) and Gram-negative bacteria (*Escherichia coli*). In this work, we introduced a chiral residue on the tetrahydropyridine nitrogen, the hitherto the less investigated position on this pharmacophore in order to explore the effect. The antibacterial results showed that the synthesized compounds were active only against Gram-positive bacteria and the (*R*)-enantiomers displayed a greater antimicrobial potency than their (*S*)-counterparts. The structure–activity relationship here investigated may provide some interesting clues for future development of tetrahydrothienopyridine derivatives with higher antimicrobial activity.

Keywords: heterocycles, tetrahydrothieno[2,3-*c*]pyridine, chiral amine, Gewald reaction, antimicrobial agents, biological activity, synthesis design.

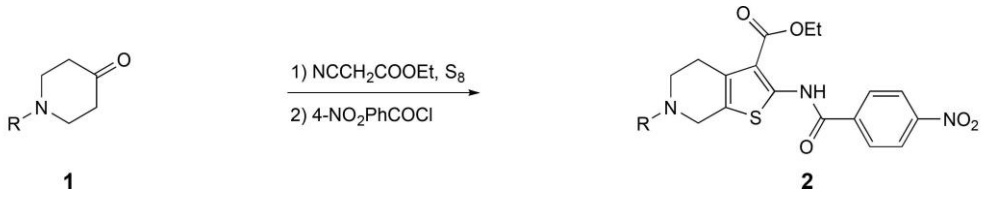
Introduction

Thiophene and its derivatives are an important class of heterocyclic compounds possessing broad biological activities;[1,2] thiophenes and thienopyridines are present in many pharmacologically active compounds and natural products,[3] e.g., the antiplatelet aggregation clopidogrel, the anticonvulsant tiagabine, antimuscarinic drugs for the treatment of asthma and bronchospasm such as tiotropium bromide, antiinflammatory,[4] antimicrobial,[5] and antiviral derivatives.[6]

For these reasons, the development of an efficient, rapid, and clean synthetic route towards focused libraries of such compounds is of great importance to both medicinal and synthetic chemists.

In the recent literature, some thienopyridines were reported as new potential antitubercular drugs, acting as inhibitors of pantothenate synthetase (Mtb-PS), an important target in tuberculosis therapy absent in mammalian cells.[7–9] Actually, the strategies directed towards targets absent in humans are considered one of the most appealing approaches to disclose new drugs potentially safer for humans.[10–13] Moreover, it is important to underline that the thienopyridine pharmacophore strongly interfered with other microbial proteins, such as dihydrofolate reductase, secreted aspartic protease and *N*-myristoyl transferase from *Candida albicans*, dihydrofolate reductase and gyrase B from *Staphylococcus aureus*.[14]

The increasing multidrug resistant (MDR) and extensively drug resistant (XDR) bacteria strains worldwide, together with the lack of new effective drugs in the last decades,[15] suggested the urgent need for the identification of innovative antimicrobial targets and inhibitors. In this perspective, the multicomponent (MCRs) and cascade approaches are of valuable interest in drug discovery as source of novel chemical starting points, since they provide a wide range of scaffold differentiations and a quick synthetic methodology for libraries production. The Gewald three component reaction (G-3CR)[16] is a one-pot multicomponent reaction that has been increasingly used due to its simplicity and ease of synthesis for the production of several thiophene scaffolds derivatives. Synthesis of *N*-substituted-2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine derivatives were reported;[7,8] nevertheless, in most cases, the tetrahydropyridine *N*substitution pattern was limited to few groups such as methyl, benzyl, or acetyl.



Scheme 1. Synthetic pathway for the preparation of target compounds **2**.

Inspired by the above research results and our experience acquired in the application of 'one-pot' techniques for the identification of novel lead compounds brought us to investigate the applicability of the G-3CR 'multicomponent approach' to generate a small library of 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*] pyridine derivatives with new different substituents on the tetrahydrothienopyridine nitrogen atom. In addition, as the nitroaromatic compounds are often active in the poisoning of bacteria,[17] we fused the tetrahydrothieno[2,3-*c*]pyridine scaffold with a second pharmacophoric subunit, the nitro-benzene moiety, following the molecular hybridization strategy, in order to rationally design novel lead compounds endowed with synergic antibacterial properties.

As enantiomers can have very different biological activities, sometimes with an opposite effect on the target,[18] we decided to explore the synthesis of new chiral tetrahydrothienopyridines employing different commercially available chiral amines as enantiopure chiral building blocks. Hence, both the enantiomers were prepared in some cases in order to evaluate the influence of the chiral residue on the microbial growth. In this work, we report the synthesis, characterization, and a preliminary SAR study of all compounds to facilitate the further development of this kind of hybrids (*Figure 1*).

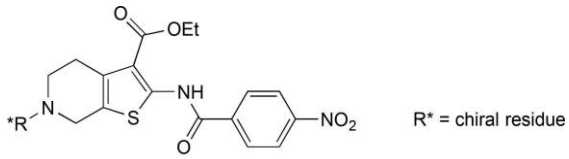


Figure 1. Molecular structures of the target compounds reported in this work.

Results and Discussion

The synthetic pathway is reported in *Scheme 1*. The desired chiral *N*-substituted piperidone was reacted under Gewald multicomponent conditions to afford the aminothiophene product (*Scheme 1*). Further reaction with 4-nitrobenzoyl chloride yielded the final products.

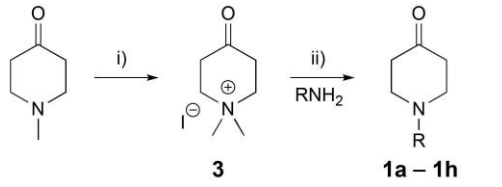
The synthesis of piperidone **1** started with the methylation of the commercially available *N*-methylpiperidone (*Scheme 2*) giving the intermediate salt **3**, which was then reacted with different chiral and achiral primary amines to yield the desired piperidones **1a**–**1h**.[19]

Firstly, we prepared three achiral derivatives bearing an aromatic substituent: the benzyl derivative **1a** and the fluorine derivative **1b**. The aromatic pyridine ring in **1c** was selected to investigate the effect of the presence of a potentially coordinating nitrogen atom capable of further interactions within the target active

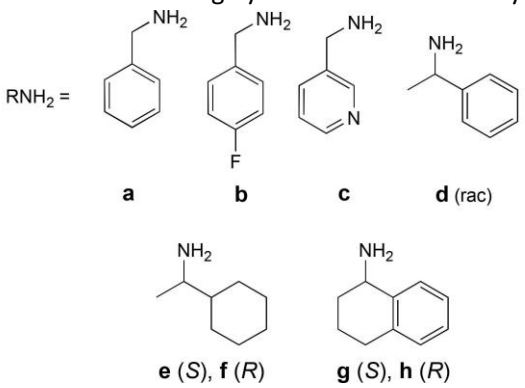
site. Then, we introduced in the scaffold different chiral substituents. The racemic phenylethyl derivative **1d** was firstly prepared, then two other chiral amines were used in both enantiomers (**1e**–**1h**).

The piperidones **1a**–**1h** were then reacted with ethyl cyanoacetate and sulfur, in the presence of morpholine as a base (*Scheme 3*), to give the Gewald 2-aminothiophene adduct **4a**–**4h**.

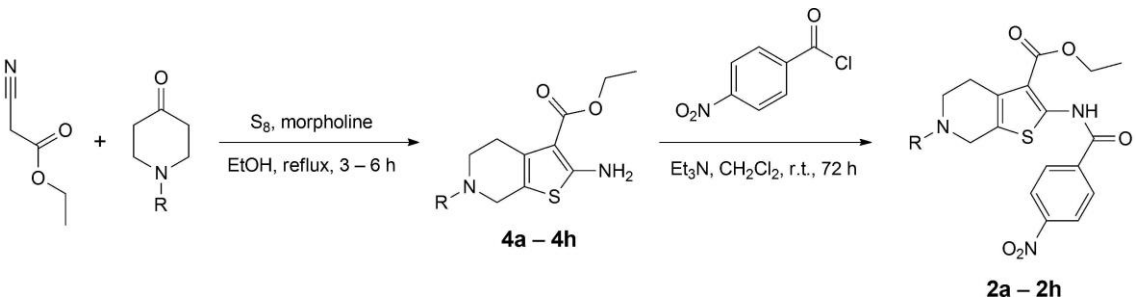
Finally, the target compounds were achieved by reaction with the highly reactive 4-nitrobenzoyl



i) MeI, acetone, r.t., 24 h. ii) EtOH, K_2CO_3, H_2O, reflux, 3 h.



Scheme 2. Preparation of the *N*-substituted piperidones **1a**–**1h** and list of the employed amines.



Scheme 3. General scheme for the synthesis of compounds **2a**–**2h**.

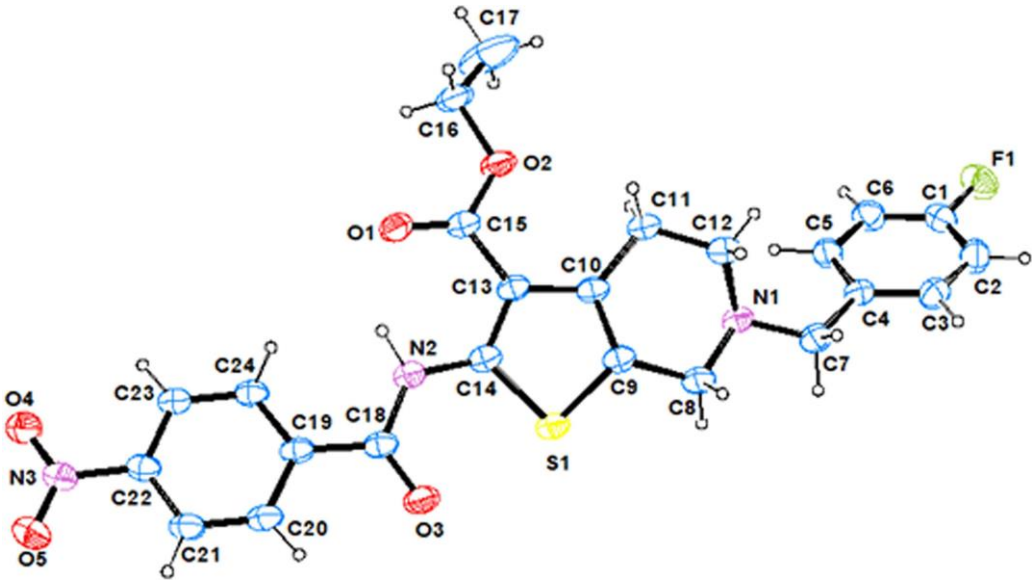


Figure 2. ORTEP drawing of **2b** with the arbitrary atom numbering (ellipsoids are at 40% probability and H atoms are as spheres of arbitrary radii).

chloride in presence of Et_3N. The desired final products **2a**–**2h** were obtained in satisfactory yields.

For compound **2b**, we obtained single crystals suitable for X-ray analysis, which allowed us to determine its 3D structure. The crystallographic structure of **2b** is shown in *Figure 2* as ORTEP[20] view. In the tetrahydrothienopyridine moiety, the six-membered ring has a flattened-boat conformation and its puckering parameters are: Q_T=0.504(3) Å, φ=150(1)°, and *#*=130(1)°. The fluoro-substituted benzene and thiophene forms a dihedral angle of 48(1)°, while the nitrobenzyl-amido moiety is nearly coplanar to the bicyclic moiety. The carbonyl oxygen of the ethyl carboxylate lateral chain is involved in an intramolecular hydrogen bond with the amidic hydrogen
(N2 H···O1, distance 1.900(1) Å, angle 139.3(1)°). The presence of this strong hydrogen bond is confirmed by ^{1}H-NMR spectrum. In the NMR spectra, in fact, the amidic NH hydrogen resonates at 12.48 ppm due to the strong deshielding effect of the coordination with the carbonyl oxygen. In the crystals, the molecules are arranged into a zigzag chain parallel to the face diagonal of the *ac* plane (*Figure 2*).

Antibacterial tests were performed to assess the *in vitro* activity of compounds **2a**–**2h** against two bacterial strains, namely *Sarcina lutea* ATCC 9341 (*S. lutea*; Gram-positive bacteria) and *Escherichia coli* ATCC 8739 (*E. coli*; Gram-negative bacteria). Results are reported in *Table 1*.

For each compound, the concentration required to suppress 50% bacterial growth (IC_{50}) is reported. All the 4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines did not inhibit the *E. coli* growth (data not shown), while they were able to interfere with the *S. lutea cell* growth. Of interest, the effects generally detected for our compounds against *S. lutea* allowed us to derive preliminary structure–activity relationships (SAR) among this class, which indicated that a change in the substituent might also affect the antibacterial activity of title compounds.

The highest inhibition activity among the achiral **2a**–**2d** derivatives was displayed by **2d**, the compound having the less extended structure. The presence of the pyridine ring in **2c** led to detect an appreciable activity, suggesting a possible interaction of the sp^2 nitrogen with the biological target. For what concerns the chiral products, the comparison between the configuration and the activity of the two pairs of stereoisomers **2e**/**2f** and **2g**/**2h** showed that (*R*)enantiomers (**2f**, **2h** IC_{50} =145 μM and IC_{50} =330 μM, respectively) displayed greater antimicrobial activity with respect to their (*S*)-counterparts (**2e**, **2g** IC_{50} = 521 μM and IC_{50} =509 μM, respectively). These outcomes are in agreement with our working hypothesis regarding the probable influence of the chirality on the biological effects; namely, it appears that the absolute configuration of the compounds plays a key role for the activity. It is worth noting that **2f** exhibited promising activity and could act as a starting point for further optimization.

Conclusions

In this work, we described the fast and efficient synthesis and the characterization of a small library of new 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine
derivatives, as new potential antimicrobial agents. The antibacterial activity of the compounds, investigated *in vitro* by means of a phenotypic screening on both Gram-negative (*E. coli*) and Gram-positive (*S. lutea*) bacteria, evidenced that all the derivatives showed a moderate antibacterial activity only against *S. lutea* growth. When both enantiomers of a same candidate were prepared, the biological results of the (*R*) and (*S*) couples proved the influence of the chiral residue on the activity. The two stereoisomers exhibited differences in their microbiological activity, as shown by their IC50 values, indicating a significant preference for the (*R*)-enantiomers as the most active ones. These observations evidenced the importance of having a specific spatial orientation of the functional groups in the enantiomers pairs for eliciting high binding affinities with the target and this strategy deserves to be considered for further investigations. Further microbiological investigations are underway to confirm these results, which could pave the way for further improvements on this promising scaffold, with the aim to develop a new class of antimicrobial agents.

Experimental Section

General

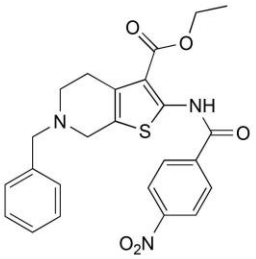
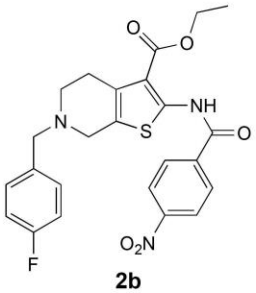
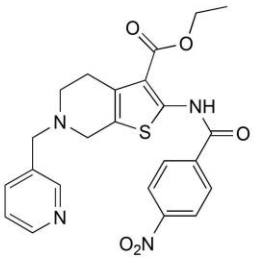
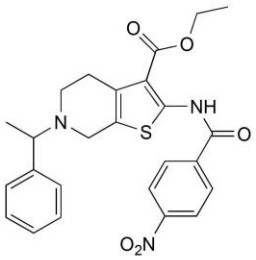
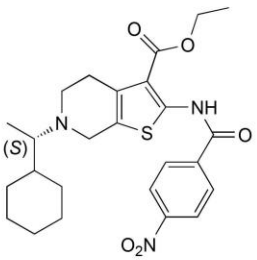
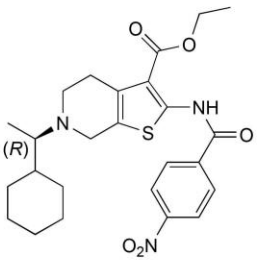
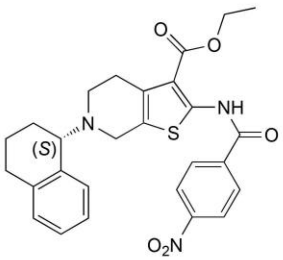
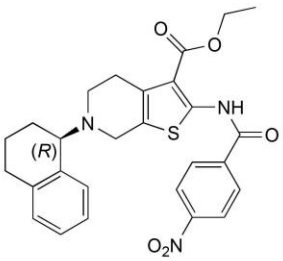
Reactions were monitored mostly by thin-layer chromatography (TLC), performed on Merck Kieselgel 60 F_{254} plates. Visualization was accomplished by UV irradiation at 254 nm and subsequently by treatment with alkaline $KMnO_4$ reactant (an oxidant mixture of $KMnO_4$, K_2CO_3 and 5% NaOH in water) or with phosphomolybdic

reagent. Each compound has been purified by silica gel column chromatography (230– 400 mesh).^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker ARS 400 spectrometer (^{1}H-NMR, 400 MHz; ^{13}CNMR, 100 MHz). Spectra are registered at room temperature, unless otherwise indicated, in $CDCl_3$, with tetramethylsilane (TMS, δ=0.0 ppm) used as internal standard. Chemical shifts are reported as δ values in parts per million (ppm) in comparison to internal standards; the coupling constants *J* are reported in Hz.

Synthesis

1,1-Dimethyl-4-oxopiperidinium Iodide (**3**). 1-methyl-4-piperidone (23.9 g, 26 ml, 0.2 mmol) was dissolved in acetone (130 ml). The resulting solution was cooled to 0°C and iodomethane (35.1 g; 15.4 ml; 1.2 equiv.) was slowly added. The mixture was stirred for 24 h at room temperature. White precipitate was formed and the product was isolated by filtration in 98% yield. ^{1}HNMR (400 MHz, D_2O): 3.56 (dd, *J*=6.6, 5.2, 4H), 3.24 (s, 6H), 2.18 (q, *J*=5.6, 4.8, 4H).

Table 1. Antibacterial activity of compounds **2a**–**2h**.

Compound	IC_{50} [μM]	Compound	IC_{50} [μM]
 **2a**	469	 **2b**	750
 **2c**	296	 **2d**	189
 **2e**	521	 **2f**	145
 **2g**	509	 **2h**	330

General Procedure for Synthesis of Compounds **1a**–**1h**

A mixture of the desired amine (10 mmol) and K_2CO_3 (25 mmol) in EtOH/H_2O 2:1 (60 mL) was heated to 110°C. Then, an aqueous solution of **3** (10 mmol) was added dropwise and the resulting solution was heated for 1 h. After completion of reaction, EtOH was removed by reduced pressure and the aqueous layer was extracted with diethyl ether (3×100 ml). The organic layer was dried over Na_2SO_4, filtered and the solvent was then removed under vacuum to afford the crude product, which was purified by column chromatography on silica gel (eluent: hexane/AcOEt 4:1).

1-(4-Fluorobenzyl)piperidin-4-one (**1b**). Yield: 69%. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.32 (dd, *J*=8.9, 5.6, 2H, Ar H), 7.05–6.98 (m, 2H, Ar H), 3.58 (s, 2H, CH_2), 2.73 (t, *J*=6.1, 4H, CH_2), 2.45 (t, *J*=6.2, 4H, CH_2). ^{13}CNMR (101 MHz, $CDCl_3$): 209.0 (C=O), 163.3–160.9 (d, *J*=240, Ar), 133.9 (d, *J*=3.8, 1 C, Ar), 130.3 (d, *J*=8.2, 2C, Ar), 115.3 (d, *J*=22.4, 2C, Ar), 61.2 (CH_2), 52.8 (2C, C3,5), 41.3 (2C, C2,6). Anal. calc. for $C_{12}H_{14}FNO$: C, 69.55; H, 6.81; F, 9.17; N, 6.76; O, 7.72; found: C, 69.57; H, 6.84; N, 6.79.

1-(Pyridin-3-ylmethyl)piperidin-4-one (**1c**). Yield: 74%. ^{1}H-NMR (400 MHz, $CDCl_3$): 8.58 (d, *J*=2.2, 1H, Ar H), 8.52 (dd, *J*=4.8, 1.7, 1H, Ar H), 7.70 (dt, *J*=7.8, 2.1, 1H, Ar H), 7.30–7.24 (m, 1H, Ar H), 3.62 (s, 2H, CH_2), 2.74 (t, *J*=6.1, 4H, CH_2), 2.45 (t, *J*=6.1, 4H, CH_2). ^{13}CNMR (101 MHz, $CDCl_3$): 208.6 (C=O), 150.2 (Ar), 148.9
(Ar), 136.4 (Ar), 133.6 (Ar), 123.4 (Ar), 59.2 (CH_2), 52.9

(2C, C3,5), 41.2 (2C, C2,6). Anal. calc. for $C_{11}H_{14}N_2O$: C, 69.45; H, 7.42; N, 14.73; O, 8.41; found: C, 69.48; H, 7.40; N, 14.70.

1-(1-Phenylethyl)piperidin-4-one (**1d**). Yield: 64%. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.36–7.29 (m, 4H, Ar H), 7.27–7.23 (m, 1H, Ar H), 3.62 (q, *J*=6.7, 1H, CH), 2.86–2.61 (m, 4H, CH_2), 2.44–2.39 (m, 4H, CH_2), 1.42 (d, *J*=6.7, 3H, Me). ^{13}C-NMR (101 MHz, $CDCl_3$): 209.5 (C=O), 143.5 (Ar), 128.3 (2C, Ar), 127.3 (2C, Ar), 125.7
(Ar), 63.4 (CH), 50.0 (2C, C3,5), 41.6 (2C, C2,6), 19.4
(Me). Anal. calc. for $C_{13}H_{17}NO$: C, 76.81; H, 8.43; N, 6.89; O, 7.87; found: C, 76.84; H, 8.45; N, 6.91.

1-(1-Cyclohexylethyl)piperidin-4-one (**1e**, **1f**). Yield: 71%. $[\alpha]_D^{20}$ (*c*=1.00, $CHCl_3$):+47.6 ((*S*)-enantiomer). ^{1}H-NMR (400 MHz, $CDCl_3$): 2.90–2.79 (m, 2H, CH_2), 2.65–2.56 (m, 2H, CH_2), 2.47–2.32 (m, 5H, Cy), 2.11–2.02 (m, 1H, Cy), 1.78–1.60 (m, 4H, Cy), 1.34–1.04 (m, 4H, Cy), 0.97–0.86 (m, 5H, Cy, Me). ^{13}C-NMR (101 MHz, $CDCl_3$): 210.2 (C=O), 64.0 (CH), 48.5 (2C, C3,5), 42.2 (2C, C2,6), 41.4 (CH_2), 31.0 (CH_2), 30.5 (CH_2), 26.7 (CH_2), 26.4 (CH_2), 26.4 (CH_2), 10.3 (Me). Anal. calc. for $C_{13}H_{23}NO$: C, 74.59; H, 11.08; N, 6.69; O, 7.64; found:
C, 74.61; H, 11.06; N, 6.70. **1-(1,2,3,4-Tetrahydronaphthalen-1-yl)piperidin4-one** (**1g**, **1h**). Yield: 56%. $[\alpha]_D^{20}$ (*c*=1.00, $CHCl_3$):+ 116.6 ((*S*)-enantiomer). ^{1}H-NMR (400 MHz, $CDCl_3$): 7.77 (d, *J*=7.4, 1H, Ar H), 7.23–7.11 (m, 2H, Ar H), 7.07 (d, *J*=7.6, 1H, Ar H), 4.02 (dd, *J*=10.0, 4.1, 1H, CH), 3.01– 2.83 (m, 2H, CH_2), 2.84–2.71 (m, 4H, CH_2), 2.54–2.35 (m, 4H, CH_2), 2.06–1.91 (m, 2H, CH_2), 1.79–1.61 (m, 2H, CH_2). ^{13}C-NMR (101 MHz, $CDCl_3$): 209.9 (C=O), 138.2
(Ar), 137.9 (Ar), 128.9 (Ar), 127.5 (Ar), 126.5 (Ar), 125.9 (Ar), 62.8 (CH), 48.4 (2C, C3,5), 42.4 (2C, C2,6), 29.7 (CH_2), 22.0 (CH_2), 21.9 (CH_2). Anal. calc. for $C_{15}H_{19}NO$: C,78.56; H, 8.35; N, 6.11; O, 6.98; found: C, 78.59; H, 8.37; N, 6.12.

General Procedure for Synthesis of Compounds **4a**–**4h**

To a stirred mixture of *N*-substituted-4-piperidone, ethyl cyanoacetate (1 equiv.) and sulfur (S_8; 1 equiv.) in EtOH at 0°C, morpholine (1 equiv.) was dripped slowly. The resulting mixture was then heated at 110°C for at least 4 h (or until complete dissolution of S_8) and stirred for other 24 h. The reaction was monitored by GC. At completion of reaction, the mixture was cooled in an ice bath: EtOH and ice were added to promote precipitation of the product that was then isolated by filtration under vacuum (*a*). Instead if it did not precipitate, EtOH was evaporated and the aqueous mixture was extracted three times with AcOEt. The organic layer was dried over Na_2SO_4, filtered and then concentrated under vacuum (*b*). The crude product was purified by silica gel column chromatography (eluent: hexane/AcOEt 3:1).

Ethyl 2-Amino-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**4a**). Yield: 84%. ^{1}HNMR (400 MHz, $CDCl_3$): 7.41–7.26 (m, 5H, Ar H), 5.92 (s, 2H, NH_2), 4.25 (q, *J*=7.1, 2H, OCH_2Me), 3.67 (s, 2H, CH_2), 3.43–3.35 (m, 2H, CH_2), 2.86–2.79 (m, 2H,

CH_2), 2.78–2.70 (m, 2H, CH_2), 1.31 (t, *J*=7.1, 3H, OCH_2*Me*). ^{13}C-NMR (101 MHz, $CDCl_3$): 166.3 (C=O), 162.1 (C2), 138.3 (Ar), 131.2 (C3a), 129.1 (2C, Ar), 128.3 (2C, Ar), 127.2 (Ar), 115.0 (C3), 105.5 (C7a), 62.1 (CH_2), 59.5 (CH_2), 51.4 (C7), 50.3 (C5), 27.4 (C4), 14.5 (Me). Anal. calc. for $C_{17}H_{20}N_2O_2S$: C 64.53, H 6.37, N 8.85; found: C 64.49, H 6.36, N 8.87.

Ethyl 2-Amino-6-(4-fluorobenzyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**4b**). Yield: 81%. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.33 (dd, *J*=8.5, 5.6, 2H, Ar H), 7.01 (t, *J*=8.7, 2H, Ar H), 5.93 (s, 2H, NH_2), 4.25 (q, *J*=7.1, 2H, O*CH*$_2$Me), 3.63 (s, 2H, CH_2), 3.39 (s, 2H, CH_2), 2.85–2.78 (m, 2H, CH_2), 2.73 (t, *J*=5.7, 2H, CH_2), 1.32 (t, *J*=7.1, 3H, OCH_2*Me*). ^{13}C-NMR (101 MHz, $CDCl_3$): 165.9 (C=O), 162.1–159.7 (d, *J*=240, Ar), 160.7 (C2), 131.2 (C3a), 130.6–130.5 (d, *J*=8.1, 2C, Ar), 115.3–115.0 (d, *J*=20.9, 2C, Ar), 114.0 (C7a), 103.4 (C3), 61.2 (CH_2), 59.5 (CH_2), 51.3 (C7), 50.2 (C5), 27.3 (C4), 14.5 (Me). Anal. calc. for $C_{17}H_{19}FN_2O_2S$: C 61.06, H 5.73, N 8.38; found: C 61.09, H 5.71, N 8.40.

Ethyl 2-Amino-6-(pyridin-3-ylmethyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**4c**). Yield: 68%. ^{1}H-NMR (400 MHz, $CDCl_3$): 8.58 (d, *J*=2.1, 1H, Ar H), 8.53 (dd, *J*=4.8, 1.7, 1H, Ar H), 7.73 (dt, *J*= 7.8, 2.0, 1H, Ar H), 7.27 (q, *J*=4.5, 1H, Ar H), 5.97 (s, 2H, NH_2), 4.25 (q, *J*=7.1, 2H, O*CH*$_2$Me), 3.69 (s, 2H, CH_2), 3.44–3.40 (m, 2H, CH_2), 2.83 (td, *J*=5.4, 2.5, 2H, CH_2), 2.75 (t, *J*=5.5, 2H, CH_2), 1.32 (t, *J*=7.1, 3H, OCH_2*Me*). ^{13}C-NMR (101 MHz, $CDCl_3$): 165.9 (C=O), 162.2 (C2), 150.4 (Ar), 148.8 (Ar), 136.7 (Ar), 133.8 (Ar), 131.1 (C3a), 123.5 (Ar), 114.5 (C7a), 104.1 (C3), 59.5 (CH_2), 51.4 (C7), 50.2 (C5), 27.2 (C4), 14.5 (Me). Anal. calc. for $C_{16}H_{19}N_3O_2S$: C 60.55, H 6.03, N 13.24; found: C 60.57, H 6.06, N 13.27.

Ethyl 2-Amino-6-(1-phenylethyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**4d**). Yield: 64%. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.41–7.26 (m, 5H, Ar H), 5.92 (s, 2H, NH_2), 4.24 (q, *J*=7.1, 2H, O*CH*$_2$Me), 3.60–3.51 (m, 2H, CH_2), 3.35 (d, *J*=14.1, 1H, CH), 2.82–2.73 (m, 3H, CH_2), 2.64–2.57 (m, 1H, CH_2), 1.43 (d, *J*= 6.7, 3H, Me), 1.30 (t, *J*=7.1, 3H, OCH_2*Me*). ^{13}C-NMR (101 MHz, $CDCl_3$): 166.1 (C=O), 162.0 (C2), 144.3 (Ar), 131.3 (C3a), 128.4 (2C, Ar), 127.5 (2C, Ar), 127.0 (Ar), 116.1 (C7a), 105.3 (C3), 64.0 (CH_2), 59.4 (CH), 49.2 (C7), 47.8 (C5), 27.6 (C4), 20.5 (Me), 14.4 (Me). Anal. calc. for $C_{18}H_{22}N_2O_2S$: C 65.43, H 6.71, N 8.48; found: C 65.38, H 6.67, N 8.55.

Ethyl 2-Amino-6-(1-cyclohexylethyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**4e**, **4f**). Yield: 70%. $[\alpha]_D^{20}$ (*c*=1.00, $CHCl_3$):+12.7 ((*S*)-enantiomer). ^{1}H-NMR (400 MHz, $CDCl_3$): 5.92 (s, 2H, NH_2), 4.25 (q, *J*=7.1, 2H, O*CH*$_2$Me), 3.84–3.60 (m, 1H, CH), 3.54 (d, *J*=14.1, 1H, CH_2), 3.36 (d, *J*=14.1, 1H, CH_2), 2.82–2.71 (m, 3H, CH_2), 2.59–2.50 (m, 1H, Cy), 2.37 (dq, *J*=8.1, 6.6, 1H, Cy), 1.98 (d, *J*=13.4, 1H, Cy), 1.80–1.65 (m, 1H, Cy), 1.45–1.35 (m, 2H, Cy), 1.33 (t, *J*=7.1, 3H, OCH_2*Me*), 1.23–1.10 (m, 4H, Cy), 0.97 (d, *J*=6.6, 3H, Me), 0.96–0.82 (m, 2H, Cy). ^{13}C-NMR (101 MHz, $CDCl_3$): 168.3 (C=O), 163.3 (C2), 133.4 (C3a), 115.7 (C7a), 103.2 (C3), 63.7 (CH), 59.4 (CH_2), 47.5 (C7), 45.7 (C5), 41.1 (Cy), 31.1 (Cy), 30.9 (Cy), 30.1 (Cy), 28.4 (C4), 26.8 (Cy), 26.5 (Cy), 14.5 (Me), 10.3 (Me). Anal. calc. for $C_{18}H_{28}N_2O_2S$: C, 64.25; H, 8.39; N, 8.33; found: C, 64.30; H, 8.33; N, 8.36.

Ethyl 2-Amino-6-(1,2,3,4-tetrahydronaphthalen1-yl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**4g**, **4h**). Yield: 61%. $[\alpha]_D^{20}$ (*c*=1.00, $CHCl_3$): +54.8 ((*S*)-enantiomer). ^{1}H-NMR (400 MHz, $CDCl_3$): 7.75–7.69 (m, 1H, Ar H), 7.17–7.11 (m, 2H, Ar H), 7.09–7.04 (m, 1H, Ar H), 5.92 (s, 2H, NH_2), 4.26 (q, *J*=7.1, 2H, O*CH*$_2$Me), 4.00 (dd, *J*=9.3, 4.4, 1H, CH), 3.63 (d, *J*= 14.1, 1H, CH_2), 3.49 (d, *J*=14.1, 1H, CH_2), 2.92–2.59 (m, 6H, CH_2), 2.02 (dd, *J*=10.5, 4.0, 2H, CH_2), 1.79–1.68 (m, 2H, CH_2), 1.32 (t, *J*=7.1, 3H, OCH_2*Me*). ^{13}C-NMR (101 MHz, $CDCl_3$): 166.0 (C=O), 161.9 (C2), 138.3 (Ar), 138.1 (Ar), 131.4 (C3a), 128.8 (Ar), 128.2 (Ar), 126.4 (Ar), 125.8 (Ar), 116.1 (C7a), 105.6 (C3), 62.6 (CH), 59.5 (CH_2), 47.1 (C7), 46.0 (C5), 29.7 (CH_2), 28.7 (C4), 21.8 (CH_2), 21.7 (CH_2), 14.5 (Me). Anal. calc. for $C_{20}H_{24}N_2O_2S$: C, 67.39; H, 6.79; N, 7.86; found: C, 67.44; H, 6.75; N, 7.81.

General Procedure for Synthesis of Compounds **2a**–**2h**

To a stirred solution of amino thiophene (1 equiv.) dissolved in dichloromethane (10 ml), triethylamine (2 equiv.) and 4-nitrobenzoyl chloride (3 equiv.) were added. The resulting mixture was stirred at room temperature for 72 h and the reaction was monitored by TLC. At completion of the reaction, the mixture was extracted

three times with sodium bicarbonate ($NaHCO_3$) solution. The organic layer was then dried over Na_2SO_4, filtered and concentrated in vacuo. A crude compound was obtained that was purified by silica gel column chromatography (eluent: hexane/AcOEt 4:1).

Ethyl 6-Benzyl-2-(4-nitrobenzamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**2a**). Yield: 84%. ^{1}H-NMR (400 MHz, $CDCl_3$): 12.48 (s, 1H, NH), 8.37 (d, *J*=8.7, 2H, Ar H), 8.18 (d, *J*=8.7, 2H, Ar H), 7.44–7.29 (m, 5H, Ar H), 4.39 (q, *J*=7.1, 2H, OC*H*$_2$Me), 3.73 (s, 2H, CH_2), 3.63 (s, 2H, CH_2), 2.93 (d, *J*=6.0, 2H, CH_2), 2.81 (t, *J*=5.8, 2H, CH_2), 1.40 (t, *J*=7.1, 3H, OCH$_2$*Me*). ^{13}C-NMR (101 MHz, $CDCl_3$): 167.0 (C3), 163.1 (C=O), 153.3 (C=O), 151.9 (Ar), 149.0 (Ar), 144.1 (Ar), 138.3 (Ar), 137.9 (Ar), 129.2 (2C, Ar), 128.7 (2C, Ar), 128.4 (2C, Ar), 127.4 (2C, Ar, C3a), 124.2 (C7a), 118.1 (C3), 62.1 (CH_2), 61.0 (CH_2), 51.4 (C7), 50.1 (C5), 26.9 (C4), 14.3 (Me). Anal. calc. for $C_{24}H_{23}N_3O_5S$: C, 61.92; H, 4.98; N, 9.03; found: C, 61.86; H, 5.02; N, 9.00.

Ethyl 6-(4-Fluorobenzyl)-2-(4-nitrobenzamido)4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**2b**). Yield: 88%. ^{1}H-NMR (400 MHz, $CDCl_3$): 12.48 (s, 1H, NH), 8.37 (d, *J*=8.6, 2H, Ar H), 8.18 (d, *J*=8.6, 2H, Ar H), 7.38–7.30 (m, 2H, Ar H), 7.03 (t, *J*=8.6, 2H, Ar H), 4.39 (q, *J*=7.1, 2H, OC*H*$_2$Me), 3.68 (s, 2H, CH_2), 3.61 (s, 2H, CH_2), 2.92 (s, 2H, CH_2), 2.78 (t, *J*=5.8, 2H, CH_2), 1.40 (t, *J*=7.1, 3H, OCH$_2$*Me*). ^{13}C-NMR (101 MHz, $CDCl_3$): 169.9 (C2), 167.6 (C=O), 163.1–161.5 (d, *J*= 240, Ar), 162.2 (C=O), 160.4 (Ar), 148.3 (Ar), 143.2 (Ar), 137.8 (Ar), 130.6 (d, *J*=8.1, 2C, Ar), 129.7 (C3a), 128.7 (2C, Ar), 125.3 (C7a), 124.2 (2C), 115.3(d, *J*=20.9, 2C, Ar), 113.2 (C3), 61.2 (CH_2), 61.1 (CH_2), 51.4 (C7), 50.0 (C5), 26.9 (C4), 14.3 (Me). Anal. calc. for $C_{24}H_{22}FN_3O_5S$: C, 59.62; H, 4.59; N, 8.69; found: C, 59.71; H, 5.02; N, 8.60.

Ethyl 2-(4-Nitrobenzamido)-6-(pyridin-3-ylmethyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**2c**). Yield: 81%. ^{1}H-NMR (400 MHz, $CDCl_3$): 12.49 (s, 1H, NH), 8.61 (d, *J*=2.1, 1H, Ar H), 8.55 (dd, *J*=4.7, 1.7, 1H, Ar H), 8.40–8.36 (m, 2H, Ar H), 8.18 (d, *J*=8.8, 2H, Ar H), 7.74 (d, *J*=8.1, 1H, Ar H), 7.32–7.27 (m, 1H, Ar H), 4.39 (q, *J*=7.2, 2H, OC*H*$_2$Me), 3.74 (s, 2H, CH_2), 3.65 (s, 2H, CH_2), 2.93 (d, *J*=5.9, 2H, CH_2), 2.80 (t, *J*=5.8, 2H, CH_2), 1.40 (t, *J*=7.1, 3H, OCH$_2$*Me*). ^{13}C-NMR (101 MHz, $CDCl_3$): 168.2 (C2), 165.5 (C=O), 162.1 (C=O), 152.2 (Ar), 150.3 (Ar), 148.7 (Ar), 144.0 (Ar), 139.9 (Ar), 136.8 (Ar), 133.8 (Ar), 130.7 (C3a), 128.7 (2C, Ar), 124.2 (2C, Ar), 123.1 (C7a), 113.7 (C3), 61.1 (CH_2), 59.2 (CH_2), 51.5 (C7), 49.9 (C5), 26.8 (C4), 14.8 (Me). Anal. calc. for $C_{23}H_{22}N_4O_5S$: C, 59.22; H, 4.75; N, 12.01; found: C, 59.30; H, 4.79; N, 12.10.

Ethyl 2-(4-Nitrobenzamido)-6-(1-phenylethyl)4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**2d**). Yield: 79%. ^{1}H-NMR (400 MHz, $CDCl_3$): 12.46 (s, 1H,NH), 8.37 (d, *J*=8.9, 2H, Ar H), 8.17 (d, *J*=8.8, 2H, Ar H), 7.51–7.30 (m, 5H, Ar H), 4.38 (q, *J*=7.1, 2H, OC*H*$_2$Me), 3.91 (d, *J*=14.8, 1H, CH_2), 3.85 (q, *J*=6.8, 1H, CH), 3.75 (d, *J*=14.9, 1H, CH_2), 3.11–2.71 (m, 4H, CH_2), 1.59 (d, *J*=6.7, 3H, CH_2), 1.40 (t, *J*=7.1, 3H, OC*H*$_2$Me). ^{13}C-NMR (101 MHz, $CDCl_3$): 167.0 (C2), 161.2 (C=O), 158.2 (C=O), 150.2 (Ar), 147.6 (Ar), 143.9 (Ar), 130.2 (C3a), 128.7 (2C, Ar), 128.5 (2C, Ar), 127.5 (2C, Ar), 124.2 (2C, Ar), 123.4 (C7a), 121.1 (Ar), 112.4 (C3), 64.0 (CH_2), 61.0 (CH), 49.1 (C7), 47.7 (C5), 27.1 (C4), 20.4 (Me), 14.3 (Me). Anal. calc. for $C_{25}H_{25}N_3O_5S$: C, 62.62; H, 5.25; N, 8.76; found: C, 62.55; H, 5.21; N, 8.80.

Ethyl 6-(1-Cyclohexylethyl)-2-(4-nitrobenzamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (**2e**/**2f**). Yield: 88%. $[\alpha]_D^{20}$ (*c*=1.00, $CHCl_3$):+ 16.1 ((*S*)-enantiomer). ^{1}H-NMR (400 MHz, $CDCl_3$): 12.48 (s, 1H, NH), 8.37 (d, *J*=8.8, 2H, Ar), 8.18 (d, *J*=8.8, 2H, Ar), 4.39 (q, *J*=7.1, 2H, OC*H*$_2$Me), 3.74 (d, *J*=14.3, 1H, CH_2), 3.56 (d, *J*=14.5, 1H, CH_2), 2.99–2.76 (m, 4H, CH_2), 2.67–2.54 (m, 1H, Cy), 2.50–2.39 (m, 1H, Cy), 1.88–1.59 (m, 2H, Cy), 1.42 (t, *J*=7.1, 3H, OCH$_2$*Me*), 1.33–1.11 (m, 4H, Cy), 1.01 (d, *J*=6.6, 3H, Me), 0.96–0.75 (m, 2H, CH_2). ^{13}C-NMR (101 MHz, $CDCl_3$): 167.0 (C2), 161.2 (C=O), 150.1 (C=O), 147.4 (Ar), 137.9 (Ar), 130.3 (C3a), 128.6 (2C, Ar), 124.1 (2C, Ar), 123.2 (C7a), 112.5 (C3), 65.8 (CH), 63.7 (CH_2), 60.9 (CH_2), 47.5 (C7), 45.6 (C5), 41.1 (CH_2), 31.1 (CH_2), 30.1 (CH_2), 27.8 (CH_2), 26.7 (C4), 26.5 (CH_2), 14.3 (Me), 10.3 (Me). Anal. calc. for $C_{25}H_{31}N_3O_5S$: C, 61.84; H, 6.43; N, 8.65; found: C, 61.93; H, 6.47; N, 8.60.

Ethyl 2-(4-Nitrobenzamido)-6-(1,2,3,4-tetrahydronaphthalen-1-yl)-4,5,6,7-tetrahydrothieno[2,3*c*]pyridine-3-carboxylate (**2g**/**2h**). Yield: 79%. $[\alpha]_D^{20}$ (*c*=1.00, $CHCl_3$):+31.2 ((*S*)-enantiomer).

^{1}H-NMR (400 MHz, $CDCl_3$): 12.50 (s, 1H, NH), 8.37 (d, *J*=8.8, 2H, Ar H), 8.18 (d, *J*=8.9, 2H, Ar H), 7.85–7.62 (m, 1H, Ar H), 7.22–7.01 (m, 3H, Ar H), 4.39 (q, *J*=7.1, 2H, O*CH*$_2$Me), 4.06 (dd, *J*=9.5, 4.6, 1H, CH), 3.84 (d, *J*= 14.3, 1H, CH_2), 3.71 (d, *J*=14.3, 1H, CH_2), 3.02–2.57 (m, 6H, CH_2), 2.05 (d, *J*=5.4, 2H, CH_2), 1.85–1.66 (m, 2H, CH_2), 1.40 (t, *J*=7.1, 3H, OCH_2*Me*). ^{13}C-NMR (101 MHz, $CDCl_3$): 167.1 (C2), 163.1 (C=O), 161.2 (C=O), 150.1, 147.4, 143.6, 138.3, 130.3, 129.4, 128.9, 128.6 (2C), 128.0, 126.5 (C3a), 125.8 (C7a), 124.1 (2C), 118.6 (C3), 64.1 (CH) 62.6 (CH_2), 61.0 (C7), 47.3 (C5), 29.8 (CH_2), 28.2 (C4), 21.9 (CH_2), 21.7 (CH_2), 14.3 (Me). Anal. calc. for $C_{27}H_{27}N_3O_5S$: C, 64.14; H, 5.38; N, 8.31; found: C, 64.10; H, 5.44; N, 8.37.

Diffraction data for the crystal **2b** have been collected by means of a Bruker-Axs CCD-based three circle diffractometer, working at ambient temperature with graphite-monochromatic MoK_α X-radiation (λ= 0.7107 Å). X-ray diffraction data in the θ range 2–25° were collected acquiring four sets of 600 bidimensional CCD frames with the following operative conditions: omega rotation axis, scan width 0.2°, acquisition time 20 s, sample-to-detector distance 60 mm, phi angle fixed at four different values (0°, 270°, 160°, 40°) for the four different sets. Omegarotation frames were processed with the SAINT software[21] for data reduction (including intensity integration, background, Lorentz and polarization corrections) and for determination of accurate unit-cell dimensions, obtained by least-squares refinement of the positions of 3571 independent reflections with I >10σ(I) in the θ range 3–21°. Absorption effects were empirically evaluated by the SADABS software[22] and absorption correction was applied to the data (0.805 and 0.994 min and max transmission factor).

Crystal Data for **2b**. $C_{24}H_{22}FN_3O_5S$, M_r =483.51 g/ mol, Monoclinic, Space group $P2_1/n$, *a*=15.4546(15) Å, *b*=5.6367(6) Å, *c*=26.566(3) Å, β=98.429(2)°, *V*= 2289.2(4) Å^3, *Z*=4, $D_{calc.}$ =1.403 Mg/m^3, *R*=0.0494 (2735 reflections), wR_2 =0.1466, *T*=293(2) K, GOF= 1.016. The reflections were collected in the range 2.43°θ25.03° employing a 0.50×0.18×0.03 crystal. CCDC number: 1822131

In Vitro *Antibacterial Activity*

Antibacterial tests were performed to assess the *in vitro* antibacterial activity of every compound against two bacterial strains, namely *Sarcina lutea* ATCC 9341 (*S. lutea*; Gram-positive bacteria) and *Escherichia coli* ATCC 8739 (*E. coli*; Gram-negative bacteria).

Solutions at different compound concentrations were prepared as described hereinabove. Briefly, 50 × stock solution of every compound was serially diluted in Luria–Bertani broth (LB) to give working concentrations ranging from 1,000 to 0.5 μM (2-fold serial dilution; *n*=12 concentrations for each compound).

S. lutea and *E. coli* bacterial strains were precultured in 5 mL of LB at 37°C under shaking at 130 rpm for 20 h, until reaching an optical density at λ_{max} =600 nm (OD_{600nm})1, corresponding to 10^9 bacteria/mL. Bacterial suspensions were then diluted to obtain a final concentration of
10^6 bacteria/mL, hereafter used as the test inoculum. Afterwards, bacterial suspensions (50 μL/well) were inoculated in 96-well plates at a density of 1.5× 10^5 bacteria/cm^2, then 50 μL/well of each compound dilutions (*n*3 per condition) in LB were added. Bacteria inoculated in 50 μL/well of LB were used as internal reference for growth (CTRL; *n*3 per condition), while bacteria inoculated in 50 μL/well of gentamicin and neomycin solutions (gentamicin: IC_{50} =2.2 μM; neomycin: IC_{50} =4.3 μM) were used as positive controls of antibacterial activity against *S. lutea* and *E. coli*, respectively. After 24-h incubation, OD_{600nm} of each well was read with a Sunrise microplate reader (Tecan, Italy). Viability of bacterial controls was assigned as 100%. For each compound, the bacterial percent survival was calculated as follows:

Bacterial survival ð%Þ ¼ ð$OD_{600nm,sample}$=ð$OD_{600nm,CTRL}$Þ

100

For each compound tested, the concentration required to suppress 50% bacterial growth (IC_{50}) was obtained by non-linear curve fitting plots of OD_{600nm} vs. log[compound] using GraphPad version 6 (GraphPad software, La Jolla, CA, USA).

All reagents and solvents were purchased from commercial sources and used without further purification. The reactions were carried out under

atmospheric air unless otherwise indicated, such as moisturesensitive ones, for which a static nitrogen atmosphere was used.

Author Contribution Statement

Arianna Rossetti and Alessandro Sacchetti performed the research, conceived and designed the experiments and wrote the article. Gabriella Roda assisted in writing the manuscript and analyzing the data. Fiorella Meneghetti performed the X-ray analysis and assisted in writing the manuscript. Nina Bono and Gabriele Candiani performed the biological assays and assisted in writing the manuscript.

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