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Synthesis and biological evaluation of tetracyclic thienopyridones as antibacterial and antitumor agents

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ABSTRACT

A facile synthesis of model 4-oxopyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylic acids **9a**–**e** was achieved via Stille arylation of 2-chloro-3-nitro-4-oxothieno[2,3-*b*]pyridine-5-carboxylate and a subsequent microwave-assisted phosphite-mediated Cadogan reaction. The new compounds were tested for their in vitro antimicrobial and antiproliferative activity. Compounds **9a**–**c** and **9e** exhibited very high potency against Gram positive *Bacillus subtilis* and *Bacillus megaterium* at concentrations 0.000015–0.007 μg/mL. They also displayed excellent activity towards other Gram positive bacilli and staphylococci and Gram negative *Haemophilus influenzae*, being in most cases superior or equal to commercial fluoroquinolones. Both **9a** and **9c** were inhibitors of the DNA gyrase activity. As concerns antitumor properties, compounds **9b**–**e** showed growth inhibition of MCF-7 breast tumor and A549 non-small cell lung cancer cells with IC_{50} 1.6–2.8 μM and 2.6–6.9 μM, respectively, coupled with absence of cytotoxicity towards normal cells. These compounds are promising as dual acting chemotherapeutics.

1. Introduction

The fluoroquinolones constitute a major class of antibacterial chemotherapeutic agents which have a broad spectrum against Gram positive and Gram negative bacteria.[1–3] Examples include norfloxacin **1a**[2] and ciprofloxacin **1b**[3] (Fig. 1) which were the first two quinolones marketed consecutively in 1986 and 1987 for human use. Since 1986, more than twenty fluoroquinolones have been approved by FDA and most of them remain on the market.[1f]

On the other hand, several 4-oxothieno[2,3-*b*]pyridine-5-carboxylic acids (e.g. **2a**–**e**, Fig. 1), potential bioisosters of quinolone antibacterials, were prepared and bioassayed.[4–11] Substitution at the N(7)-position of thienopyridones has been reported for alkyl groups, for example, compounds **2a**[4] and **2b**[5,6] which exhibited good level of activity against Gram negative bacteria. *N*(7)-Aryl and -heteroaryl substitution has also been achieved, exemplified by compounds **2c**[12] and **2d**[13] that exhibited good level of activity against Gram negative and Gram positive bacterial strains. In addition, selected *N*(7)-azacyclohexyl derivatives and related congeners, such as 7-(*N*,*N*-dimethylamino) derivative **2e**, have been prepared;[14] the latter exhibited good level of activity, especially against *Klebsiella pneumoniae* and *Salmonella paratyphi A* (MIC 0.5 and 1.0 μg/mL, respectively).[14]

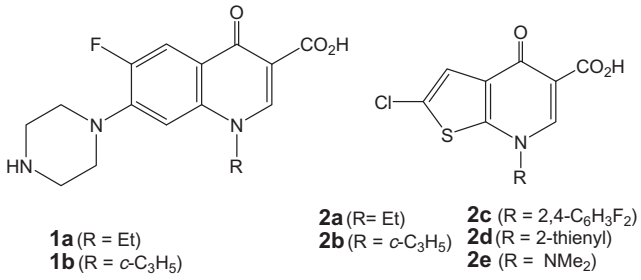

Figure 1. Structures of some fluoroquinolones (**1a**–**b**) and thieno[2,3-*b*]pyridones (**2a**–**e**).

Quite recently, we have also explored the synthesis of some tetracyclic fluoroquinolones (Fig. 2) such as 4-oxopyrido[2,3-*a*]carbazole-3-carboxylic acids (**3**),[15] and 4-oxothieno[2′,3′:4,5]-pyrrolo[3,2-*h*]quinoline-3-carboxylic acid (**4**).[15,16]

As part of an ongoing program aimed at developing facile synthesis of novel antibacterial agents, we directed our attention

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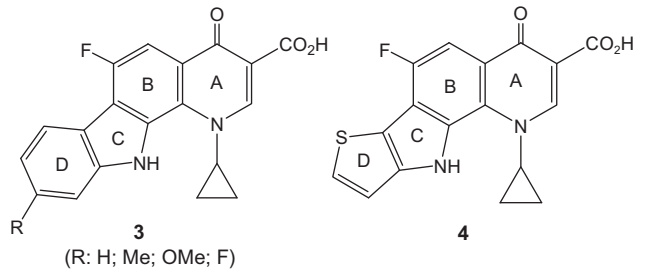


Figure 2. Structures of some tetracyclic fluoroquinolones.

towards 4-oxothieno[2,3-*b*]pyridine-5-carboxylic acid derivatives that are bioisosters of fluoroquinolone antibacterials. To the best of our knowledge, fused rings with a bridge between the critical C-2 and C-3 positions have not yet been investigated in this 4-oxothieno[2,3-*b*]pyridine system. At the same time, the designed compounds are isosteric with pyridocarbazoles, a class to which the natural ellipticine antitumor drug belongs.[15] Accordingly, we report herein the synthesis and in vitro evaluation of the antimicrobial and antitumor properties of novel tetracyclic thienopyridones such as the variously substituted 4-oxopyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylic acids **9a**–**e**, shown in Scheme 1.

2. Result and discussion

2.1. Synthesis

Microwave-enhanced Cadogan cyclization of *ortho*-nitrobiaryls, in combination with phosphite reagent, has provided easy access to substituted carbazoles and other fused heterocyclic systems.[17,18] Based on the above methodology, the synthesis of the target 4-oxopyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylic acids **9a**–**e** and their ethyl esters **8a**–**e** was achieved by utilizing ethyl 2-chloro-7-cyclopropyl-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate (**5**)[5,6] as the starting material, and constructing the fused thieno[*b*]indole nuclei via a two-step procedure as illustrated in Scheme 1. The first key-step involves palladium-catalyzed carbon–carbon cross coupling between **5** and the appropriate 2-thienyltrimethyl stannane (**6a**–**e**)[19–21] (Stille reaction)[22–30] with ultimate production of the respective ethyl 2-aryl-7-cyclopropyl-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate **7a**–**e**. In a subsequent step, the latter compounds undergo phosphite-mediated reductive cyclization (Cadogan reaction) assisted by microwave irradiation to furnish the respective ethyl 4-oxopyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylates **8a**–**e**. This reaction entails the generation of a nitrene intermediate that undergoes insertion into an 7-thienyl C–H bond to form a fused pyrrole ring (C) embedded in the tetracyclic product. It is of note that in absence of microwave irradiation, the phosphite-mediated nitro group-deoxygenation step, leading to **8**, does not proceed to any detectable extent at 160 °C for 96 h. Acid-catalyzed hydrolysis of the esters **8a**–**e** produced the corresponding target 4-oxothieno[2′,3′:4,5]pyrrolo[3,2-*h*]quinoline-3-carboxylic acids **9a**–**e**.

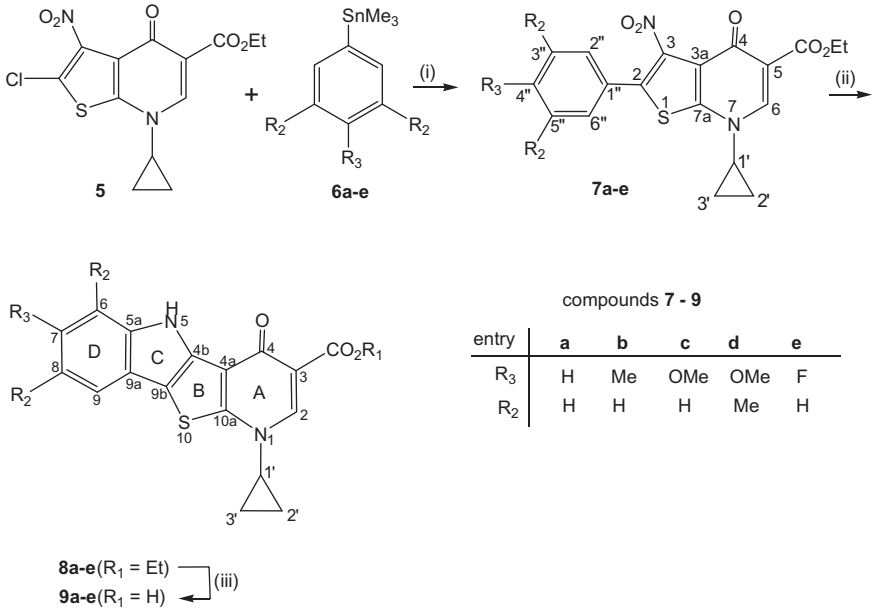
The new compounds **7**–**9** were characterized by elemental analyses, IR, MS and NMR spectral data. These data, detailed in the experimental part, are consistent with the suggested structures. Thus, the mass spectra display the correct molecular ion peaks for which the measured high resolution (HRMS) data are in good agreement with the calculated values. DEPT and 2D (COSY, HMQC, HMBC) experiments showed correlations that helped in the ^{1}H- and ^{13}C-signal assignments to the different carbons and their attached, and/or neighboring hydrogens. In compounds **7e**–**9e**, the carbons of the benzo-fused ring D are readily identified by their doublet signals (with varying J_{C-F}-values) originating from coupling with the nearby fluorine atom at C-7.

2.2. Biology

2.2.1. Antimicrobial activity

The new thienopyridones **9a**–**e** were screened for their in vitro antimicrobial activity against model Gram positive and Gram negative bacteria, including multidrug-resistant species, yeasts and mould. The minimum inhibitory concentrations are listed in Table 1 and are compared with the results obtained for standard antibacterial quinolones ciprofloxacin, levofloxacin, moxifloxacin and gemifloxacin.

All the investigated compounds presented remarkable antibacterial properties against Gram positive bacteria, both bacilli and



compounds **7 - 9**

entry	a	b	c	d	e
R_3	H	Me	OMe	OMe	F
R_2	H	H	H	Me	H

Scheme 1. Reagents and conditions: (i) $Pd(OAc)_2$, CsF, DMF, 50–65 °C; (ii) $P(OEt)_3$, 175–200 °C (MW); (iii) 10% aq HCl, EtOH, reflux.

Table 1
Inhibitory activity of compounds **9a–e** against bacteria and fungi, expressed as MIC (μg/mL)

Microorganism[a]	Compound[b]								
	9a	**9b**	**9c**	**9d**	**9e**	CIP	LEV	MOX	GEM
Gram positive bacteria									
BC	0.007	0.3	0.03	3	0.007	0.3	0.15	0.3	0.07
BM	0.000015	0.007	0.00003	3	0.000015	0.007	0.03	0.003	0.0003
BS	0.000015	0.0003	0.0003	3	0.000015	0.03	0.03	0.015	0.007
BTK	0.007	0.15	0.007	1.5	0.003	0.03	0.07	0.03	0.015
SA	0.003	0.07	0.015	6	0.003	0.3	0.07	0.03	0.015
SAR 72	0.7	12	1.5	3	0.7	100	25	12	6
SAR 4790	>100	>100	>100	>100	>100	>100	100	50	50
SE	0.03	0.3	0.15	3	0.03	0.07	0.15	0.07	0.015
SER	3	25	6	3	1.5	100	>100	50	50
Gram negative bacteria									
AB	0.07	0.7	3	>100	0.15	0.7	0.15	0.15	0.3
ABR	>100	>100	>100	—[c]	>100	>100	>100	50	100
EC	25	>100	>100	>100	50	0.015	0.03	0.03	0.015
ECR	>100	—	—	—	>100	100	25	50	50
HI	0.03	1.5	0.15	6	0.15	0.15	0.15	0.03	0.015
PA	>100	>100	>100	>100	>100	0.07	0.3	0.7	0.07
Fungi									
SC	>100	>100	>100	>100	12	>100	>100	>100	>100
CT	>100	>100	>100	>100	>100	>100	>100	>100	>100
AN	>100	>100	>100	>100	>100	>100	>100	>100	>100

[a] BC, *Bacillus cereus* ATCC 11778; BM, *Bacillus megaterium* ATCC 19213; BS, *Bacillus subtilis* ATCC 6633; BTK, *Bacillus thuringiensis* var. *kurstaki* BGSC 4D1; SA, *Staphylococcus aureus* ATCC 6538; SAR 72 and SAR 4790, *Staphylococcus aureus* quinolone- and penicillin-resistant clinical isolates; SE, *Staphylococcus epidermidis* ATCC 12228; SER, *Staphylococcus epidermidis* quinolone- and penicillin-resistant clinical isolate; AB, *Acinetobacter baumannii* ATCC 19606; ABR, *Acinetobacter baumannii* quinolone- and penicillin-resistant clinical isolate; EC, *Escherichia coli* ATCC 8739; ECR, *Escherichia coli* quinolone- and penicillin-resistant clinical isolate; HI, *Haemophilus influenzae* ATCC 19418; PA, *Pseudomonas aeruginosa* ATCC 9027; SC, *Saccharomyces cerevisiae* ATCC 9763; CT, *Candida tropicalis* ATCC 1369; AN, *Aspergillus niger* ATCC 6275.
[b] CIP, ciprofloxacin; LEV, levofloxacin; MOX, moxifloxacin; GEM, gemifloxacin.
[c] Not tested because inactive against the corresponding quinolone-sensitive microorganism.

staphylococci, in most cases at concentrations lower than reference drugs. Interestingly, compounds **9a** and **9e** exhibited unprecedented powerful activity at 0.000015 μg/mL against *Bacillus megaterium* and *Bacillus subtilis*. These microorganisms were also inhibited by **9b** and **9c** at 0.00003–0.007 μg/mL, so appearing to be the most sensitive strains. Furthermore, compounds **9a–c** and **9e** displayed excellent activity against other tested bacilli and wild staphylococci. The inhibition of *Bacillus cereus*, *Bacillus thuringiensis* and *Staphylococcus aureus* was achieved by compounds **9a**, **9c** and **9e** at concentrations of 0.003–0.03 μg/mL, generally lower than those of standard quinolones (MICs 0.015–0.3 μg/mL), and also against *Staphylococcus epidermidis* compounds **9a** and **9e** at 0.03 μg/mL were superior to reference ciprofloxacin, levofloxacin and moxifloxacin (MICs 0.07–0.15 μg/mL). As concerns compound **9d**, it showed significant activity against all tested bacteria at concentrations 1.5–6 μg/mL.

Of particular relevance is that thienopyridones exerted strong antibacterial effectiveness against both multidrug-resistant strains of *S. aureus* (SAR 72) and *S. epidermidis*, their minimum inhibitory concentrations ranging from 0.7 to 25 μg/mL and displaying for compounds **9a** and **9c–e** higher potency as compared to the standard quinolones used in this study.

Almost all of the studied thienopyridones exhibited also a marked degree of activity against Gram negative bacteria. Among these, *Acinetobacter baumannii* and, especially, *Haemophilus influenzae* were found to be more sensitive in comparison to *Escherichia coli* and *Pseudomonas aeruginosa*. Unsubstituted **9a** displayed the highest activity, inhibiting the growth of *H. influenzae* and *A. baumannii* at concentrations of 0.03 and 0.07 μg/mL, respectively, comparable or lower than those of the reference quinolones. Strong effect was shown by fluoro derivative **9e** at 0.15 μg/mL, while compounds **9b** and **9c** were found to exhibit high to good antibacterial properties (MIC 0.15–3 μg/mL). A significant inhibition was also observed for **9d** towards *H. influenzae*. Only **9a** and **9e** showed activity against *E. coli* with MIC values 25–50 μg/mL, higher than those of standard substances. However, none of the tested thienopyridones inhibited *P. aeruginosa* and multidrug-resistant Gram negative strains even at the higher concentration of 100 μg/mL.

It is worthwhile to note that **9e** was the only compound exhibiting antifungal properties, when compared to the other thienopyridones and quinolones tested. This chemical displayed against yeast *Saccharomyces cerevisiae* an effect equal to that of standard antifungal miconazole (MIC 12 μg/mL), but it was found to be less active than the reference drug against *Candida tropicalis* and *Aspergillus niger* (MIC of miconazole 6 and 3 μg/mL, respectively).

The structure–activity relationships analysis of the data reported in Table 1 shows that the thiophene isosteric replacement of the fluorobenzene (ring B) in the tetracyclic structure of fluoroquinolones **3**[15] played a positive role in the antibacterial effectiveness, with a general increase of activity, especially against bacilli, and including resistant *S. aureus* SAR 72 and *S. epidermidis* strains. Overall, **9a**, unsubstituted, and **9e**, carrying an electron-withdrawing fluorine group with low bulkiness and a comparable size to the hydrogen atom, showed higher effectiveness against both Gram positive and Gram negative bacteria, as compared to methyl and methoxy substituted thienopyridones **9b–d**. MIC values exhibited by **9e** were equal to those of **9a** with a slight enhancement in the activity against Gram positive *B. thuringiensis* and resistant *S. epidermidis* strain and a weak reduction against Gram negative *A. baumannii*, *E. coli* and *H. influenzae*. It is worth noting that fluoro substituted **9e** is also endowed with a certain antifungal activity. The introduction in the *para* position of the benzene ring D of bulky and electron-donating substituents, such as a hydrophilic methoxy group and, in a more extent, a lipophilic methyl group, leads to comparatively less active compounds **9c** and **9b**. The antibacterial properties of the latters are further considerably reduced in three-substituted **9d**, except when multidrug-resistant *S. aureus* SAR 72 and *S. epidermidis* strains were tested. In the studied thienopyridones the presence of a fluorine atom on the benzene ring D of

Table 2
Inhibitory activity of compounds **9a** and **9c** against DNA gyrase and topoisomerase IV of *E. coli*, expressed as 50% inhibitory concentration (μg/mL)

Compound	IC_{50}	
	Gyrase[a]	Topoisomerase IV[b]
9a	1.0	>24
9c	1.1	>24
Ciprofloxacin	0.34	4.60
Moxifloxacin	0.85	5.00

[a] *E. coli* DNA gyrase supercoiling assay.
[b] *E. coli* topoisomerase IV decatenation assay.

the tetracyclic ring system has not the significant impact on the antibacterial properties as exhibited in the fluoroquinolones **3**, where the *para*-fluoroderivative is more active than the unsubstituted analogue, and the most active derivative among the compounds **3**. The introduction of bulky and multiple substituents seems detrimental for the antimicrobial activity tested, as already observed for the tetracyclic isosters **3**.

2.2.2. DNA gyrase and topoisomerase IV inhibition

Bacterial DNA gyrase of Gram negative strains and topoisomerase IV of Gram positive ones are well-characterized clinically validated targets of the quinolone antibacterials.[1] Therefore, the enzymatic inhibition of compounds **9a** and **9c**, possessing potent antibacterial activity against a wide spectrum of microorganisms and structurally related to quinolones, was tested against DNA gyrase and topoisomerase IV isolated from *E. coli*. Data reported in Table 2 show that both the tested compounds displayed inhibitory properties towards DNA gyrase, with IC_{50} values 1.0–1.1 μg/mL (ciprofloxacin and moxifloxacin IC_{50} 0.34 and 0.85 μg/mL, respectively), whereas they did not inhibit topoisomerase IV up to the concentration of 24 μg/mL. The different target affinity suggests that the great antibacterial potency exhibited by thienopyridones against Gram positive organisms was not due to inhibition of topoisomerase IV and hints that other mechanisms are involved in the antibacterial effect.

2.2.3. Antitumor activity

The antitumor activity of compounds **9a**–**e** was assayed with respect to ellipticine by evaluating cell proliferation in MCF-7 breast cancer, in A549 non-small cell lung cancer (NSCLC) cell lines and in normal human-derm fibroblasts (HuDe). After 72 h cell viability was determined: against HuDe cells no effect was detected until 10 μM, while against both the tumor cell lines all the tested compounds showed significant inhibition of cell proliferation in a dose-dependent manner (Table 3). Ellipticine showed IC_{50} of 1.6 μM and 3.4 μM against MCF-7 and A549 cells, respectively. The most potent compounds **9c** and **9e** had comparable IC_{50} to the reference substance, ranging between 1.6 and 1.9 μM against MCF-7 cells, and between 2.6 and 2.7 μM against A549 cells. Compound **9a** showed the lowest effect on cell proliferation against both cell lines (IC_{50} 10 and 5.2 μM against A549 and MCF-7 cells, respectively). Replacement of the B benzene ring of compounds **3** with its heterocyclic bioisoster thiophene (**9a**–**e**) did not significantly modify the antiproliferative activity as a further evidence for the bioisosteric equivalence between benzene and thiophene rings.

Table 3
Effects of compounds **9a**–**e** and ellipticine on MCF-7, A549 and HuDE cell lines

Compound	IC_{50} MCF-7, μM (breast cancer)	IC_{50} A549, μM (NSCLC)	IC_{50} HuDe, μM
9a	5.2 ± 1.09	10 ± 1.04	>10
9b	2.8 ± 1.05	6.9 ± 1.06	>10
9c	1.6 ± 1.04	2.6 ± 1.02	>10
9d	2.5 ± 1.05	4.7 ± 1.05	>10
9e	1.9 ± 1.04	2.7 ± 1.02	>10
Ellipticine	1.6 ± 1.06	3.4 ± 1.04	>10

Cells were treated with the indicated compounds at concentrations ranging from 0.1 to 20 μM for 72 h and then viability was determined by MTT assay. Concentration that inhibits 50% (IC_{50}) (e.g., the point at which viability is 50%) was extrapolated from the dose–response curves. Representative results of at least 3 independent experiments are reported.

3. Conclusion

Novel 1-cyclopropyl-4-oxopyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylic acids **9a**–**e** were synthesized by efficient synthetic routes and their antimicrobial and antitumor properties assessed. Four of these five tetracyclic thieno[2,3-*b*]pyridones showed unprecedented powerful activity against *B. megaterium* and *B. subtilis*, excellent activity against *S. aureus* and also against Gram negative *H. influenzae*, in addition to activity against few resistant Gram positive bacteria. Furthermore, these compounds had high anticancer activity against breast MCF-7 and lung A549 tumor cell lines, and were devoid of cytotoxicity against normal cells.

The combination of potent activity against bacteria and cancer cell lines, together with the absence of cytotoxicity against normal cells, makes these agents of interest in the search for novel potential antimicrobials able to reduce the danger of bacterial infections in the frequently immunocompromised cancer patients.[31]

4. Experimental

4.1. Chemistry

Synthetic starting material, reagents and solvents were purchased from Aldrich Chemical Co. Silica gel for column chromatography was purchased from Macherey-Nagel GmbH & Co (Germany). Melting points (uncorrected) were determined on a Stuart scientific melting point apparatus in open capillary tubes. ^1H and ^{13}C NMR spectra were recorded on a 300 MHz spectrometer (Bruker DPX-300) with TMS as the internal standard. Chemical shifts are expressed in δ units; ^1H–^1H, H–F and C–F coupling constants are given in hertz (Hz). Electron-impact mass spectra (EIMS) were obtained using a Finnegan MAT TSQ-70 spectrometer at 70 eV; ion source temperature 200 °C. High resolution mass spectra (HRMS) were measured by electrospray ionization (ESI) technique on a Bruker APEX-2 instrument. The samples were dissolved in acetonitrile, diluted in spray solution (methanol/water 1:1 v/v + 0.1% formic acid) and infused using a syringe pump with a flow rate of 2 μL/min. External calibration was conducted using Arginine cluster in a mass range *m/z* 175–871. IR spectra were recorded as KBr discs on a Nicolet Impact-400 FT-IR spectrophotometer. Microwave irradiation experiments, conducted for the Cadogan–nitrene insertion reactions, were carried out with a Biotage Initiator 2.0 Microwave Synthesizer Instrument. Elemental analyses were performed on a Euro Vector Elemental Analyzer (EA 3000 A).

4.1.1. Ethyl 2-chloro-7-cyclopropyl-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate (5)

This compound was prepared from 3-acetyl-2,5-dichlorothiophene, dimethyl carbonate, triethyl orthoformate and cyclopropylamine by following the literature procedures.[5,6]

4.1.2. General procedure for the synthesis of ethyl 2-aryl-7-cyclopropyl-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylates (7a–e)

The appropriate trimethyl(aryl)stannane (**6a**–**e**)[19–21] (3.9–6.9 mmol), $Pd(OAc)_2$ (0.065 g, 0.29 mmol), and CsF (0.44 g,

2.9 mmol) were successively added to a stirred solution of ethyl 2-chloro-7-cyclopropyl-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate (**5**) (1.0 g, 2.9 mmol) in DMF (4 mL). The reaction mixture was heated at 50–65 °C under nitrogen atmosphere for 12–15 h. The resulting solution was then cooled to rt and quenched with water (10 ml), whereby a black gummy precipitate was obtained. The latter product was treated with $CHCl_3$ and filtered to remove the insoluble matter. The filtrate was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give a brown residue. This crude product was purified by column chromatography using silica gel and eluting with chloroform/ethyl acetate to give **7a–e** as yellow solid products.

4.1.2.1. Ethyl 7-cyclopropyl-3-nitro-4-oxo-2-phenyl-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate (7a). Reaction temperature: 60 °C; reaction time: 14 h; ratio of the eluting mixture: 1:2, v/v. Yield 65%; mp 220–223 °C (dec). Anal. Calcd for $C_{19}H_{16}N_2O_5S$ (384.41): C, 59.37; H, 4.19; N, 7.29; S, 8.34. Found: C, 59.35; H, 4.27; N, 6.98; S, 8.21. IR: ν_{max} (KBr)/cm^{-1} 2971, 1728, 1699, 1623, 1535, 1499, 1452, 1372, 1321, 1241, 1150, 1074, 1034, 801, 799, 760 and 694; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.16 (m, 2H) and 1.28 (m, 2H) (H_2-2′/H_2-3′), 1.24 (t, J = 7.1 Hz, 3H, CH_3CH$_2$O–), 3.75 (m, 1H, H-1′), 4.20 (q, J = 7.1 Hz, 2H, –OCH_2Me), 7.48 (m, 5H, H-2″/H-6″, H-3″/H-5″, H-4″), 8.34 (s, 1H, H-6). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 14.7 (*C*H$_3$CH$_2$O–), 37.2 (C-1′), 60.8 (–O*C*H$_2$Me), 116.1 (C-5), 128.3 (C-2), 128.5 (C-2″/C-6″), 130.2 (C-3″/C-5″), 141.6 (C-3), 120.7 (C-1″), 131.1 (C-3a), 130.9 (C-4″), 146.4 (C-6), 149.9 (C-7a), 164.3 (*C*O$_2$Et), 168.2 (C-4). HRMS (ESI): found 385.08583 ([M+H]$^+$), $C_{19}H_{17}N_2O_5S$ requires 385.08527; found 407.06777 ([M+Na]$^+$), $C_{19}H_{16}N_2O_5SNa$ requires 407.06721; found 791.14567 ([2M+Na]$^+$), $C_{38}H_{32}N_4O_{10}S_2$-Na requires 791.14521; *m*/*z* (EI): 384 (M$^+$, 7), 339 (8), 312 (100), 296 (6), 267 (23), 250 (22), 236 (18), 225 (11), 185 (7), 165 (6), 105 (8).

4.1.2.2. Ethyl 7-cyclopropyl-2-(4-methylphenyl)-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate (7b). Reaction temperature: 55 °C; reaction time: 12 h; ratio of the eluting solvent mixture: 1:2, v/v. Yield 70%; mp 282–284 °C (dec) Anal. Calcd for $C_{20}H_{18}N_2O_5S$ (398.44): C, 60.29; H, 4.55; N, 7.03; S, 8.05. Found: C, 59.98; H, 4.41; N, 6.91; S, 7.76. IR: ν_{max} (KBr)/cm^{-1} 2924, 1728, 1615, 1535, 1499, 1452, 1325, 1248, 1150, 1070 and 805; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.14 (m, 2H) and 1.27 (m, 2H) (H_2-2′/H_2-3′), 1.23 (t, J = 7.1 Hz, 3H, CH_3CH$_2$O–), 2.33 (s, 3H, C(4″)–CH_3), 3.74 (m, 1H, H-1′), 4.19 (q, J = 7.1 Hz, 2H, –OCH_2Me), 7.32 (d, J = 8.2 Hz, 2H, H-3″/H-5″), 7.36 (d, J = 8.2 Hz, 2H, H-2″/H-6″), 8.34 (s, 1H, H-6). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 14.7 (*C*H$_3$CH$_2$O–), 21.3 (C(4″)–CH$_3$), 37.2 (C-1′), 60.8 (–O*C*H$_2$Me), 116.1 (C-5), 123.1 (C-3a), 125.4 (C-1″), 128.2 (C-2″/C-6″), 128.4 (C-2), 130.8 (C-3″/C-5″), 141.0 (C-3), 141.0 (C-4″), 146.4 (C-6), 149.6 (C-7a), 164.4 (*C*O$_2$Et), 168.2 (C-4). HRMS (ESI): found 421.08389 ([M+Na]$^+$), $C_{20}H_{18}N_2O_5SNa$ requires 421.08286; found 819.17760 ([2M+Na]$^+$), $C_{40}H_{36}N_4O_{10}S_2Na$ requires 819.17651; *m*/*z* (EI): 398 (M$^+$, 10), 353 (3), 350 (7), 326 (100), 279 (6), 250 (6), 195 (6), 182 (14), 165 (11), 152 (6), 135 (9), 107 (7), 83 (9).

4.1.2.3. Ethyl 7-cyclopropyl-2-(4-methoxyphenyl)-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate (7c). Reaction temperature: 65 °C; reaction time: 15 h; ratio of the eluting solvent mixture: 1:2, v/v. Yield 42%; mp 285–288 °C (dec). Anal. Calcd for $C_{20}H_{18}N_2O_6S$ (414.44): C, 57.96; H, 4.38; N, 6.76; S, 7.74. Found: C, 57.64; H, 4.08; N, 6.84; S, 7.97%. IR: ν_{max} (KBr)/cm^{-1} 2997, 1699, 1630, 1605, 1539, 1499, 1445, 1248, 1183, 1027, 841 and 798; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.15 (m, 2H) and 1.27 (m, 2H) (H_2-2′/H_2-3′), 1.24 (t, J = 7.1 Hz, 3H, CH_3CH$_2$O–), 3.76 (m, 1H, H-1′), 3.79 (s, 3H, OCH_3), 4.20 (q, J = 7.1 Hz, 2H, –OCH_2Me), 7.07 (d, J = 8.7 Hz, 2H, H-3″/H-5″), 7.42 (d, J = 8.7 Hz, 2H, H-2″/H-6″), 8.33 (s, 1H, H-6). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 14.7 (*C*H$_3$CH$_2$O–), 37.2 (C-1′), 56.0 (O*C*H$_3$), 60.8 (–O*C*H$_2$Me), 115.7 (C-3″/C-5″), 116.1 (C-5), 120.3 (C-3a), 120.6 (C-1″), 130.0 (C-2″/C-6″), 130.5 (C-2), 140.3 (C-3), 146.2 (C-6), 149.3 (C-7a), 161.4 (C-4″), 164.4 (*C*O$_2$Et), 168.1 (C-4). HRMS (ESI): found 415.09591 ([M+H]$^+$), $C_{20}H_{19}N_2O_6S$ requires 415.09583; found 829.18334 ([2M+H]$^+$), $C_{40}H_{37}N_4O_{12}S_2$ requires 829.18439; *m*/*z* (EI): 414 (M$^+$, 23), 369 (6), 342 (100), 338 (6), 326 (6), 297 (12), 270 (17), 252 (6), 151 (7), 124 (9), 123 (12), 109 (9).

4.1.2.4. Ethyl 7-cyclopropyl-2-(4-methoxy-3,5-dimethylphenyl)-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate (7d). Reaction temperature: 55 °C; reaction time: 15 h; ratio of the eluting solvent mixture: 1:1, v/v. Yield 61%; mp 290–292 °C (dec). Anal. Calcd for $C_{22}H_{22}N_2O_6S$ (442.49): C, 59.72; H, 5.01; N, 6.33; S, 7.25. Found: C, 60.02; H, 5.13; N, 6.52; S, 7.16. IR: ν_{max} (KBr)/cm^{-1} 3022, 2968, 2949, 1743, 1626, 1535, 1448, 1372, 1237, 1216, 1008 and 799; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.15 (m, 2H) and 1.27 (m, 2H) (H_2-2′/H_2-3′), 1.24 (t, J = 7.1 Hz, 3H, CH_3CH$_2$O–), 2.23 (s, 6H, C(3″)–CH_3/C(5″)–CH_3), 3.68 (s, 3H,-OCH_3), 3.73 (m, 1H, H-1′), 4.20 (q, J = 7.1 Hz, 2H, –OCH_2Me), 7.15 (s, 2H, H-2″/H-6″), 8.33 (s, 1H, H-6). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 14.7 (*C*H$_3$CH$_2$O–), 16.3 (C(3″)–*C*H$_3$/C(5″)–*C*H$_3$), 37.2 (C-1′), 60.0 (–O*C*H$_3$), 60.8 (–O*C*H$_2$Me), 116.1 (C-5), 123.0 (C-3a), 123.6 (C-1″), 128.8 (C-2″/C-6″), 129.1 (C-2), 132.6 (C-3″/C-5″), 138.2 (C-3), 146.3 (C-6), 149.5 (C-7a), 159.0 (C-4″), 164.4 (*C*O$_2$Et), 168.2 (C-4). HRMS (ESI): found 465.10901 ([M+Na]$^+$), $C_{22}H_{22}N_2O_6SNa$ requires 465.10908; *m*/*z* (EI): 442 (M$^+$, 23), 412 (6), 397 (12), 370 (100), 325 (8), 310 (6), 280 (5), 163 (3), 115 (2).

4.1.2.5. Ethyl 7-cyclopropyl-2-(4-fluorophenyl)-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate (7e). Reaction temperature: 50 °C; reaction time: 15 h; ratio of the eluting solvent mixture: 1:1, v/v. Yield 65%; mp 275–277 °C (dec). Anal. Calcd for: $C_{19}H_{15}N_2FO_5S$ (402.40) C, 56.71; H, 3.76; N, 6.96; S, 7.97. Found: C, 56.61; H, 3.66; N, 7.14; S, 7.78. IR: ν_{max} (KBr)/cm^{-1} 3011, 2968, 1724, 1615, 1543, 1492, 1452, 1372, 1321, 1252, 1230, 1168, 1077, 864, 837, 801 and 784; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.15 (m, 2H) and 1.28 (m, 2H) (H_2-2′/H_2-3′), 1.24 (t, J = 7.1 Hz, 3H, CH_3CH$_2$O–), 3.74 (m, 1H, H-1′), 4.20 (q, J = 7.1 Hz, 2H, –OCH_2Me), 7.37 (dd, $^3J_{H-F}$ = 8.8 Hz, J = 8.7 Hz, 2H, H-3″/H-5″), 7.54 (dd, $^4J_{C-F}$ = 5 Hz, J = 8.7 Hz, 2H, H-2″/H-6″), 8.35 (s, 1H, H-6). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 14.6 (*C*H$_3$CH$_2$O–), 37.2 (C-1′), 60.8 (-O*C*H$_2$Me), 116.1 (C-5), 117.3 (d, $^2J_{C-F}$ = 22 Hz, C-3″/C-5″), 122.9 (C-3a), 124.7 (d, $^4J_{C-F}$ = 2 Hz, C-1″), 129.3 (C-2), 131.1 (d, $^3J_{C-F}$ = 8.7 Hz, C-2″/C-6″), 141.5 (C-3), 146.5 (C-6), 149.9 (C-7a), 163.6 (d, $^1J_{C-F}$ = 246 Hz, C-4″), 164.3 (*C*O$_2$Et), 168.2 (C-4). HRMS (ESI): found 403.07631([M+H]$^+$), $C_{19}H_{16}N_2FO_5S$ requires 403.07585; found 425.05799 ([M+Na]$^+$), $C_{19}H_{15}N_2FO_5SNa$ requires 425.05779; found 827.12762 ([2M+Na]$^+$), $C_{38}H_{30}N_4F_2O_{10}S_2Na$ requires 827.12636; *m*/*z* (EI): 402 (M$^+$, 8), 357 (7), 330 (100), 314 (6), 285 (11), 254 (15), 214 (6), 163 (6), 139 (10), 123 (11), 95 (6).

4.1.3. General procedure for the synthesis of ethyl 1-cyclopropyl-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylates (8a–e)

A mixture of the appropriate ethyl 2-aryl-7-cyclopropyl-3-nitro-4-oxo-4,7-dihydrothieno[2,3-*b*]pyridine-5-carboxylate (**7a–e**) (0.3 mmol) and P(OEt)$_3$ (3–4 mL) was placed in a 10 mL glass vial. The vial was sealed tightly with an aluminum Teflon crimp® top and the mixture therein irradiated with microwaves (power level,

400 W) for 1.75 h at preselected temperature of 175–200 °C. Thereafter, the vial was cooled to 20 °C by gas jet cooling system and excess $P(OEt)_3$ was evaporated in vacuo. The residual solid was treated with $CHCl_3$ (3 × 4 mL) whereby the respective title products (**8a–e**) were obtained as light yellow solids.

4.1.3.1. Ethyl 1-cyclopropyl-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylate (8a). Irradiation time: 1.75 h. Yield 45%; mp 278–280 °C. Anal. Calcd for $C_{19}H_{16}N_2O_3S$ (352.41): C, 64.76; H, 4.58; N, 7.95. Found: C, 64.43; H, 4.22; N, 7.97. IR: ν_{max} (KBr)/cm^{-1} 3421, 2985, 1733, 1619, 1545, 1501, 1441, 1381, 1359, 1317, 1263, 1240, 1209, 1170, 1130, 1111, 1041, 1022 and 797; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.19 (m, 2H) and 1.26 (m, 2H) (H_2-2′/H_2-3′), 1.28 (t, J = 7.0 Hz, 3H, CH_3CH$_2$O–), 3.76 (m, 1H, H-1′), 4.23 (q, J = 7.0 Hz, 2H, –OCH_2Me), 7.10 (dd, J = 7.2, 7.8 Hz, 1H, H-8), 7.20 (dd, J = 7.2, 8.0 Hz, 1H, H-7), 7.58 (d, J = 8.0 Hz, 1H, H-6), 7.78 (d, J = 7.8 Hz, 1H, H-9), 8.38 (s, 1H, H-2), 11.88 (s, 1H, N–H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 14.7 (*C*H$_3$CH$_2$O–), 36.9 (C-1′), 60.4 (–OCH$_2$Me), 107.4 (C-3), 113.5 (C-6), 115.1 (C-9b), 118.6 (C-9), 119.6 (C-4a), 120.0 (C-8), 121.1 (C-9a), 122.7 (C-7), 137.3 (C-4b), 140.6 (C-5a), 145.2 (C-2), 153.0 (C-10a), 164.9 (*C*O$_2$Et), 169.7 (C-4). HRMS (ESI): found 353.11007 ([M+H]$^+$), $C_{19}H_{17}N_2O_3S$ requires 353.11011.

4.1.3.2. Ethyl 1-cyclopropyl-7-methyl-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylate (8b). Irradiation time: 1.75 h. Yield 63%; mp 315–317 °C (dec). Anal. Calcd for $C_{20}H_{18}N_2O_3S$ (366.44): C, 65.56; H, 4.95; N, 7.64. Found: C, 65.64; H, 4.74; N, 7.41. IR: ν_{max} (KBr)/cm^{-1} 3378, 2971, 1721, 1601, 1557, 1481, 1445, 1372, 1350, 1310, 1270, 1237, 1208, 1154, 1136, 1107, 1041 and 801; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.19 (m, 2H) and 1.26 (m, 2H) (H_2-2′/H_2-3′), 1.28 (t, J = 7.1 Hz, 3H, CH_3CH$_2$O–), 2.41 (s, 3H, C(7)–CH_3), 3.75 (m, 1H, H-1′), 4.23 (q, J = 7.1 Hz, 2H, –OCH_2Me), 6.94 (d, J = 8.1 Hz, 1H, H-8), 7.37 (s, 1H, H-6), 7.65 (d, J = 8.1 Hz, 1H, H-9), 8.31 (s, 1H, H-2), 11.72 (s, 1H, N–H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.5 (C-2′/C-3′), 14.7 (*C*H$_3$CH$_2$O–), 22.0 (C(7)–*C*H$_3$), 36.9 (C-1′), 60.4 (-OCH$_2$Me), 109.0 (C-9b), 111.8 (C-3), 113.3 (C-6), 118.3 (C-9), 119.0 (C-9a), 119.2 (C-4a), 121.7 (C-8), 132.1 (C-4b), 141.4 (C-7), 141.5 (C-5a), 145.0 (C-2), 155.2 (C-10a), 166.3 (*C*O$_2$Et), 173.3 (C-4). HRMS (ESI): found 367.11107 ([M+H]$^+$), $C_{20}H_{19}N_2O_3S$ requires 367.11109; found 733.21527 ([2M+H]$^+$), $C_{40}H_{37}N_4O_6S_2$ requires 733.21490.

4.1.3.3. Ethyl 1-cyclopropyl-7-methoxy-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylate (8c). Irradiation time: 1.75 h. Yield 40%; mp 303–305 °C (dec). Anal. Calcd for $C_{20}H_{18}N_2O_4S$ (382.44): C, 62.81; H, 4.74; N, 7.32; S, 8.38. Found: C, 62.61; H, 4.58; N, 7.27; S, 8.19. IR: ν_{max} (KBr)/cm^{-1} 3320, 3077, 2982, 2931, 2840, 1728, 1601, 1565, 1499, 1485, 1463, 1419, 1346, 1306, 1274, 1256, 1216, 1154, 1107, 1034, 801, 787 and 696; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.17 (m, 2H) and 1.25 (m, 2H) (H_2-2′/H_2-3′), 1.28 (t, J = 7.1 Hz, 3H, CH_3CH$_2$O–), 3.75 (m, 1H, H-1′), 3.77 (s, 3H, –OCH_3), 4.22 (q, J = 7.1 Hz, 2H, –OCH_2Me), 6.77 (dd, J = 8.7, 1.2 Hz, 1H, H-8), 7.10 (d, J = 1.2 Hz, 1H, H-6), 7.66 (d, J = 8.7 Hz, 1H, H-9), 8.29 (s, 1H, H-2), 11.67 (s, 1H, N–H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.5 (C-2′/C-3′), 14.7 (*C*H$_3$CH$_2$O–), 36.9 (C-1′), 55.8 (–OCH$_3$), 60.4 (–OCH$_2$Me), 96.9 (C-6), 109.9 (C-8), 114.8 (C-9b), 115.2 (C-3), 115.4 (C-9a), 119.3 (C-9), 120.0 (C-4a), 136.4 (C-4b), 141.7 (C-5a), 144.8 (C-2), 151.8 (C-10a), 156.8 (C-7), 165.0 (*C*O$_2$Et), 169.6 (C-4). HRMS (ESI): found 405.08817([M+Na]$^+$), $C_{20}H_{18}N_2O_4SNa$ requires 405.08795; *m*/*z* (EI): 382 (M$^+$, 100), 367 (17), 336 (14), 321 (20), 310 (30), 295 (60), 281 (10), 267 (9), 252 (6), 243 (9), 168 (7).

4.1.3.4. Ethyl 1-cyclopropyl-6,8-dimethyl-7-methoxy-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno-[3,2-*b*]indole-3-carboxylate (8d). Irradiation time: 1.75 h. Yield 61%; mp 255-257 °C (dec). Anal. Calcd for $C_{22}H_{22}N_2O_4S$ (410.49): C, 64.37; H, 5.40; N, 6.82; S, 7. Found: C, 64.33; H, 5.38; N, 6.78; S, 7.68. IR: ν_{max} (KBr)/cm^{-1} 3288, 2942, 1732, 1594, 1567, 1455, 1346, 1303, 1263, 1150, 1096, 1023, 897 and 805; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.18 (m, 2H) and 1.25 (m, 2H) (H_2-2′/H_2-3′), 1.28 (t, J = 7.1 Hz, 3H, CH_3CH$_2$O–), 2.31 (s, 3H, C(8)–CH_3), 2.48 (s, 3H, C(6)–CH_3), 3.66 (s, 3H, –OCH_3), 3.73 (m, 1H, H-1′), 4.23 (q, J = 7.1 Hz, 2H, –OCH_2Me), 7.40 (s, 1H, H-9), 8.29 (s, 1H, H-2), 11.31 (s, 1H, N–H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.5 (C-2′/C-3′), 11.1 (C(6)–*C*H$_3$), 14.7 (*C*H$_3$CH$_2$O–), 17.0 (C(8)–*C*H$_3$), 36.9 (C-1′), 60.5 (-OCH$_3$), 60.7 (–OCH$_2$Me), 108.0 (C-9b), 114.9 (C-3), 116.9 (C-9), 117.5 (C-9a), 123.4 (C-4a), 137.5 (C-4b), 139.6 (C-5a), 141.4 (C-6), 143.8 (C-8), 145.0 (C-2), 152.2 (C-10a), 153.4 (C-7), 165.2 (*C*O$_2$Et), 169.5 (C-4). HRMS (ESI): found 411.13792 ([M+H]$^+$), $C_{22}H_{23}N_2O_4S$ requires 411.13730; found 821.27228 ([2M+H]$^+$), $C_{44}H_{45}N_4O_8S_2$ requires 821.26733.

4.1.3.5. Ethyl 1-cyclopropyl-7-fluoro-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylate (8e). Irradiation time: 1.75 h. Yield 54%; mp 305–307 °C (dec). Anal. Calcd for $C_{19}H_{15}N_2FO_3S$ (370.40): C, 61.61; H, 4.08; N, 7.56 S, 8. Found: C, 61.52; H, 3.93; N, 7.49 S, 8. IR: ν_{max} (KBr)/cm^{-1} 3331, 3080, 1721, 1605, 1557, 1481, 1448, 1346, 1303, 1266, 1208, 1161, 1110, 1034, 932, 801 and 699; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.19 (m, 2H) and 1.26 (m, 2H) (H_2-2′/H_2-3′), 1.28 (t, J = 7.1 Hz, 3H, CH_3CH$_2$O–), 3.74 (m, 1H, H-1′), 4.22 (q, J = 7.1 Hz, 2H, –OCH_2Me), 6.96 (ddd, $^3J_{H-F}$ = 9.7 Hz, J = 8.7, 2.1 Hz, 1H, H-8), 7.29 (dd, $^3J_{H-F}$ = 10.1 Hz, J = 2.1 Hz, 1H, H-6), 7.81 (dd, J = 8.7 Hz, $^4J_{H-F}$ = 3.1 Hz, 1H, H-9), 8.31 (s, 1H, H-2), 11.97 (s, 1H, N–H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.5 (C-2′/C-3′), 14.7 (*C*H$_3$CH$_2$O–), 36.9 (C-1′), 60.4 (–OCH$_2$Me), 99.5 (d, $^2J_{C-F}$ = 26.2 Hz, C-6), 116.6 (C-3), 108.4 (d, $^2J_{C-F}$ = 24.1 Hz, C-8), 115.0 (C-4a), 107.5 (C-9b), 118.0 (C-9a), 119.8 (d, $^3J_{C-F}$ = 11.0 Hz, C-9), 137.8 (C-4b), 140.7 (d, $^3J_{C-F}$ = 13.1 Hz, C-5a), 145.3 (C-2), 152.9 (C-10a), 159.6 (d, $^1J_{C-F}$ = 234 Hz, C-7), 164.9 (*C*O$_2$Et), 169.7 (C-4). HRMS (ESI): found 371.08608 ([M+H]$^+$), $C_{19}H_{16}N_2FO_3S$ requires 371.08602; found 393.06802 ([M+Na]$^+$), $C_{19}H_{15}N_2FO_3SNa$ requires 393.06796; found 763.14670 ([2M+Na]$^+$), $C_{38}H_{30}N_4F_2O_6S_2Na$ requires 763.14670.

4.1.4. General procedure for the synthesis of 1-cyclopropyl-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-b]indole-3-carboxylic acids (9a–e)

A suspension of the corresponding ester (**8a–e**) (1 mmol) in 10% aq HCl (8 mL) and ethanol (10 mL) was refluxed for 20–24 h. The solvents were evaporated in vacuo from the reaction mixture, the residual yellow solid product was soaked in methanol (3–5 mL) and collected by suction filtration.

4.1.4.1. 1-Cyclopropyl-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylic acid (9a). Yield 96%; mp 295–297 °C (dec). Anal. Calcd for $C_{17}H_{12}N_2O_3S$ (324.36): C, 62.95; H, 3.73; N, 8.64; S, 9.89. Found: C, 62.65; H, 3.96; N, 8.31; S, 9.83. IR: ν_{max} (KBr)/cm^{-1} 3309, 1706, 1601, 1554, 1477, 1335, 1303, 1034, 805, 736 and 712; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.26 (m, 2H) and 1.37 (m, 2H) (H_2-2′/H_2-3′), 3.95 (m, 1H, H-1′), 7.17 (dd, J = 7.2, 8.0 Hz, 1H, H-8), 7.26 (dd, J = 7.2, 8.1 Hz, 1H, H-7), 7.62 (d, J = 8.1 Hz, 1H, H-6), 7.89 (d, J = 8.0 Hz, 1H, H-9), 8.61 (s, 1H, H-2), 12.11 (s, 1H, N–H), 15.51(s, 1H, CO$_2$*H*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 38.2 (C-1′), 109.3 (C-9b), 111.9 (C-3), 113.6 (C-6), 119.1 (C-9), 120.3 (C-8), 120.9 (C-9a), 123.6 (C-7), 124.1 (C-4a), 135.4 (C-4b), 141.0 (C-5a), 145.2 (C-2), 155.7 (C-10a), 166.3 (–*C*O$_2$H), 173.4 (C-4).

HRMS (ESI): found 323.05015 ([M−H]$^-$), $C_{17}H_{11}N_2O_3S$ requires 323.04959.

4.1.4.2. 1-Cyclopropyl-7-methyl-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylic acid (9b). Yield 95%; mp 350–352 °C (dec). Anal. Calcd for $C_{18}H_{14}N_2O_3S$ (338.38): C, 63.89; H, 4.17; N, 8.28; S, 9.48. Found: C, 63.62; H, 4.06; N, 8.15; S, 9.41. IR: ν_{max} (KBr)/cm^{-1} 3320, 3029, 2968, 2957, 1743, 1714, 1608, 1568, 1506, 1481, 1452, 1408, 1372, 1336, 1314, 1223, 1037, 808 and 790; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.25 (m, 2H) and 1.36 (m, 2H) (H_2-2′/H_2-3′), 2.43 (s, 3H, –CH_3), 3.94 (m, 1H, H-1′), 6.99 (d, J = 8.1 Hz, 1H, H-8), 7.41 (s, 1H, H-6), 7.75 (d, J = 8.1 Hz, 1H, H-9), 8.60 (s, 1H, H-2), 11.95 (s, 1H, N(5)–H), 15.54 (s, 1H, CO_2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 22.0 (–CH_3), 38.2 (C-1′), 109.0 (C-9b), 111.8 (C-3), 113.4 (C-6), 118.8 (C-9), 119.0 (C-9a), 119.2 (C-4a), 122.1(C-8), 133.1 (C-4b), 141.4 (C-7), 141.5 (C-5a), 144.9 (C-2), 155.2 (C-10a), 166.3 (CO_2H), 173.3 (C-4). HRMS (ESI): found 337.06569 ([M−H]$^-$), $C_{18}H_{13}N_2O_3S$ requires 337.06524.

4.1.4.3. 1-Cyclopropyl-7-methoxy-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indo-le-3-carboxylic acid (9c). Yield 96%; mp 317–319 °C (dec). Anal. Calcd for $C_{18}H_{14}N_2O_4S$ (354.38): C, 61.01; H, 3.98; N, 7.90; S. 9.05. Found: C, 60.77; H, 3.76; N, 7.86; S. 9.04. IR: ν_{max} (KBr)/cm^{-1} 3333, 3039, 2977, 2951, 1750, 1719, 1601, 1566, 1511, 1478, 1448, 1396, 1377, 1341, 1305, 1233, 1042, 799 and 781; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.25 (m, 2H) and 1.36 (m, 2H) (H_2-2′/H_2-3′), 3.79 (s, 3H, –OCH_3), 3.93 (m, 1H, H-1′), 6.81 (d, J = 8.1 Hz, 1H, H-8), 7.11 (s, 1H, H-6), 7.77 (d, J = 8.1 Hz, 1H, H-9), 8.75 (s, 1H, H-2), 11.92 (s, 1H, N-H), 15.58 (s, 1H, CO_2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 38.2 (C-1′), 55.8 (–OCH_3), 96.8 (C-6), 109.7 (C-9b), 110.4 (C-8), 111.7 (C-3), 115.2 (C-9a), 117.8 (C-4a), 119.9 (C-9), 134.5 (C-4b), 142.2 (C-5a), 144.6 (C-2), 154.5 (C-10a), 157.3 (C-7), 166.4 (–CO_2H), 173.2 (C-4). HRMS (ESI): found 353.07756 ([M−H]$^-$), $C_{18}H_{13}N_2O_4S$ requires 353.07743.

4.1.4.4. 1-Cyclopropyl-6,8-dimethyl-7-methoxy-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylic acid (9d). Yield 94%; mp 320–322 °C (dec). Anal. Calcd for $C_{20}H_{18}N_2O_4S$ (382.44) requires C, 62.81; H, 4.74; N, 7.33; S, 8.38. Found: C, 62.78; H, 4.74; N, 7.26; S, 8.24. IR: ν_{max} (KBr)/cm^{-1} 3397, 2935, 1706, 1608, 1554, 1463, 1408, 1328, 1303, 1256, 1154, 1088, 1030, 998, 852, 801 and 744; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.24 (m, 2H) and 1.35 (m, 2H) (H_2-2′/H_2-3′), 2.32 (s, 3H, C(8)–CH_3), 2.50 (s, 3H, C(6)–CH_3), 3.68 (s, 3H, –OCH_3), 3.92 (m, 1H, H-1′), 7.51 (s, 1H, H-9), 8.58 (s, 1H, H-2), 11.58 (s, 1H, N-H), 15.72(s, 1H, CO_2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 11.3 (C(6)–CH_3), 17.0 (C(8)–CH_3), 38.2 (C-1′), 60.5 (–OCH_3), 109.9 (C-9b), 111.7 (C-3), 117.4 (C-9), 118.0 (C-9a), 124.0 (C-4a), 135.6 (C-4b), 140.2 (C-5a), 140.3 (C-6), 140.6 (C-8), 144.7 (C-2), 154.0 (C-7), 155.0 (C-10a), 166.4 (CO_2H), 173.2 (C-4). HRMS (ESI): found 381.09207 ([M−H]$^-$), $C_{20}H_{17}N_2O_4S$ requires 381.09145.

4.1.4.5. 1-Cyclopropyl-7-fluoro-4-oxo-1,5-dihydro-4*H*-pyrido[3′,2′:4,5]thieno[3,2-*b*]indole-3-carboxylic acid (9e). Yield 96%; mp 335–337 °C (dec). Anal. Calcd for $C_{17}H_{11}N_2FO_3S$ (342.35) requires C, 59.64; H, 3.24; N, 8.18; S, 9.37. Found: C, 59.44; H, 3.21; N, 8.06; S, 9.28. IR: ν_{max} (KBr)/cm^{-1} 3331, 3048, 1714, 1612, 1583, 1565, 1499, 1481, 1339, 1310, 1267, 1114, 1038 and 794; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.26 (m, 2H) and 1.37 (m, 2H) (H_2-2′/H_2-3′), 3.94 (m, 1H, H-1′), 7.02 (ddd, J = 2.0, J = 8.8, Hz, $^3J_{H-F}$ = 9.8 Hz, 1H, H-8), 7.33 (dd, J = 2.0 Hz, $^3J_{H-F}$ = 9.9 Hz, 1H, H-6), 7.93 (dd, J = 8.8 Hz, $^4J_{H-F}$ = 3.3 Hz, 1H, H-9), 8.60 (s, 1H, H-2), 12.20 (s, 1H, N(5)-H), 15.45 (s, 1H, CO_2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.6 (C-2′/C-3′), 38.2 (C-1′), 99.6 (d, $^2J_{C-F}$ = 26.3 Hz, C-6), 108.8 (d, $^2J_{C-F}$ = 24.4 Hz, C-8), 109.5 (C-9b), 111.9 (C-3), 116.8 (C-4a), 117.9 (C-9a), 120.6 (d, $^3J_{C-F}$ = 9.8 Hz, C-9), 136.0 (C-4b), 141.2 (d, $^3J_{C-F}$ = 12.7 Hz, C-5a), 145.2 (C-2), 155.5 (C-10a), 160.0 (d, $^1J_{C-F}$ = 236 Hz, C-7), 166.2(CO_2H), 173.3 (C-4). HRMS (ESI): found 341.04062 ([M−H]$^-$), $C_{17}H_{10}N_2FO_3S$ requires 341.04016.

4.2. Biology

4.2.1. Antimicrobial activity

The in vitro antimicrobial assay was carried out against several bacteria and fungi by using the twofold serial broth dilution method.[32] Antibacterial activity was tested towards Gram positive bacteria (*B. cereus* ATCC 11778, *B. megaterium* ATCC 19213, *B. subtilis* ATCC 6633, *B. thuringiensis* var. *kurstaki* BGSC 4D1, *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228, quinolone- and penicillin-resistant clinical isolates of both *S. aureus* and *S. epidermidis*) and Gram negative bacteria (*A. baumannii* ATCC 19606, *E. coli* ATCC 8739, *H. influenzae* ATCC 19418, *P. aeruginosa* ATCC 9027, quinolone- and penicillin-resistant clinical isolates of both *A. baumannii* and *E. coli* strains). Antifungal activity was obtained by means of yeasts (*S. cerevisiae* ATCC 9763, *C. tropicalis* ATCC 1369) and mould (*A. niger* ATCC 6275). The minimum inhibitory concentrations (MIC, μg/mL) were determined as the lowest concentration for each compound at which no growth was observed. Ciprofloxacin, levofloxacin, moxifloxacin and gemifloxacin were screened as reference antibacterial fluoroquinolones, miconazole as standard antifungal drug.

Dimethyl sulfoxide solutions of the test compounds were prepared and then diluted in the applicable media in a concentration range from 100 μg/mL to 0.0000015 μg/mL. Haemophilus Test Medium, Mueller Hinton Broth and Sabouraud Dextrose Broth were used as specific culture media for the growth of *H. influenzae*, other bacteria and fungi, respectively. Tubes were inoculated with a suspension of the different bacteria (5×10^5 CFU/mL) and fungi (1×10^3 CFU/mL). At the end of an incubation period of 24 h at 37 °C (bacteria) or 48 h at 30 °C (fungi), the minimum inhibitory concentrations were detected. In addition, for each microorganism, drug-free tubes and tubes containing only dimethyl sulfoxide were tested as growth and solvent controls, respectively. Organism-free tubes were kept as sterility controls.

Three replicates were done with each microorganism and at least three independent experiments were performed.

4.2.2. DNA gyrase and topoisomerase IV inhibition

Test compounds were evaluated for their inhibitory activity against quinolone target enzymes by using *E. coli* DNA gyrase and topoisomerase IV (Inspiralis, Norwich, UK) and were compared to reference drugs ciprofloxacin and moxifloxacin.

To detect the inhibition of DNA gyrase activity, supercoiling assay was carried out with relaxed pBR322 DNA as a substrate. Several amounts of each compound were incubated at 37 °C with 35 mM Tris–HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM dithiothreitol, 1.8 mM spermidine, 1 mM ATP, 6.5% w/v glycerol, 0.1 mg/mL albumin, 16 ng/μL relaxed pBR322 DNA and 0.026 U/μL of gyrase protein. After 30 min the reactions were stopped by the addition of a chloroform/isoamyl alcohol 24:1 solution and bromophenol blue dye. The blue aqua phase was then loaded on 1.0% w/v agarose gel and analyzed by electrophoresis.

The inhibition of topoisomerase IV activity was detected in the decatenation assay using kinetoplast DNA as a substrate. Compounds were treated in different amounts with reaction mixtures containing 40 mM HEPES-KOH pH 7.6, 100 mM potassium glutamate, 10 mM $Mg(OAc)_2$, 10 mM dithiothreitol, 1 mM ATP, 50 μg/mL albumin, 6.6 ng/μL kDNA and 0.016 U/μL topoisomerase IV protein.

After 30 min incubation at 37 °C, a chloroform/isoamyl alcohol 24:1 solution and bromophenol blue dye were added to stop the reactions. The blue aqua phases were analyzed by electrophoresis in 1.0% w/v agarose gel.

A Bio-Rad gel documentation system was employed to quantitate the products of the reactions. Results were expressed as 50% inhibitory concentrations (IC_{50}, μg/mL), that is the drug concentrations that reduced by 50% the supercoiling and the decatenation activity observed for the enzymes in drug-free controls.

Representative results of at least three independent experiments were recorded.

4.2.3. Cell proliferation assay

MCF-7 breast cancer cells and A549 NSCLC cell lines were obtained from ATCC and were cultured in RPMI; human-derm fibroblasts (HuDe) were obtained from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Italy, and were cultured in DMEM. All media were supplemented with 2 mM glutamine and 10% Fetal Bovin Serum (FBS, Gibco Life Technologies) and cells were maintained under standard cell culture conditions at 37 °C in a water-saturated atmosphere of 5% CO_2 in air.

Cells were treated with the indicated compounds at concentrations ranging from 0.1 to 20 μM for 72 h. Cell viability was assessed with tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), obtained from Sigma (Dorset, UK), and assayed as previously described.[33] Representative results of at least three independent experiments were used for evaluation of dose–response curves, calculated from experimental points using single or double Hill functions (Graph Pad Prism Software 5, San Diego California USA, www.graphpad.com). Concentrations that inhibit 50% (IC_{50}) (e.g., the point at which viability is 50%) were obtained from the dose–response curves by extrapolation.

In all assays, the drugs were dissolved in DMSO immediately before the addition to cell cultures. The final concentration of DMSO never exceeded 0.1% (v/v), and equal amounts of the solvent were added to control cells.

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