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Design and synthesis of mycobacterial pks13 inhibitors: Conformationally rigid tetracyclic molecules

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ABSTRACT

We previously reported a series of coumestans—a naturally occurring tetracyclic scaffold containing a δ-lactone—that effectively target the thioesterase domain of polyketide synthase 13 (Pks13) in *Mycobacterium tuberculosis* (*Mtb*), resulting in superior anti-tuberculosis (TB) activity. Compared to the corresponding 'open-form' ethyl benzofuran-3-carboxylates, the enhanced anti-TB effects seen with the conformationally restricted coumestan series could be attributed to the extra π-π stacking interactions between the benzene ring of coumestans and the phenyl ring of F1670 residue located in the Pks13-TE binding domain. To further probe this binding feature, novel tetracyclic analogues were synthesized and evaluated for their anti-TB activity against the *Mtb* strain $H_{37}Rv$. Initial comparison of the 'open-form' analogueues against the tetracyclic counterparts again showed that the latter is superior in terms of anti-TB activity. In particular, the δ-lactam-containing 5*H*-benzofuro [3,2-*c*]quinolin-6-ones gave the most promising results. Compound **65** demonstrated potent activity against *Mtb* $H_{37}Rv$ with MIC value between 0.0313 and 0.0625 μg/mL, with high selectivity to Vero cells (64—128 fold). The thermal stability analysis supports the notion that the tetracyclic compounds bind to the Pks13-TE domain as measured by nano DSF, consistent with the observed SAR trends. Compound **65** also showed excellent selectivity against actinobacteria and therefore unlikely to develop potential drug resistance to nonpathogenic bacteria.

1. Introduction

Tuberculosis (TB) which is caused by *Mycobacterium tuberculosis* (*Mtb*) still poses a major threat to human health. Millions of people continue to suffer from TB each year worldwide, with cases that can be found in virtually all countries and age groups. The improper use of TB drugs in combination with the long treatment regimen has led to the emergence of drug-resistant strains of *Mtb*. As presented in the 2019 World Health Organization Global Tuberculosis Report, approximately 0.5 million people developed TB in 2018 that were resistant to rifampicin (**1**, Fig. 1), a first-line TB drug widely considered to be the most effective [1]. Approximately 78% of these patients had the multidrug-resistant TB (MDR-TB), which is defined by resistance to at least isoniazid and rifampicin. Moreover, the TB-HIV co-infection exacerbates the problem, adding a layer of complexity with respect to designing an effective treatment regimen that is devoid of unwanted drug-drug interactions. The development of new drugs for TB has also been slow. For the past decade, only two new drugs, bedaquiline (**2**) and delamanid (**3**) have been introduced into the market (Fig. 1). Consequently, there is still an urgent, unmet need for novel, more effective drugs that are efficacious against drug-resistant strains of *Mtb*.

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Abbreviations used

TB	tuberculosis
Mtb	*Mycobacterium tuberculosis*
MDR	multidrug-resistant
N-ACP	N terminus acyl carrier protein
C-ACP	C terminus acyl carrier protein
KS	ketoacyl synthase
AT	acyl transferase
TE	thioesterase
SIT	serum inhibition titration
XDR	extensively drug-resistant
SARs	structure-activity relationships
SIT	serum inhibition titration
THF	tetrahydrofuran
DMF	dimethylformamide
NMP	N-methyl pyrrolidinone
MABA	microplate alamar blue assay
MIC	minimum inhibitory concentration
EWG	electron-withdrawing group
EDG	electron-donating group
SI	selectivity index
CC	column chromatography
TLC	thin layer chromatography
LCMS	liquid chromatography mass spectrometry
TMS	tetramethylsilane
HRMS	high-resolution mass spectra
nanoDSF	Nano differential scanning fluorimetry

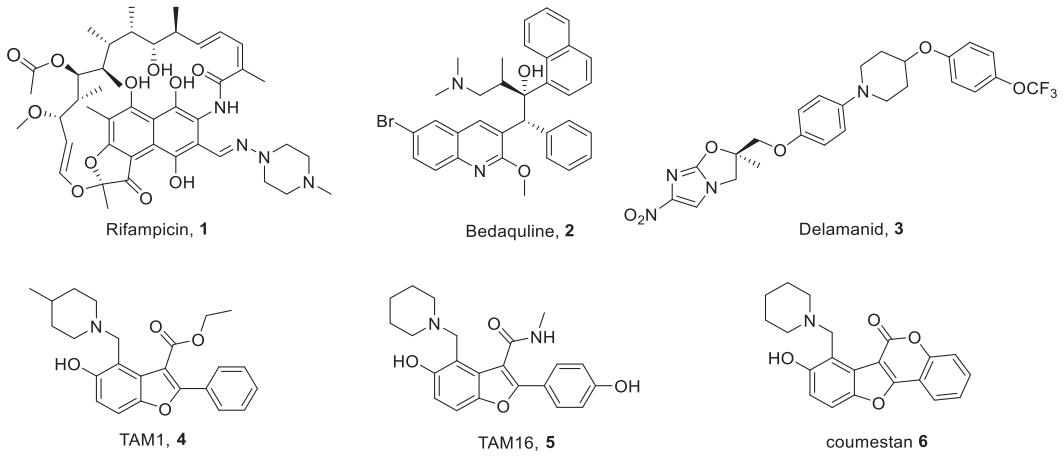


Fig. 1. Chemical structures of anti-TB drugs rifampicin, bedaquiline and delamanid; benzofuran-based Pks13 inhibitors TAM1, TAM16, and coumestan **6**.

Mycolic acids are the essential building blocks used to assemble the unique cell wall of *Mtb*, which is responsible for the pathogen's viability and virulence [2]. Inhibition of mycolic acid biosynthesis has been widely accepted as a highly effective strategy in the anti-TB drug discovery [3,4], with polyketide synthase 13 (Pks13) recently emerging as an attractive and viable target. Briefly, Pks13 performs the Claisen-type condensation step in the mycolic acid synthesis [5], and this activity has been shown to be essential both *in vitro* and *in vivo* for the bacteria survival [6]. Pks13 is encoded by the *FadD32-Pks13-AccD4* gene cluster and contains 1733 amino acid residues divided into five distinct domains, including: two acyl carrier protein domains located at the *N* terminus (*N*-ACP) and *C* terminus (*C*-ACP), a ketoacyl synthase (KS), an acyl transferase (AT) and a thioesterase domain (TE) located at the C terminus [7,8]. There has been an increasing number of reported small molecule inhibitors for mycobacterial Pks13 in recent years [6,9–13]. In 2017, the Sacchettini group reported the benzofuran compound TAM1 (**4**, Fig. 1) as an initial lead molecule, and later solved an X-ray co-crystal structure of Pks13 complexed with the benzofurans binding to the thioesterase domain of Pks13 (Pks13-TE) [10]. The more refined compound TAM16 (**5**, Fig. 1) emerged through a structure-guided approach as a highly potent Pks13-TE inhibitor with good pharmacological properties. At the same time, our own group identified coumestan derivatives represented by compound **6** (Fig. 1), a natural product-inspired tetracyclic δ-lactone with modest activity against *Mtb* strain $H_{37}Rv$ [13,14]. While the structural change from benzofuran to the coumestans may be minimal, a head-to-head comparison showed that the coumestans possess superior bioavailability in a mouse serum inhibition titration (SIT) assay [14]. Whole genome deep sequencing of the wild-type and coumestan-resistant mutants identified mutation sites A1667V, D1644G, N1640K, and N1640S, all of which are co-localized within the active site of the Pks13-TE, consistent with the X-ray co-crystal structure reported by the Sacchettini group. Taken together, these observations substantiate Pks13 as a druggable target and highlight its potential for the development of new TB drugs.

1.1. Compound design

During our medicinal chemistry campaign on the coumestans, one prominent trend that we observed was that the coumestans generally exhibited enhanced anti-TB activity compared to the corresponding ethyl benzofuran-3-carboxylates [13,14]. Utilizing a structure-based drug design approach, it is hypothesized that the enhanced potency could be attributed to the extra π-π stacking between the benzene ring of coumestans and the phenyl ring of F1670 residue located in the Pks13-TE domain as shown in Fig. 2. The conformational rigidity brought about by the coumestan is predicted to enhance the π-π interactions between the tetracyclic heteroaromatic ring structure and the F1670 residue. To verify this hypothesis, we proposed that the coumestan scaffold could be replaced by other related tetracycles, which are aimed to further enhance the

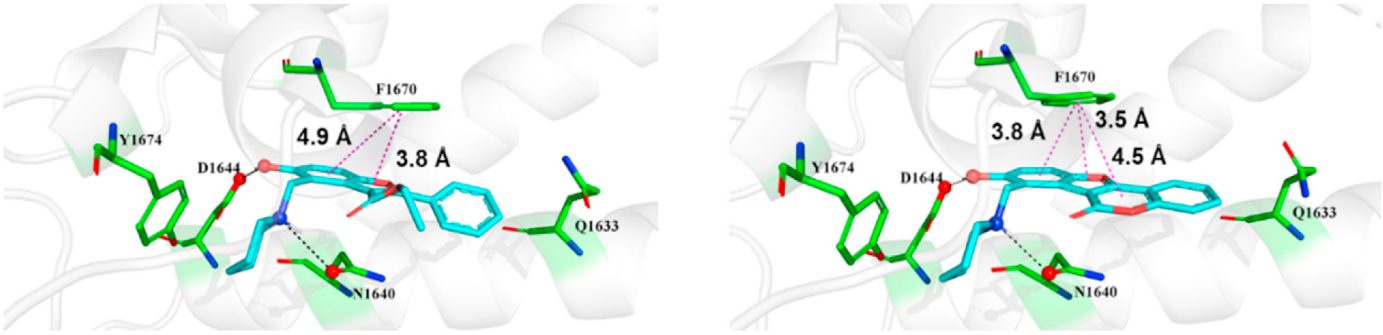


Fig. 2. Proposed binding modes of compounds **7** (colored cyan, left figure) and **6** (colored cyan, right figure). The key amino acid residues located in the active site of Pks13 (PDB ID 5V3Y) were colored green.

π-π stacking interactions. Consequently, a series of various benzothiophene-3-carboxylates, indole-3-carboxylates, benzofuran-3-carboxylates, *N*-ethylbenzofuran-3-carboxamides and their corresponding 6*H*-benzothieno [3,2-*c*] [1]benzopyran-6-ones [1],benzopyrano [4,3-*b*]indol-6(11*H*)-ones, and 5*H*-benzofuro [3,2-*c*]quinolin-6-ones were synthesized and evaluated for their anti-TB activity against *Mtb* $H_{37}Rv$.

2. Results and discussion

2.1. Chemistry

The synthetic routes for reference compounds **5**–**12** (structures shown in Table 1) were described previously [13]. [10] For the synthesis of compound **16** (Scheme 1), the key intermediate ethyl (*Z*)-3-amino-3-(2-methoxyphenyl)acrylate **14** was obtained from ester **13** using ammonium formate in refluxing ethanol as described in the literature [15]. Cyclization of acrylate **14** with 1,4-benzoquinone in the presence of zinc bromide resulted in the formation of indole derivative **15.** Subsequent lactonization and Mannich reaction with piperidine and 37% aqueous formaldehyde gave the desired product **16** [16]. The corresponding 'open-form' compound **18** was obtained using ester **17** as the starting material in a similar fashion to the synthesis of **16**. Cyclization of the intermediate ethyl (*Z*)-3-amino-3-phenylacrylate with 1,4-benzoquinone followed by Mannich reaction gave compound **18**. To access compounds **21** and **22**, basic hydrolysis of esters **19** [17] and **20** was performed, followed by sequential amide bond formation and Mannich reaction with piperidine and formaldehyde.

Compound **24** was formed via a copper-catalyzed cyclization of benzoquinone and ethyl 3-(2-bromophenyl)-3-oxopropanoate **23**. Lactam **25** was obtained via amination of **24** followed by a cyclization reaction. The reaction took place in a closed vessel using Cu_2O as a catalyst in the presence of bromobenzene **24**, *N*-methyl pyrrolidinone (NMP) and excess of ammonia at 80 °C according to a reported protocol [18]. Compound **25** underwent a subsequent Mannich reaction to yield compound **26**. The synthesis of compounds **31**, **32**, and **34** followed the methods similar to compound **12**. Intermediate **27** was methylated and the aldehyde group was installed using dichloromethyl methyl ether and $TiCl_4$ to give isomers **29** and **30**, which could be readily separated using flash chromatography. Each isomer was oxidized under Pinnick condition and sulfamic acid as hypochlorite scavenger to its corresponding carboxylic acid. Amide bond formation with piperidine gave the desired amides **31** and **32**. Similarly, compound **34** was synthesized using starting material **33** [13]. Compound **35** was obtained via bromination of previously synthesized compound **7** using molecular bromine in dichloromethane at room temperature.

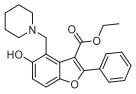
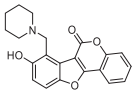
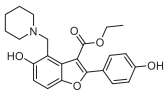
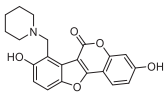
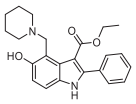
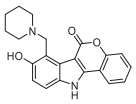
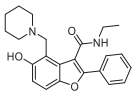
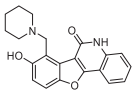
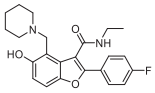
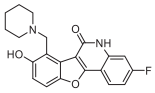
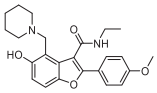
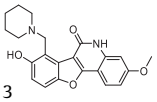
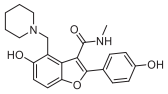
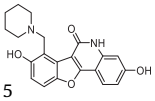
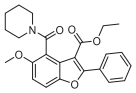
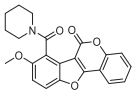
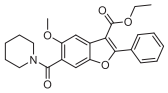
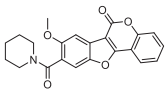
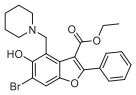
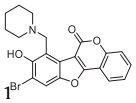
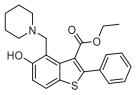
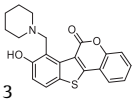
The synthesis of compounds **41** and **45** were shown in Scheme 2. 2-Bromo-5-methoxybenzaldehyde, sulfur powder, and methyl 2-(2-methoxyphenyl)acetate **36** were refluxed in DMF for 16 h to give benzothiophene **37**. Introduction of an aldehyde group at the 3-position was achieved using dichloromethyl methyl ether and $TiCl_4$ to give intermediate **38**. Pinnick oxidation afforded the carboxylic acid **39**, which was subjected to sequential lactonization and Mannich reaction to give the desired compound **41**. Similarly, compound **45** was synthesized in 6 steps utilizing the commercially available starting material methyl phenylacetate **42**.

The synthesis of 5*H*-benzofuro [3,2-*c*]quinolin-6-one analogueues **51**–**58** and **64**–**67** were achieved using a different route to the synthesis of **26** which necessitates high pressure in a closed vessel. As shown in Scheme 3, substituted *o*-iodoanilines **46** or **59** were subjected to double Sonogashira coupling employing a palladium as well as copper co-catalyst to form alkyne **48** or **61**. Following a reported protocol, the 5*H*-benzofuro [3,2-*c*]quinolin-6-one scaffolds were prepared utilizing Cs_2CO_3 as a source of both carbonyl (CO) and ethereal oxygen in the presence of 5 mol% $Cu(OAc)_2$ and 1 equiv. of Ag_2CO_3 form **49** or **62** [19]. Demethylation of the methoxy intermediate **49** followed by Mannich reaction gave the desired compound **51**. Whereas compound **62** was deprotected under acidic conditions and subjected to sequential Mannich reaction and demethylation to give the desired compound **65**. Compounds **52**–**58** and **66**–**67** were synthesized in a similar manner to compounds **51** and **65** respectively. All final compounds (**51**–**58**, **66**–**67**) were obtained as hydrochloride salts, except **64** (free amine) and **65** (hydrobromide salt).

2.2. Structure activity relationships

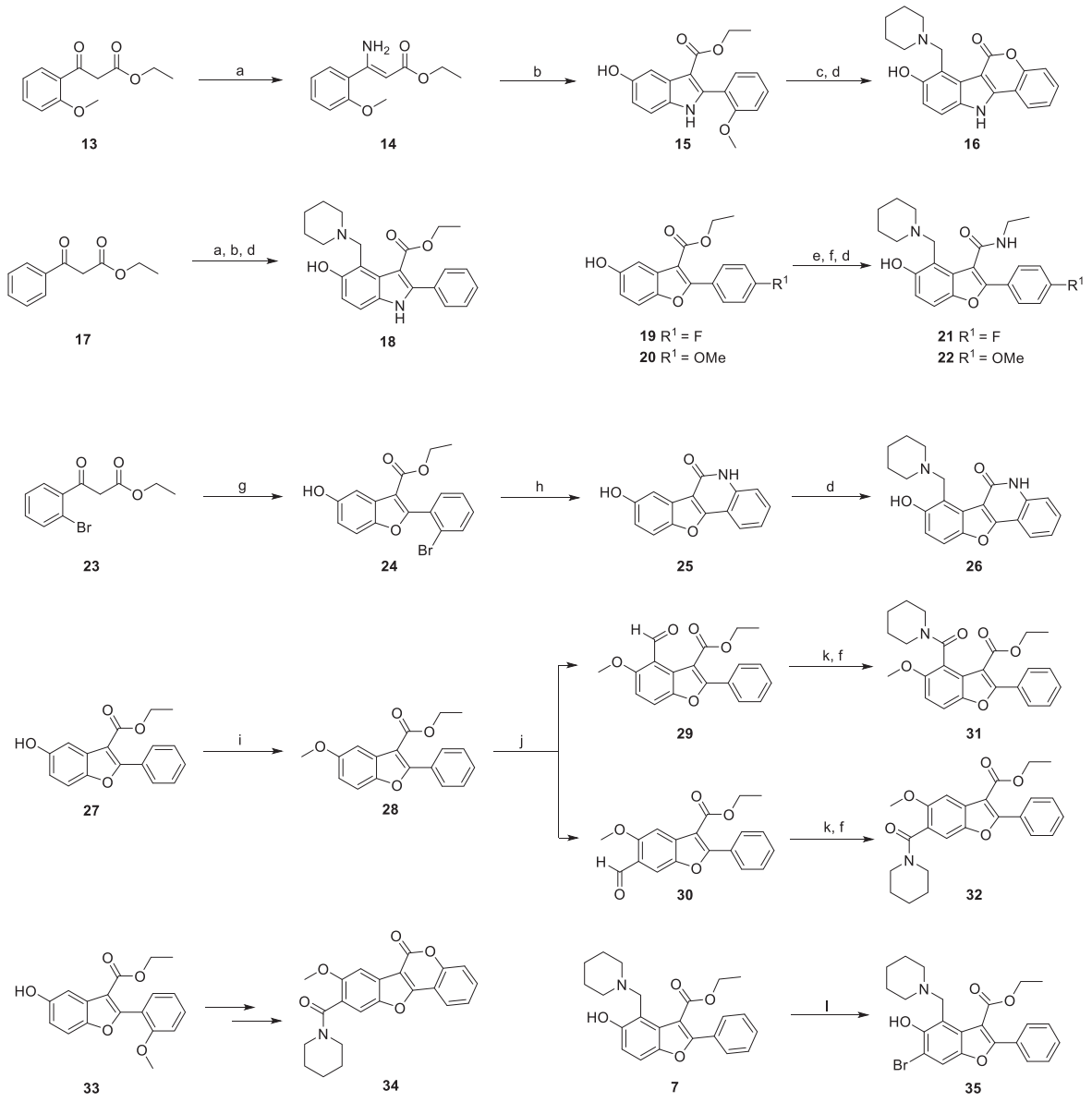
All final compounds were evaluated in a microplate alamar blue assay (MABA) [20] for anti-TB activity against *Mtb* $H_{37}Rv$ expressed as the minimum inhibitory concentration (MIC) values. As shown in Table 1, the coumestan **6** showed 16-fold increase in activity compared to the corresponding 'open-form' 2-phenylbenzofuran-3-carboxylate **7** (MIC values of 0.125 vs 2 μg/mL). Installment of the 4-OH group on the benzene ring of **6** afforded a highly potent compound **9** with MIC value less than 0.0039 μg/mL, being at least 8-fold more potent than TAM16 (compound **5**, MIC = 0.0313 μg/mL) [14]. The tetracyclic coumestan **9** again resulted in at least a 64-fold increase of activity when compared to 'open-form' ester **8**. This trend is evident for most of the synthesized compounds, notably upon the comparison of *N*-ethylbenzofuran-3-carboxamides (**10**, **21**, and **22**) vs the corresponding 5*H*-benzofuro [3,2-*c*]quinolin-6-ones (**26**, **57**, and **64**). The trend is also observed upon comparison of the benzothiophene-3-carboxylate **45** vs the corresponding 6*H*-benzothieno

Table 1
Antitubercular activity of compounds against the *M. tuberculosis* strain $H_{37}Rv$.[a]

Conformationally relaxed 'open-ring' analogues				Corresponding rigid tetracyclic analogues			
ID	Structure	MIC, μg/mL	CLogP[b]	ID	Structure	MIC, μg/mL	CLogP
7[c]		2	6.01	6[c]		0.125	4.17
8[c]		0.25	5.38	9[c]		0.0039	4.11
18		32	5.57	16		32	4.00
10[c]		4	4.15	26		0.25	3.41
21		4	4.34	57		0.5	3.70
22		2	4.10	64		0.25	3.60
5[c]		0.0313	2.98	65		0.0313–0.0625	3.14
31		>64	5.24	12[c]		>64	3.46
32		>64	5.24	34		>64	3.46
35		>32	6.70	11[c]		>64	4.86
45		16	6.56	41		1	6.79

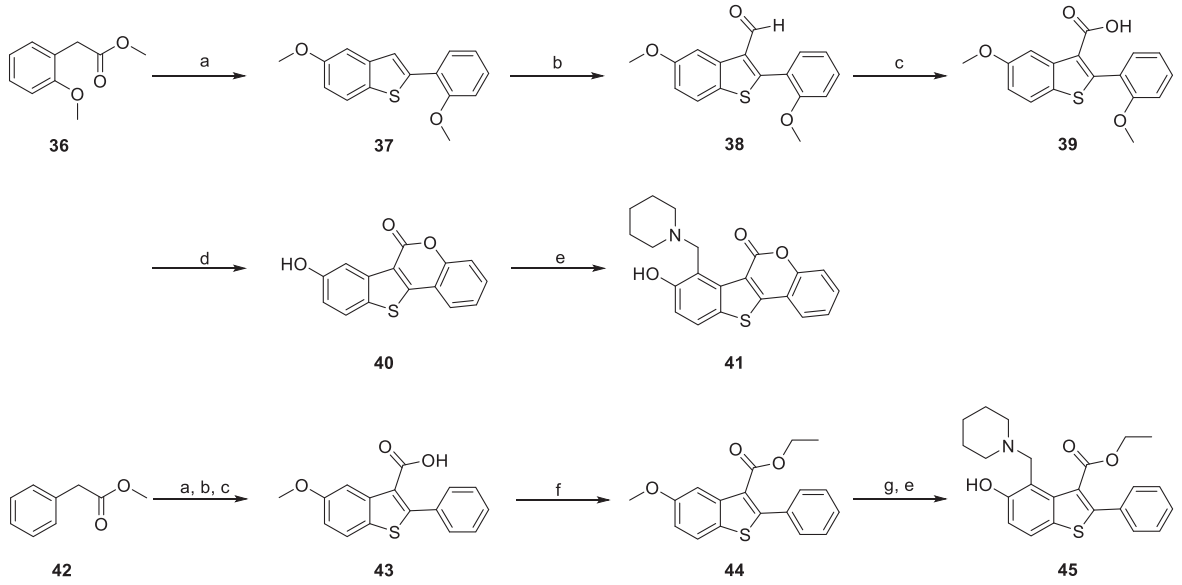
[a] See Experimental Section. The lowest concentration of compounds leading to at least 90% inhibition of bacterial growth signal by the MABA. MIC values are reported as an average of three individual measurements.
[b] CLogP was calculated using ChemBioDraw Ultra 14.0.
[c] Compounds **5**–**12** [10,14] were reference compounds for comparision.

[3,2-*c*] [1]benzopyran-6-one **41**. In these cases, at least an 8-fold improvement in activity was observed in the tetracyclic analogueues. The installment of 4-OH group on the benzene ring of compound **26** gave an 8-fold more potent analogue **65**, which is equipotent with its 'open-form' analogue **5**. However, replacing the benzofuran nucleus with an indole resulted in compounds **18** and **16**, both of which have MIC values of 32 μg/mL, indicating that the bioisosteric replacement is not favorable for activity. Six other inactive compounds (MIC > 32 μg/mL; compounds **11**, **12**, **31**, **32**, **34**, and **35**) were also displayed in Table 1. Comparison of the 'open-form' vs tetracyclic analogueues (**31** vs **12**, **32** vs **34**, and **35** vs **11**) revealed that the inactivity in the 'open-form' analogueues were also mirrored in the corresponding tetracyclic analogues. Molecular docking simulations suggested that the substituents at 6-Br of benzofurans may interfere with Arg 1641 residue causing unfavorable ligand binding. Overall, encouraged by the observation that the 5*H*-benzofuro [3,2-*c*]quinolin-6-ones displayed improved antimycobacterial properties compared to the acyclic amides, we focused our attention on the SAR of the 5*H*-benzofuro [3,2-*c*]quinolin-6-one derivatives.

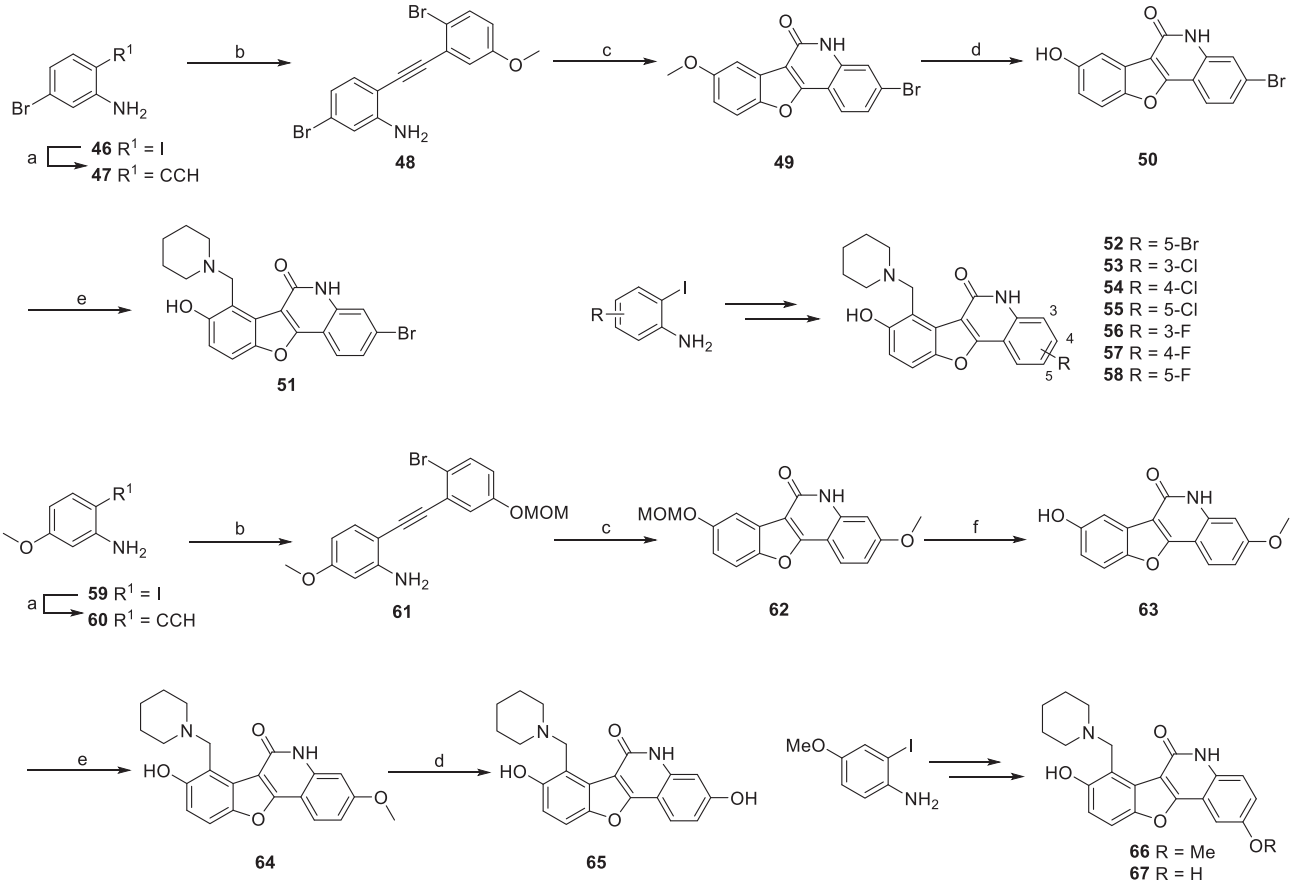


Scheme 1. Synthesis of Compounds **16**, **18**, **21**, **22**, **26**, **31**, **32**, **34** and **35**. [a].
[a] Reagents and conditions: (a) ammonium formate, 4 Å molecular sieve, EtOH, reflux, 7 h; (b) benzoquinone, $ZnBr_2$, THF, 3 h; (c) BBr_3 in CH_2Cl_2 (1 M), CH_2Cl_2, rt, overnight, then EtOH, reflux, 1 h; (d) formaldehyde (37% aq), piperidine, EtOH, reflux, 8–12 h; (e) KOH, MeOH, reflux, 3 h; (f) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 1-hydroxybenzotriazole, ethylamine or piperidine, pyridine, DMF, overnight; (g) benzoquinone, $Cu(OTf)_2$ (5 mol %), toluene, reflux, 7–12 h; (h) Cu_2O, ammonia, *N*-methylpyrrolidone, 80 °C, 15 h; (i) K_2CO_3, CH_3I, DMF, 3–5 h; (j) Cl_2CHOCH_3, $TiCl_4$, $CHCl_3$; (k) $NaClO_2$, NH_2SO_3H, dioxane, H_2O, 30 min; (l) Br_2, CH_2Cl_2, rt, overnight.

In our previous work as well as Sacchettini group's, it was established that the 4' -(methylpiperidin-1-yl)methyl and 5' −OH substituents play important roles for optimal anti-TB activity. The X-ray cocrystal structure reported by the Sacchettini group [10] revealed that both of these groups were completely buried in Pks13-TE domain while the 3′-ethyl ester and 2′-phenyl group (right-hand side benzene ring) were oriented towards the solvent. In addition, substituents at the 6′ or 7′ position of the benzofuran portion, whether electron-withdrawing groups (EWGs) or electron-donating groups (EDGs) are generally tolerable [14]. With these considerations in mind, various EWGs such as halogens (F, Cl, Br − compounds **51**–**58**) and EDGs (methoxy and hydroxyl − compounds **64**–**67**) were introduced at different positions of the right-hand side benzene ring of the 5*H*-benzofuro [3,2-*c*]quinolin-6-one (Table 2). In general, it was found that the 4-position constitutes an ideal site of substitution for optimal potency, for instance when comparing the bromides **51** vs **52**, the chlorides **53**–**55** and the fluorides **56**–**58**. Within this set of analogueues, MIC values ranged from 0.5 to 1.0 μg/mL, with the exception of bromide **52** (MIC = 32 μg/mL), being 64-fold less potent compared to the 4-bromo derivative **51**. Unfortunately, none of these eight compounds demonstrated more potent anti-TB activities compared to the unsubstituted analogueue **26** (MIC = 0.25 μg/mL). Introduction of EDGs (OMe and OH) at the 5-position of the phenyl ring gave compounds **66** (MIC = 4 μg/mL) and **67** (MIC = 1 μg/mL) respectively. Introduction of a hydroxyl group at the 4-position of the phenyl ring dramatically increase the anti-TB activity (**65**) with an MIC value of 0.0313–0.0625 μg/mL. This trend is consistent with previous findings and the improved potency was likely mediated through an optimal hydrogen bond to the carbonyl oxygen of

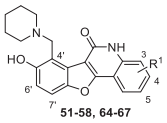


Scheme 2. Synthesis of Compounds **41** and **45**. [a].
[a] Reagents and conditions: (a) 2-bromo-5-methoxybenzaldehyde, K_2CO_3, S, DMF, 110 °C, 16 h; (b) Cl_2CHOCH_3, $TiCl_4$, $CHCl_3$, 0 °C to rt, 30 min; (c) $NaClO_2$, NH_2SO_3H, dioxane, H_2O; (d) BBr_3 in CH_2Cl_2 (1 M), CH_2Cl_2, rt, overnight, then EtOH, reflux, 1 h; (e) formaldehyde (37% aq), piperidine, EtOH, reflux, 8–12 h; (f) EtOH, concentrated sulfuric acid, 70 °C, 5 h; (g) BBr_3 in CH_2Cl_2 (1 M), CH_2Cl_2, rt, overnight.



Scheme 3. Synthesis of Compounds **51**–**58**, **64**–**67**. [a].
[a] Reagents and conditions: (a) $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, TMS-acetylene, N_2, rt, 4 h; then K_2CO_3, MeOH, rt, 3 h; (b) $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, 1-bromo-2-iodo-4-methoxybenzene or 1-bromo-2-iodo-4-(methoxymethoxy)benzene, N_2, rt, overnight; (c) $Cu(OAc)_2$ (5 mol%), Ag_2CO_3, DMSO, Cs_2CO_3, 130 °C, 24 h; (d) BBr_3 in CH_2Cl_2 (1 M), CH_2Cl_2, rt, overnight; (e) formaldehyde (37% aq), piperidine, EtOH, reflux, 8–12 h; (f) HCl, MeOH, reflux, 5 h.

Table 2
Antitubercular activity of 5*H*-benzofuro [3,2-*c*]quinolin-6-ones **51**–**58** and **64**–**67** against the *M. tuberculosis* strain $H_{37}Rv$.[a]



4.

ID	R^1	MIC, μg/mL	CLogP[b]
51	4-Br	0.5	4.42
52	5-Br	32	4.42
53	3-Cl	1.0	4.27
54	4-Cl	0.5	4.27
55	5-Cl	1.0	4.27
56	3-F	0.5	3.70
57	4-F	0.5	3.70
58	5-F	1	3.70
64	4-OMe	0.25	3.60
65	4-OH	0.0313–0.0625	3.14
66	5-OMe	4	3.60
67	5-OH	1	3.14

[a] See Experimental Section. The lowest concentration of compounds leading to at least 90% inhibition of bacterial growth signal by the MABA. MIC values are reported as an average of three individual measurements.
[b] CLogP was calculated using ChemBioDraw Ultra 14.0.

Q1633. Comparison of analogues **64**–**65** vs the corresponding 5-substituted analogues **66**–**67** further supported that the 4-position of the benzene ring was optimal site for substitution.

Given the excellent MIC values of most benzofuranoquinolinone derivatives, representative compound **65** was further evaluated its antitubercular activity against clinical drug susceptible (DS, V4207), MDR-TB (KZN494 and V2475), and XDR-TB (TF274 and R506) strains (Table 3). To our delight, benzofuranoquinolinone **65** exhibited potent activities against all tested stains.

2.3. Toxicity to vero cells

In order to evaluate their general cytotoxicity profiles, selected 5*H*-benzofuro [3,2-*c*]quinolin-6-ones (Table 2) with MIC values equal or less than 1 μg/mL were subsequently evaluated in Vero cells, which are derived from African green monkey kidney. The selectivity index (SI) values were calculated from the ratio of IC_{50} (Vero)/MIC (*Mtb*) as shown in Table 4. Comparison of Vero cell toxicity (IC_{50} value) and MIC value of *Mtb* growth inhibition gives a measure of the general toxicity profile of the target compound. Three of the selected seven compounds **51**, **64** and **65** had SI values of more than 100, with compound **64** having the highest SI value of 256. Compound **65** with excellent anti-TB activity exhibited a moderate SI value between 64 and 128 compared to **58** and **67**, which exhibited low SI values of 16.

Table 4
Vero toxicity and selectivity index of selected compounds.

ID	Toxicity to Vero cells IC_{50}, μg/mL	MIC $H_{37}Rv$, μg/mL	SI[a]
51	64	0.5	128
54	32	0.5	64
57	16	0.5	32
58	16	1	16
64	64	0.25	256
65	4	0.0313–0.0625	64–128
67	16	1	16

[a] SI, selectivity index = $IC_{50\ (Vero\ cells)}$/$MIC_{(H37Rv)}$.

2.4. Microbial selectivity

Following the cytotoxicity evaluation, antimicrobial selectivity of the most promising compound **65** was further explored. When tested against a selected panel of gram-positive and gram-negative bacteria including *S. aureus* Newman, *B. subtilis* 168, *E. coli* AB1157, and *E. coli* DH5α (Table 5), compound **65** showed no appreciable inhibition of growth (MIC values > 25 μg/mL), suggesting that it was selective against mycobacteria and unlikely to develop potential drug resistance to nonpathogenic bacteria.

2.5. Thermal stability analysis and enzyme activity

As shown previously by our group, the results from whole genome sequencing of the wild-type and coumestan-resistant mutants demonstrated that these coumestans were likely to target the Pks13-TE [13]. In order to demonstrate whether this target engagement was responsible for their observed antitubercular effects, selected tetracyclic compounds were evaluated for thermal shift assay in the presence of Pks13-TE using nano differential scanning fluorimetry (nanoDSF) method. Briefly, the binding of ligands with high affinity to proteins generally leads to thermal

Table 5
Assessment of **65** against selected gram positive and gram-negative bacteria.

Compounds	MIC, μg/mL			
	S. aureus Newman	*B. subtilis* 168	*E. coli* AB1157	*E. coli* DH5α
Vancomycin	0.39–0.78	>25	>25	>25
Tetracycline	0.2–0.39	>25	1.56–3.13	0.78–1.56
Kanamycin	2.34–4.68	9.39–18.75	9.39–18.75	2.34–4.68
65	>25	>25	>25	>25

Table 3
Antitubercular activity of compound **65** against DS, MDR, and XDR strains of *M. tuberculosis.*

Mtb MIC[a] (μg/mL)					
strain/phenotype	V4207/DS[b]	V2475/MDR[c]	KZN494/MDR	TF274/XDR[d]	R506/XDR
Isoniazid	0.04	16	16	8	8
Rifampin	0.0625	4	128	>128	>128
Levofloxacin	0.125	NT[e]	NT	1	2
Ofloxacin	0.5	0.5	0.5	8	4
Kanamycin	2	2	2	>128	>128
65	0.0313	0.0625	0.0313	0.0156–0.0313	0.0156–0.0313

[a] The lowest concentration of drug leading to at least a 90% reduction of bacterial growth signal by microplate alamar blue assay (MABA); MIC values are reported as an average of three individual measurements.
[b] Drug susceptible strain of *M. tuberculosis*.
[c] Multidrug resistant strain of *M. tuberculosis*, resistance to isoniazid and rifampin.
[d] Extensively drug resistant strain of *M. tuberculosis*, resistance to isoniazid, rifampin, levofloxacin, ofloxacin, and kanamycin.
[e] NT = not tested.

stability shift of proteins, which can be detected using nanoDSF. Stabilization of the Pks-TE protein at 30 μM concentration upon binding of high affinity ligands (at 300 μM concentration) was evaluated by comparing the melting temperatures (T_m) of Pks13-TE (56.2 ± 0.03 °C) in the absence or presence of the compounds and calculating the shift in the *T*m (ΔT_m). A significant increase in thermal stability of Pks13-TE was observed following the addition of the 10-fold tetracycle-containing compounds (ΔT_m > 3.0 °C, Table 6), indicating high-affinity binding of the compounds to the Pks13-TE. Although caution must be exercised upon correlating ΔT_m and the observed antitubercular phenotype, in our case a consistent trend was evident: the extent of the antitubercular activity of the compounds tend to be accompanied by an increase in the ΔT_m values. Collectively, the observed thermal stabilization was consistent with the notion that the tetracyclic analogues bind to the Pks13-TE, leading to the antimycobacterial effects.

Compounds **65** and **7** were next selected for enzymatic potency evaluation using a protocol reported by Sacchettini group [10]. As shown in Table 7, compound **65** inhibited the Pks13-TE activity with an IC_{50} value of 0.58 μM while the IC_{50} value of compound **7** was found to be 4.6 μM, which agreed with their MIC data.

3. Conclusions

In this report, we demonstrated that targeting Pks13 is a valid strategy when designing antitubercular compounds that work via inhibition of the synthesis of *Mtb* cell wall. Our work on the coumestans and Sacchettini's group on the benzofuran esters revealed novel scaffolds that are highly amenable for SAR and further physiochemical parameters optimization. Consequently, a total of 23 compounds were synthesized and evaluated for their antitubercular activity against the *M. tuberculosis* $H_{37}Rv$. The observed SAR comparison between the conformationally relaxed 'open-form' benzofuran esters and the corresponding rigid tetracyclic analogues showed that the latter tend to be superior anti-TB compounds. This trend is consistent with our hypothesis that the additional π-π stacking interactions between the benzene ring of the tetracyclic compounds and the phenyl ring of F1670 in the Pks13-TE domain is important for binding. We also explored biosisosteric replacements of the benzofuran nucleus to the benzothiophene and the indole ring, which unfortunately proved to be detrimental for their anti-TB activities. Lactam **65** possessed outstanding anti-TB activity, improved selectivity to Vero cells (SI = 128), and excellent selectivity to mycobacteria against nonpathogenic bacteria strains. Selected tetracyclic compounds were also demonstrated to bind with high affinities to the recombinant Pks13-TE protein as measured by nanoDSF assay, indicating their target engagement.

Table 6
Thermal stabilization of Pks13-TE upon binding with compounds measured by nanoDSF.

ID	ΔT_m[a], °C		MIC[b], μg/mL
	5X	10X	
DMSO	−1.3 ± 0.03		–
16	0.8 ± 0.14	3.1 ± 0.09	32
26	9.6 ± 0.03	10.2 ± 0.64	0.25
41	7.9 ± 0.16	9.1 ± 0.14	1
45	3.2 ± 0.04	3.3 ± 0.15	16
64	8.7 ± 0.15	9.0 ± 0.39	0.25
65	7.5 ± 0.10	9.4 ± 0.25	0.0313−0.0625
67	7.4 ± 0.00	8.5 ± 0.06	1

[a] ΔT_m was calculated as T_m (Pks13-TE) in the presence of compounds (3% DMSO final concentration) minus Tm (Pks-TE) with DMSO only. Final concentration ratio of Pks-TE and compounds is 1:5 and 1:10. T_m values are tested as an average of three individual measurements.
[b] The lowest concentration of compounds leading to at least 90% inhibition of bacterial growth signal by the MABA. MIC values are reported as an average of three individual measurements.

Table 7
IC_{50} and MIC values of compounds **65** and **7**.

Compounds	Pks13-TE IC_{50}[a], μM	$H_{37}Rv$ MIC[b], μg/mL
65	0.58 ± 0.03	0.0313−0.0625
7	4.60 ± 0.20	2

[a] IC_{50} values were tested as an average of three individual measurements.
[b] The lowest concentration of compounds leading to at least 90% inhibition of bacterial growth signal by the MABA. MIC values are reported as an average of three individual measurements.

4. Experimental section

General. Starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification, unless otherwise stated. Anhydrous toluene and CH_2Cl_2 were obtained by distillation over sodium wire or calcium hydride respectively. All non-aqueous reactions were run under a nitrogen atmosphere with exclusion of moisture from reagents, and all reaction vessels were oven-dried. The progress of reactions was monitored by thin layer chromatography on SiO_2 and LCMS. Products were purified by column chromatography on 200−300 mesh SiO_2, and an EtOAc/petroleum ether mixture or gradient was used unless stated otherwise. ^1H NMR and ^{13}C NMR spectra were recorded at 400 and 101 MHz respectively. NMR chemical shifts were reported in δ (ppm) using the δ 0 signal of tetramethylsilane, δ 7.26 signal of $CDCl_3$, δ 3.31 signal of CD_3OD or δ 2.50 signal of $(CD_3)_2SO$ as internal standards. High resolution mass spectra (HRMS) were performed using a Bruker ESI-TOF high-resolution mass spectrometer. Purities of final compounds (>95%) were established by analytical HPLC, which was carried out on a Waters HPLC system using InertSustain-C18 column (5 micron, 250 × 4.6 mm) with detection at 280 and 254 nm on a variable wavelength detector 2998 PDA. The melting points were recorded using a WRR-Y melting point instrument. See Supporting Information for NMR spectra (^1H and ^{13}C) of all final compounds and analytical HPLC traces of compound **65**.

General procedure for the synthesis of substituted 5-hydroxy-2-phenyl benzofuran from substituted ethyl benzoylacetates (method A) was reported previously [13].

General procedure for the preparation of coumestan derivatives, 8-hydroxychromeno[4,3-*b*]indol-6(11*H*)-one or 8-hydroxy-6*H*-benzo[4,5]thieno[3,2-*c*]chromen-6-one from substituted ethyl 5-hydroxy-2-(2-methoxyphenyl)benzocycloheterocycle-3-carboxylate (method B). To a solution of substituted ethyl 5-hydroxy-2-(2-methoxyphenyl)benzocycloheterocycle-3-carboxylate (1.0 mmol) in anhydrous CH_2Cl_2 (4 mL), BBr_3 (1 M in CH_2Cl_2, 4.0 mmol) was added at room temperature under N_2. After being stirred overnight, the reaction mixture was quenched with EtOH. The resulting mixture was allowed to reflux for 1 h and evaporated. The residue was purified by flash chromatography or by recrystallization in EtOH to obtain target compound.

General procedure for the Mannich reaction of substituted ethyl 2-(2-methoxyphenyl)-5-hydroxybenzocyclo-3-carboxylate or coumestan analogueue with amine and formaldehyde (method C). To a solution of substituted ethyl 2-(2-methoxyphenyl)-5-hydroxybenzocyclo-3-carboxylate or coumestan analogueues (1 mmol) in ethanol (3 mL) were added formaldehyde (37% in water, 4 mmol) and the appropriate amine (4 mmol)

at room temperature under N_2. The reaction mixture was allowed to reflux for 5–12 h and then cooled to rt. The reaction mixture was evaporated and the residue was purified by flash chromatography or recrystallized in EtOH to give the product.

General procedure for the hydrolysis of ester to afford carboxylic acid (method D). To a solution of ester (1.0 mmol) in 5 mL MeOH was added KOH (6.0 mmol) in 1 mL H_2O at rt. The reaction mixture was allowed to reflux for 3 h and then acidified with HCl (2 M) to pH 5–6. The resultant solid was filtered off to give carboxylic acid without further purification.

General procedure for the amidation of carboxylic acid (method E). To a solution of carboxylic acid to anhydrous DMF (5 mL), EDC·HCl (1.3 mmol), HOBt (1.3 mmol), pyridine (2.1 mmol), and ethylamine or piperidine (1.2 mmol) under N_2 were added at rt. After being stirred overnight, the reaction mixture was quenched with saturated NH_4Cl solution and extracted with EtOAc (2 × 30 mL). The combined organic phases were washed with HCl (5% aqueous solution), saturated aqueous $NaHCO_3$ solution (1 × 30 mL), dried over anhydrous Na_2SO_4 and evaporated. The residue was purified by flash chromatography to give the amide product.

Ethyl (*Z*)-3-amino-3-(2-methoxyphenyl)acrylate (**14**). A mixture of commercially available **13** (1.0 mmol), ammonium formate (5.0 mmol) and molecular sieves (4 Å, 0.1 g) in 10 mL of ethanol were refluxed for 7 h under N_2 and then cooled to rt. The reaction mixture was filtered through celite, and the filtrate was evaporated and the residue was extracted with ethyl acetate (3 × 20 mL). Combined organic layers were dried over anhydrous Na_2SO_4 and evaporated to give product without further purification. Yield 97%; yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (d, J = 7.6 Hz, 1H), 7.28 (t, J = 8.0 Hz, 1H), 6.92–6.83 (m, 2H), 4.76 (s, 1H), 4.09 (q, J = 6.9 Hz, 2H), 3.78 (s, 3H), 1.21 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.5, 158.7, 155.6, 129.9, 128.6, 124.9, 120.0, 110.5, 84.4, 57.7, 54.7, 13.6.

Ethyl 5-hydroxy-2-(2-methoxyphenyl)-1*H*-indole-3-carboxylate (15). To a solution of **14** (1.0 mmol) and $ZnBr_2$ (1.0 mol) in 3 mL anhydrous THF was added a solution of benzoquinone (1.0 mmol) in THF (2 mL) under N_2. After stirring for 3 h at room temperature. The reaction mixture was quenched with saturated NH_4Cl solution and extracted with EtOAc (3 × 10 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and evaporated. The residue was purified by flash chromatography to give **15**. Yield 58%; pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.65 (s, 1H), 8.95 (s, 1H), 7.46–7.39 (m, 2H), 7.34 (d, J = 7.2 Hz, 1H), 7.20 (d, J = 8.5 Hz, 1H), 7.10 (d, J = 8.2 Hz, 1H), 7.02 (t, J = 7.2 Hz, 1H), 6.69 (d, J = 8.1 Hz, 1H), 4.06 (q, J = 6.8 Hz, 2H), 3.72 (s, 3H), 1.09 (t, J = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.6, 157.2, 152.4, 141.2, 131.4, 130.2, 129.8, 128.0, 121.8, 119.7, 112.2, 112.1, 111.1, 105.1, 103.7, 58.4, 55.4, 14.1.

8-Hydroxy-7-(piperidin-1-ylmethyl)chromeno[4,3-*b*]indol-6(11*H*)-one (16). This compound was obtained from **15** employing methods B and C. Yields 64% and 80%; yellow solid; m.p > 250 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (br, 1H), 8.21 (d, J = 7.6 Hz, 1H), 7.61 (t, J = 7.6 Hz, 1H), 7.53–7.41 (m, 3H), 6.93 (d, J = 8.6 Hz, 1H), 4.79 (s, 2H), 2.80–2.66 (m, 4H), 1.67–1.55 (m, 4H), 1.54–1.45 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.3, 154.5, 152.3, 142.3, 132.2, 130.7, 124.5, 124.2, 122.5, 116.7, 115.1, 112.9, 112.8, 111.5, 99.9, 56.1, 52.7 (2C), 24.5 (2C), 23.0. HRMS (ESI) m/z: Calcd for $C_{21}H_{21}N_2O_3$ $(M + H)^+$ 349.1547, found 349.1539.

Ethyl 5-hydroxy-2-phenyl-4-(piperidin-1-ylmethyl)-1*H*-indole-3-carboxylate (18). This compound was obtained from commercially available **17** by employing amination, lactonization and Mannich reaction according to the methods described for **14**, **15**, and method C. Overall yield 38%; pale yellow semisolid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.50–7.48 (m, 2H), 7.42–7.39 (m, 3H), 7.22 (d, J = 8.7 Hz, 1H), 6.73 (d, J = 8.7 Hz, 1H), 4.17–4.08 (m, 4H), 2.75–2.48 (m, 4H), 1.66–1.61 (m, 4H), 1.51–1.46 (m, 2H), 1.06 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 169.4, 154.8, 144.3, 134.4, 132.1, 130.0 (2C), 129.4, 129.1 (2C), 127.1, 114.7, 112.5, 111.9, 106.2, 61.4, 59.2, 54.7 (2C), 26.9 (2C), 24.9, 14.2. HRMS (ESI) m/z: Calcd for $C_{23}H_{27}N_2O_3$ $(M + H)^+$ 379.2016, found 379.2002.

***N*-ethyl-2-(4-fluorophenyl)-5-hydroxy-4-(piperidin-1-ylmethyl)benzofuran-3-carboxamide (21).** This compound was obtained from **19** by employing methods D, E, and C. Yields 47%, 91% and 77%; pale yellow solid; m.p 152.6–154.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78–7.73 (m, 2H), 7.24 (d, J = 8.9 Hz, 1H), 7.13–7.09 (m, 2H), 6.79 (d, J = 8.8 Hz, 1H), 6.07 (t, J = 5.5 Hz, 1H), 3.86 (s, 2H), 3.53–3.41 (m, 2H), 3.20–2.06 (m, 4H), 1.63–1.54 (m, 6H), 1.21 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.4, 163.3 (d, $J_{C\text{-}F}$ = 250.2 Hz), 155.2, 151.8, 148.0, 128.7 (d, $J_{C\text{-}F}$ = 8.3 Hz, 2C), 126.0, 126.0 (d, $J_{C\text{-}F}$ = 3.3 Hz), 116.0 (d, $J_{C\text{-}F}$ = 21.9 Hz, 2C), 115.1, 113.2 (d, $J_{C\text{-}F}$ = 0.8 Hz), 112.2, 110.6, 57.1, 53.9 (2C), 35.2, 25.9 (2C), 24.0, 14.5. HRMS(ESI) m/z: Calcd for $C_{23}H_{26}N_2O_3$ $(M + H)^+$ 397.1922, found 397.1906.

***N*-ethyl-5-hydroxy-2-(4-methoxyphenyl)-4-(piperidin-1-ylmethyl)benzofuran-3-carboxamide (22).** This compound was obtained from **20** by employing methods D, E, and C. Yields 93%, 91%, and 61%; pale yellow solid; m.p 182.0–184.4 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 7.74 (d, J = 8.8 Hz, 2H), 7.30 (d, J = 8.8 Hz, 1H), 7.02 (d, J = 8.8 Hz, 2H), 6.75 (d, J = 8.8 Hz, 1H), 3.92 (s, 2H), 3.85 (s, 3H), 3.43 (q, J = 7.3 Hz, 2H), 2.72–2.44 (m, 4H), 1.71–1.60 (m, 4H), 1.58–1.50 (m, 2H), 1.23 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 160.5, 155.2, 153.1, 147.9, 128.4 (2C), 126.3, 122.3, 114.6, 114.3 (2C), 112.2, 112.1, 110.5, 57.2, 55.5, 54.0 (2C), 35.2, 26.0 (2C), 24.1, 14.6. HRMS(ESI) m/z: Calcd for $C_{24}H_{28}N_2O_4$ $(M + H)^+$ 409.2122, found 409.2108.

Ethyl 2-(2-bromophenyl)-5-hydroxybenzofuran-3-carboxylate (**24**). This compound was obtained from **23** employing method A. Yield 32%; yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.51 (s, 1H), 7.80 (dd, J = 7.6, 1.2 Hz, 1H), 7.64 (dd, J = 7.3, 1.9 Hz, 1H), 7.55–7.48 (m, 3H), 7.39 (d, J = 2.4 Hz, 1H), 6.88 (dd, J = 8.9, 2.5 Hz, 1H), 4.16 (q, J = 7.1 Hz, 2H), 1.11 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.3, 159.7, 154.6, 147.9, 132.4, 132.4, 131.9, 131.5, 127.4, 126.2, 123.0, 114.6, 112.0, 110.8, 106.1, 60.1, 13.7.

8-Hydroxybenzofuro[3,2-*c*]quinolin-6(5*H*)-one (**25**). To a solution of **24** (1.0 mmol), Cu_2O (0.05 mmol) and 6 mL $NH_3·H_2O$/NMP (v/v = 1:1) in a vessel with thick wall, the reaction mixture was stirring at 80 °C for 15 h under closed condition. Then cooled to room temperature. The reaction mixture was evaporated. The residue was purified by flash chromatography using silica gel to give product. Yield 21%; pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.88 (s, 1H), 9.58 (s, 1H), 8.02 (d, J = 7.9 Hz, 1H), 7.63 (d, J = 9.0 Hz, 1H), 7.59 (d, J = 7.4 Hz, 1H), 7.52 (d, J = 8.2 Hz, 1H), 7.47 (d, J = 2.3 Hz, 1H), 7.33 (t, J = 7.5 Hz, 1H), 6.93 (dd, J = 8.9, 2.3 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.1, 158.2, 154.7, 148.7, 138.3, 130.6, 124.6, 122.3, 121.0, 116.1, 114.6, 112.1, 110.9, 110.1, 105.9.

8-Hydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (26). This compound was obtained from **25** by employing method C. Yield 89%; white solid; m.p 243.8–245.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD = 4:1) δ 8.04 (d, J = 8.0 Hz, 1H), 7.52–7.46 (m, 1H), 7.41–7.35 (m, 2H), 7.27 (t, J = 7.2 Hz, 1H), 6.90–6.87 (m, 1H), 4.83 (s, 2H), 2.93–2.36 (m, 4H), 1.71–1.46 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$/CD_3OD = 4:1) δ 160.6, 159.9, 156.4, 149.6, 137.7, 130.7, 123.5, 122.8, 121.8, 115.8, 115.7, 114.9, 111.9, 111.0, 110.9, 59.1, 53.6 (2C), 25.8 (2C), 24.0. HRMS (ESI) m/z: Calcd for $C_{21}H_{21}N_2O_3$ $(M + H)^+$ 349.1547, found 349.1531.

Ethyl 5-methoxy-2-phenylbenzofuran-3-carboxylate (28). To a stirred solution of **27** (1.0 mmol) and K_2CO_3 (2.1 mmol) in anhydrous DMF (20 mL) was added CH_3I (1.2 mmol) under N_2. After being stirred overnight at rt, the reaction mixture was quenched

with H_2O. The mixture was extracted with EtOAc (2 × 20 mL), and the combined organic phases were washed with water (2 × 20 mL), dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography to give the product. Yield 98%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93–7.90 (m, 2H), 7.49 (d, J = 2.6 Hz, 1H), 7.43–7.38 (m, 3H), 7.35 (d, J = 8.9 Hz, 1H), 6.89 (dd, J = 8.9, 2.7 Hz, 1H), 4.33 (q, J = 7.1 Hz, 2H), 3.82 (s, 3H), 1.32 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.2, 161.5, 156.9, 149.0, 130.3, 129.9, 129.6 (2C), 128.2 (2C), 128.05, 114.3, 111.8, 109.1, 104.9, 60.7, 56.0, 14.4.

Ethyl 4-formyl-5-methoxy-2-phenylbenzofuran-3-carboxylate (29) and ethyl 6-formyl-5-methoxy-2-phenylbenzofuran-3-carboxylate (30). To a solution of **28** (1 mmol) and Cl_2CHOCH_3 (5.0 mmol) in $CHCl_3$ (5 mL) were added slowly $TiCl_4$ (2.5 mmol) at rt. The reaction mixture was being stirred at rt for 40 min and quenched with H_2O. The mixture was extracted with EtOAc (2 × 20 mL), washed with saturated aqueous $NaHCO_3$ solution (1 × 20 mL), dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography to give the products **29** and **30**.**29:** Yield 27%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.58 (s, 1H), 7.91 (dd, J = 8.0, 1.7 Hz, 2H), 7.69 (d, J = 9.0 Hz, 1H), 7.50–7.42 (m, 3H), 7.01 (d, J = 9.0 Hz, 1H), 4.53 (q, J = 7.2 Hz, 2H), 3.98 (s, 3H), 1.37 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.4, 166.0, 160.3, 157.5, 149.0, 130.2, 129.3, 128.9 (2C), 127.4 (2C), 125.6, 117.9, 117.2, 112.3, 109.6, 61.9, 57.0, 14.2.**30:** Yield 49%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.54 (s, 1H), 8.03–7.95 (m, 3H), 7.65 (s, 1H), 7.55–7.48 (m, 3H), 4.41 (q, J = 7.1 Hz, 2H), 4.02 (s, 3H), 1.40 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.7, 164.8, 163.6, 159.2, 148.4, 134.1, 131.1, 129.9 (2C), 129.2, 128.3 (2C), 123.0, 110.6, 109.2, 104.2, 61.0, 56.2, 14.3.

Ethyl 5-methoxy-2-phenyl-4-(piperidine-1-carbonyl)benzofuran-3-carboxylate (31). To a solution of **29** (1 mmol) in 10 mL of dioxane/H_2O (v/v = 7/3) was added $NaClO_2$ (6 mmol) and NH_2SO_3H (5.7 mmol) at rt. The reaction mixture was stirred at rt for 1 h and quenched with saturated aqueous $Na_2S_2O_3$ solution. The mixture was extracted with EtOAc (2 × 20 mL), washed with saturated aqueous NaCl solution, dried over Na_2SO_4, and evaporated. The residue and piperidine employing method E to give the product **31.** Yields 83% and 70%; white solid; m.p 121.5–123.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84–7.78 (m, 2H), 7.48–7.41 (m, 4H), 6.97 (d, J = 9.0 Hz, 1H), 4.39–4.21 (m, 2H), 4.05–3.95 (m, 1H), 3.87 (s, 3H), 3.59–3.48 (m, 1H), 3.41–3.28 (m, 1H), 3.23–3.11 (m, 1H), 1.83–1.48 (m, 6H), 1.26 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.4, 164.0, 158.8, 152.3, 149.2, 130.0, 129.8, 128.6 (2C), 128.3 (2C), 125.1, 118.5, 111.8, 110.2, 110.0, 61.2, 57.1, 48.0, 42.4, 26.1, 25.4, 24.9, 14.1. HRMS (ESI) m/z: Calcd for $C_{24}H_{25}NNaO_5$ $(M + Na)^+$ 430.1625, found 430.1630.

Ethyl 5-methoxy-2-phenyl-6-(piperidine-1-carbonyl)benzofuran-3-carboxylate (32). This compound was obtained from **30** according to the methodology described for **31.** Yields 90% and 70%; white solid; m.p 141.3–142.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99–7.97 (m, 2H), 7.58 (s, 1H), 7.52–7.45 (m, 3H), 7.40 (s, 1H), 4.40 (q, J = 7.1 Hz, 2H), 3.92 (s, 3H), 3.84–3.69 (m, 2H), 3.26–3.17 (m, 2H), 1.76–1.59 (m, 6H), 1.39 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.2, 164.0, 162.1, 153.0, 148.4, 130.5, 129.7 (2C), 129.6, 128.6, 128.2 (2C), 124.9, 110.4, 109.0, 103.8, 60.8, 56.2, 48.2, 42.8, 26.5, 25.8, 24.8, 14.3. HRMS (ESI) m/z: Calcd for $C_{24}H_{25}NNaO_5$ $(M + Na)^+$ 430.1625, found 430.1598.

8-Methoxy-9-(piperidine-1-carbonyl)-6*H*-benzofuro[3,2-*c*]chromen-6-one (34). This compound was obtained from **33** according to the methodology described for **31**, then by employing methods E, B, and methylation described for **28**. Overall yield 19%; white solid; m.p 244.2–245.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (dd, J = 7.8, 0.9 Hz, 1H), 7.64–7,59 (m, 7.1 Hz, 2H), 7.55 (s, 1H), 7.50 (d, J = 8.3 Hz, 1H), 7.42 (t, J = 7.5 Hz, 1H), 3.95 (s, 3H), 3.87–3.67 (m, 2H), 3.23–3.19 (m, 2H), 1.71–1.68 (m, 4H), 1.47–1.43 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.6, 161.1, 158.3, 153.8, 153.7, 149.9, 132.3, 126.1, 124.9, 124.8, 122.1, 117.7, 112.7, 111.1, 106.0, 102.9, 56.5, 48.2, 42.9, 26.5, 25.7, 24.7. HRMS (ESI) m/z: Calcd for $C_{22}H_{19}NNaO_5$ $(M + Na)^+$ 400.1155, found 400.1165.

Ethyl 6-bromo-5-hydroxy-2-phenyl-4-(piperidin-1-ylmethyl) benzofuran-3-carboxylate (35). To the solution of **7** (1.0 mmol) in CH_2Cl_2 (5 mL), Br_2 (1.2 mmol) under N_2 was added at room temperature. After stirring overnight at room temperature, the reaction mixture was quenched with saturated aqueous $Na_2S_2O_3$ solution and extracted with CH_2Cl_2 (2 × 30 mL). The combined organic phases were washed saturated aqueous $NaHCO_3$ solution (1 × 20 mL), brine (1 × 20 mL), dried over anhydrous Na_2SO_4 and evaporated. The residue was purified by flash chromatography to give the product. Yield 38%; yellow solid; m.p 134.5–135.7 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72–7.71 (m, 2H), 7.64 (s, 1H), 7.45–7.44 (m, 3H), 4.35 (q, J = 7.1 Hz, 2H), 4.01 (s, 2H), 2.60–2.27 (m, 4H), 1.70–1.50 (m, 6H), 1.28 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 157.5, 152.7, 147.7, 130.0, 129.8, 128.5 (2C), 128.1 (2C), 125.1, 114.3, 113.0, 110.0, 109.2, 61.7, 58.2, 53.9 (2C), 25.8 (2C), 23.9, 14.1. HRMS (ESI) m/z: Calcd for $C_{23}H_{25}NO_4Br$ $(M + H)^+$ 458.0967, found 458.0980.

5-Methoxy-2-(2-methoxyphenyl)benzo[*b*]thiophene (37). A mixture of **36** (1.0 mmol), 2-bromo-5-methoxybenzaldehyde (1.5 mmol), K_2CO_3 (3.0 mmol), S (4.0 mmol) were dissolved in 3 mL of dimethylformamide and stirred for 16 h at 110 °C under nitrogen. The mixture was filtered and the filtrate was evaporated to remove solvent. The residue was purified by flash chromatography using silica gel to give product. Yield 43%; red oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72–7.65 (m, 3H), 7.34–7.27 (m, 1H), 7.24 (d, J = 2.1 Hz, 1H), 7.05–6.98 (m, 2H), 6.95 (dd, J = 8.8, 2.5 Hz, 1H), 3.95 (s, 3H), 3.87 (s, 3H).

5-Methoxy-2-(2-methoxyphenyl)benzo[*b*]thiophene-3-carbaldehyde (38). This compound was obtained from **37** according to the method described for **29**. Yield 47%; white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.73 (s, 1H), 8.12 (d, J = 2.5 Hz, 1H), 7.98 (d, J = 8.9 Hz, 1H), 7.60–7.54 (m, 1H), 7.52 (dd, J = 7.5, 1.4 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 7.18–7.11 (m, 2H), 3.86 (s, 3H), 3.81 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 186.4, 158.5, 157.0, 156.4, 137.3, 132.1, 132.0, 130.3, 129.5, 123.2, 120.9, 119.4, 115.5, 112.2, 106.1, 55.8, 55.3.

5-Methoxy-2-(2-methoxyphenyl)benzo[*b*]thiophene-3-carboxylic acid (39). To a solution of **38** (1 mmol) in 10 mL of dioxane/H_2O (v/v = 7:3) was added $NaClO_2$ (6 mmol) and NH_2SO_3H (5.7 mmol) at rt. The reaction mixture was stirred at rt for 1 h and quenched with saturated aqueous $Na_2S_2O_3$ solution. The mixture was extracted with EtOAc (2 × 20 mL), washed with saturated aqueous NaCl solution, dried over Na_2SO_4, and evaporated to give **39**. Yield 57%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, J = 2.4 Hz, 1H), 7.68 (d, J = 8.8 Hz, 1H), 7.43–7.39 (m, 2H), 7.08–7.01 (m, 2H), 6.97 (d, J = 8.6 Hz, 1H), 3.91 (s, 3H), 3.79 (s, 3H).

8-Hydroxy-6*H*-benzo[4,5]thieno[3,2-*c*]chromen-6-one (40). This compound was obtained from **39** employing method B. Yield 85%; yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.93 (s, 1H), 8.06–7.91 (m, 3H), 7.68 (t, J = 7.6 Hz, 1H), 7.57 (d, J = 8.3 Hz, 1H), 7.46 (t, J = 7.5 Hz, 1H), 7.07 (dd, J = 8.7, 2.2 Hz, 1H).

8-Hydroxy-7-(piperidin-1-ylmethyl)-6*H*-benzo[4,5]thieno[3,2-*c*]chromen-6-one (41). This compound was obtained from **40** by employing method C. Yield 84%; white solid; m.p 214.9–216.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD = 4/1) δ 7.89–7.80 (m, 2H), 7.61 (t, J = 7.8 Hz, 1H), 7.48–7.36 (m, 2H), 7.33 (d, J = 8.9 Hz, 1H), 5.04 (s, 2H), 3.59–3.56 (m, 2H), 3.13–3.07 (m, 2H), 1.97–1.94 (m, 2H), 1.86–1.80 (m, 3H), 1.59–1.48 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$/CD_3OD = 4/1) δ 159.2, 157.8, 155.5, 151.1, 137.3, 132.7, 130.5, 126.2, 125.6, 124.6, 118.8, 117.1 (2), 116.8, 110.8, 53.4, 53.2 (2C), 23.3 (2C), 22.04. HRMS (ESI) m/z: Calcd for $C_{21}H_{20}NO_3S$ $(M + H)^+$ 366.1158, found 366.1148.

5-Methoxy-2-phenylbenzo[*b*]thiophene-3-carboxylic acid (43). This compound was obtained from **42** according to the methodology described for **39.** Yields 30%, 20%, and 42%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, J = 2.2 Hz, 1H), 7.69 (d, J = 8.8 Hz, 1H), 7.58–7.48 (m, 2H), 7.45–7.34 (m, 3H), 7.07 (dd, J = 8.8, 2.4 Hz, 1H), 3.92 (s, 3H).

Ethyl 5-methoxy-2-phenylbenzo[*b*]thiophene-3-carboxylate (**44**). To a solution of **43** (1.0 mmol) in 20 mL EtOH followed by a dropwise addition of concentrated sulfuric acid (6 mL). The mixture was further stirred for 5 h under reflex condition, the mixture was diluted with water (20 mL) and extracted with 200 mL ethyl acetate. The organic layer was washed with water (3 × 20 mL) and evaporated. The residue was purified by flash chromatography to give **44**. Yield 95%; transparent oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, J = 2.3 Hz, 1H), 7.66 (d, J = 8.7 Hz, 1H), 7.51–7.46 (m, 2H), 7.44–7.40 (m, 3H), 7.00 (dd, J = 8.7, 2.4 Hz, 1H), 4.20 (q, J = 7.1 Hz, 2H), 1.05 (t, J = 7.1 Hz, 3H).

Ethyl 5-hydroxy-2-phenyl-4-(piperidin-1-ylmethyl) benzo[*b*] thiophene-3-carboxylate (45). To a solution of **44** (1.0 mmol) in 4 mL anhydrous dichloromethane, BBr_3 (1 M in CH_2Cl_2, 4.0 mmol) was added at room temperature under N_2. After stirring overnight, the reaction mixture was quenched with EtOH. After evaporation of the solvent, the crude mixture then employing method C to give **45**. Overall yield 71%; yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (d, J = 8.7 Hz, 1H), 7.53–7.51 (m, 2H), 7.43–7.40 (m, 3H), 6.94 (d, J = 8.7 Hz, 1H), 4.25 (q, J = 7.1 Hz, 2H), 3.87 (s, 2H), 1.70–1.59 (m, 4H), 1.27–1.22 (m, 6H), 1.20 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.4, 157.4, 145.4, 136.5, 133.7, 130.8, 129.0 (2C), 128.9, 128.7 (2C), 125.6, 122.0, 116.6, 114.1, 61.8, 58.6, 54.0 (2C), 25.9 (2C), 24.0, 14.0. HRMS(ESI) m/z: Calcd for $C_{23}H_{26}NO_3S$ $(M + H)^+$ 396.1628, found 396.1649.

5-Bromo-2-ethynylaniline (47). To a solution of **46** (1.0 mmol), $Pd(PPh_3)_2Cl_2$ (0.02 mmol) and CuI (0.04 mmol) in 5 mL triethylamine followed by a dropwise addition of trimethylsilylacetylene (1.5 mmol) under an atmosphere of nitrogen. The resultant reaction mixture was stirred at rt about 4 h when **46** was consumed, the mixture was diluted with ethyl acetate (10 mL) and filtered through the celite and washed with ethyl acetate (2 × 5 mL). The filtrate was evaporated. The TMS-ethynylaniline so obtained was dissolved in 5 mL MeOH followed by addition of anhydrous K_2CO_3 (2.0 mmol) and the reaction mixture was stirred at rt for 5 h. Upon completion of the reaction MeOH was evaporated. The residue was dissolved in 20 mL of ethyl acetate and was washed with water (3 × 20 mL). Organic phase washed with saturated aqueous NaCl solution, dried over Na_2SO_4 then evaporated and the compound was purified using silica column. Yield 66%; yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16 (d, J = 8.2 Hz, 1H), 6.85 (s, 1H), 6.79 (dd, J = 8.2, 1.1 Hz, 1H), 4.30 (s, 2H), 3.42 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 149.6, 133.8, 124.1, 120.9, 117.0, 105.6, 83.6, 79.8.

5-Bromo-2-((2-bromo-5-methoxyphenyl)ethynyl)aniline (48). To a solution of 1-bromo-2-iodo-4-methoxybenzene (1.2 mmol), $Pd(PPh_3)_2Cl_2$ (0.02 mmol) and CuI (0.04 mmol) in 3 mL triethylamine followed by a dropwise addition of **47** (1.0 mmol) under N_2. The resultant reaction mixture was being stirred at rt overnight. The mixture was diluted with ethyl acetate (10 mL) and filtered through the celite and washed with ethyl acetate (2 × 5 mL). The organic phase was added 20 mL water then extracted with EtOAc (2 × 20 mL), washed with saturated aqueous NaCl solution, dried over Na_2SO_4 then evaporated. The residue was purified by silica gel column chromatography to give the product. Yield 64%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, J = 8.9 Hz, 1H), 7.23 (d, J = 8.2 Hz, 1H), 7.07 (d, J = 2.3 Hz, 1H), 6.89 (s, 1H), 6.83 (d, J = 8.2 Hz, 1H), 6.77 (dd, J = 8.8, 2.3 Hz, 1H), 4.57 (s, 2H), 3.81 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.7, 149.6, 133.2, 133.1, 125.9, 124.3, 120.9, 117.5, 117.0, 116.7, 115.8, 106.2, 94.4, 90.0, 55.7.

3-Bromo-8-methoxybenzofuro[3,2-*c*]quinolin-6(5*H*)-one (**49**)**.** To a solution of **48** (1.0 mmol), $Cu(OAc)_2$ (10 mol%), Ag_2CO_3 (1.0 mmol) and Cs_2CO_3 (4.0 mmol) in 5 mL of DMSO was stirred in a preheated oil bath at 130 °C for 24 h. After cooled to rt, the reaction mixture was diluted with EtOAc (10 mL) and filtered through a celite pad. Filtrate was washed with water (2 × 10 mL) and water layer was further extracted with ethyl acetate (2 × 10 mL). After evaporation of the solvent, the crude mixture was purified by column chromatography to give product. Yield 13%; pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.03 (s, 1H), 7.92 (d, J = 8.4 Hz, 1H), 7.72 (d, J = 9.0 Hz, 1H), 7.64 (s, 1H), 7.50 (s, 1H), 7.46 (d, J = 8.5 Hz, 1H), 7.07 (dd, J = 9.0, 0.9 Hz, 1H), 3.85 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.0, 157.8, 156.8, 149.5, 139.2, 125.3, 124.3, 123.7, 123.0, 118.4, 115.0, 112.6, 110.5, 110.0, 103.3, 55.7.

3-Bromo-8-hydroxybenzofuro[3,2-c]quinolin-6(5H)-one (50). This compound was obtained from **49** according to the methodology described for **45**. Yield 75%; white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.96 (s, 1H), 9.61 (s, 1H), 7.95 (d, J = 8.5 Hz, 1H), 7.68 (s, 1H), 7.64 (d, J = 8.8 Hz, 1H), 7.48 (d, J = 8.4 Hz, 1H), 7.44 (s, 1H), 6.94 (d, J = 8.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.1, 157.7, 154.8, 148.8, 139.2, 125.3, 124.4, 123.6, 123.0, 118.4, 115.0, 112.3, 110.5, 110.1, 105.9.

3-Bromo-8-hydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (51). This compound was obtained from **50** by employing method C. Yield 67%; white solid; m.p > 250 °C. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD = 1/1) δ 7.99 (d, J = 8.5 Hz, 1H), 7.70 (s, 1H), 7.63 (d, J = 8.9 Hz, 1H), 7.49 (d, J = 8.6 Hz, 1H), 7.11 (d, J = 8.9 Hz, 1H), 4.90 (s, 2H), 3.70–3.48 (m, 2H), 3.15–2.98 (m, 2H), 2.05–1.91 (m, 2H), 1.85–1.76 (m, 3H), 1.63–1.52 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.8, 158.3, 154.8, 148.7, 139.0, 125.8, 125.6, 124.3, 123.3, 118.4, 115.4, 114.1, 111.8, 109.8, 109.0, 52.5, 52.1 (2C), 22,4 (2C), 21.4. HRMS (ESI) m/z: Calcd for $C_{21}H_{20}N_2O_3Br$ $(M + H)^+$, 427.0657, found 427.0646.

2-Bromo-8-hydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (52). This compound was obtained from 4-bromo-2-ethynylaniline according to the methodology described for **51**. Overall yield 2.2%; white solid; m.p > 250 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.24 (d, J = 1.2 Hz, 1H), 7.75 (dd, J = 9.0, 1.9 Hz, 1H), 7.72 (d, J = 9.0 Hz, 1H), 7.43 (d, J = 8.8 Hz, 1H), 7.13 (d, J = 8.9 Hz, 1H), 4.97 (s, 2H), 3.70–3.57 (m, 2H), 3.21–3.12 (m, 2H), 2.02–1.91 (m, 2H), 1.84–1.71 (m, 3H), 1.65–1.56 (m, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 162.0, 150.0, 156.2, 151.2, 138.4, 135.3, 126.6, 125.1, 119.1, 116.8, 116.3, 115.6, 114.1, 113.1, 109.8, 54.1, 53.8 (2C), 24.1 (2C), 22.9. HRMS (ESI) m/z: Calcd for $C_{21}H_{20}N_2O_3Br$ $(M + H)^+$, 427.0657, found 427.0646.

4-Chloro-8-hydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (53). This compound was obtained from 2-chloro-6-ethynylaniline according to the methodology described for **51**. Overall yield 0.6%; white solid; m.p > 250 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (d, J = 7.8 Hz, 1H), 7.71 (d, J = 7.5 Hz, 1H), 7.64 (d, J = 8.9 Hz, 1H), 7.35 (t, J = 7.9 Hz, 1H), 7.09 (d, J = 8.9 Hz, 1H), 4.91 (s, 2H), 3.64–3.61 (m, 2H), 3.19–3.14 (m, 2H), 1.98–1.95 (m, 2H), 1.79–1.75 (m, 3H), 1.61–1.58 (m, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 161.7, 160.6, 156.2, 151.2, 135.7, 132.5, 126.4, 124.8, 121.8, 121.3, 116.4, 115.6, 114.1, 112.9, 109.7, 54.0, 53.8 (2C), 24.1 (2C), 22.8. HRMS (ESI) m/z: Calcd for $C_{21}H_{20}N_2O_3Cl$ $(M + H)^+$, 383.1162, found 383.1138.

3-Chloro-8-hydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (54). This compound was obtained from 5-chloro-2-ethynylaniline according to the methodology described for **51**. Overall yield 2.8%; white solid; m.p > 250 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (d, J = 8.5 Hz, 1H), 7.64 (d, J = 8.9 Hz, 1H), 7.47 (d, J = 1.2 Hz, 1H), 7.35 (dd, J = 8.6, 1.4 Hz, 1H), 7.09 (d, J = 8.9 Hz, 1H), 4.91 (s, 2H), 3.72–3.47 (m, 2H), 3.26–3.03 (m, 2H), 2.04–1.88 (m, 2H), 1.86–1.69 (m, 3H), 1.67–1.55 (m, 1H). ^{13}C NMR

(101 MHz, CD_3OD) δ 162.1, 160.6, 156.1, 151.0, 140.2, 138.3, 126.5, 124.7, 124.2, 116.7, 116.1, 115.4, 112.3, 111.2, 109.7, 54.1, 53.8 (2C), 24.1 (2C), 22.8. HRMS (ESI) *m/z*: Calcd for $C_{21}H_{20}N_2O_3Cl$ $(M + H)^+$, 383.1162, found 383.1138.

2-Chloro-8-hydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (55). This compound was obtained from 4-chloro-2-ethynylaniline according to the methodology described for **51**. Overall yield 0.6%; white solid; m.p > 250 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.03 (d, J = 2.0 Hz, 1H), 7.68 (d, J = 8.9 Hz, 1H), 7.59 (dd, J = 8.9, 2.2 Hz, 1H), 7.45 (d, J = 8.9 Hz, 1H), 7.10 (d, J = 8.9 Hz, 1H), 4.93 (s, 2H), 3.62−3.59 (m, 2H), 3.18−3.12 (m, 2H), 1.98−1.94 (m, 2H), 1.84−1.70 (m, 3H), 1.63−1.57 (m, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 162.0, 160.0, 156.2, 151.1, 138.0, 132.6, 129.7, 126.5, 121.9, 118.9, 116.3, 115.6, 113.6, 113.1, 109.8, 54.0, 53.8 (2C), 24.1 (2C), 22.8. HRMS (ESI) *m/z*: Calcd for $C_{21}H_{20}N_2O_3Cl$ $(M + H)^+$, 383.1162, found 383.1138.

4-Fluoro-8-hydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (56). This compound was obtained from 2-ethynyl-6-fluoroaniline according to the methodology described for **51**. Overall yield 0.4%; white solid; m.p > 250 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 7.91 (d, J = 8.0 Hz, 1H), 7.69 (d, J = 8.9 Hz, 1H), 7.51−7.43 (m, 1H), 7.39−7.34 (m, 1H), 7.12 (d, J = 9.0 Hz, 1H), 4.96 (s, 2H), 3.65−3.62 (m, 2H), 3.20−3.14 (m, 2H), 1.98−1.95 (m, 2H), 1.79−1.72 (m, 3H), 1.64−1.58 (m, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 161.9, 160.6 (d, $J_{C\text{-}F}$ = 4.2 Hz), 156.2, 151.3 (d, $J_{C\text{-}F}$ = 247.7 Hz), 151.1, 128.3 (d, $J_{C\text{-}F}$ = 14.6 Hz), 126.6, 124.5 (d, $J_{C\text{-}F}$ = 7.2 Hz), 118.5 (d, $J_{C\text{-}F}$ = 3.9 Hz), 117.4 (d, $J_{C\text{-}F}$ = 17.8 Hz), 116.3, 115.6, 114.7 (d, $J_{C\text{-}F}$ = 2.02 Hz), 1132, 109.8, 54.0, 53.8 (2C), 24.1 (2C), 22.8. HRMS (ESI) *m/z*: Calcd for $C_{21}H_{20}N_2O_3F$ $(M + H)^+$, 367.1458, found 367.1470.

3-Fluoro-8-hydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (57). This compound was obtained from 2-ethynyl-5-fluoroaniline according to the methodology described for **51**. Overall yield 3.0%; white solid; m.p > 250 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.10−8.07 (m, 1H), 7.68−7.56 (m, 1H), 7.25−7.12 (m, 2H), 7.12−6.99 (m, 1H), 4.91 (s, 2H), 3.72−3.47 (m, 2H), 3.25−2.98 (m, 2H), 2.00−1.62 (m, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 165.8 (d, J = 250.0 Hz), 162.3, 161.0, 156.1, 150.9, 141.1 (d, J = 12.3 Hz), 126.6, 125.3 (d, J = 10.6 Hz), 115.8, 115.3, 112.7 (d, J = 24.3 Hz), 111.4, 109.7, 109.5, 103.3 (d, J = 26.4 Hz), 54.1, 53.8 (2C), 24.1 (2C), 22.9. HRMS (ESI) *m/z*: Calcd for $C_{21}H_{20}N_2O_3F$ $(M + H)^+$, 367.1458, found 367.1470.

2-Fluoro-8-hydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (58). This compound was obtained from 2-ethynyl-4-fluoroaniline according to the methodology described for **51**. Overall yield 5.0%; white solid; m.p > 250 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 7.73 (dd, J = 8.3, 2.3 Hz, 1H), 7.65 (d, J = 8.9 Hz, 1H), 7.49 (dd, J = 9.0, 4.4 Hz, 1H), 7.43−7.39 (m, 1H), 7.09 (d, J = 8.9 Hz, 1H), 4.91 (s, 2H), 3.61−3.58 (m, 2H), 3.17−3.12 (m, 2H), 2.01−1.94 (m, 2H), 1.84−1.69 (m, 3H), 1.60−1.55 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.6, 158.0 (d, $J_{C\text{-}F}$ = 3.2 Hz), 157.5 (d, $J_{C\text{-}F}$ = 240.5 Hz), 154.7, 148.7, 134.8, 125.5, 119.5 (d, $J_{C\text{-}F}$ = 23.7 Hz), 118.3 (d, $J_{C\text{-}F}$ = 8.2 Hz), 115.5, 114.1, 112.2, 111.2, 108.8, 106.5 (d, $J_{C\text{-}F}$ = 24.8 Hz), 52.3, 51.9 (2C), 22.2 (2C), 21.2. HRMS (ESI) *m/z*: Calcd for $C_{21}H_{20}N_2O_3F$ $(M + H)^+$, 367.1458, found 367.1470.

2-Ethynyl-5-methoxyaniline (60). This compound was obtained from **59** according to the methodology described for **47**. Yield 67%; reddish solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (d, J = 8.5 Hz, 1H), 6.26 (dd, J = 8.5, 2.4 Hz, 1H), 6.22 (d, J = 2.3 Hz, 1H), 4.26 (s, 2H), 3.76 (s, 3H), 3.32 (s, 1H).

2-((2-Bromo-5-(methoxymethoxy)phenyl)ethynyl)-5-methoxyaniline (61). This compound was obtained from **60** with 1-bromo-2-iodo-4-(methoxymethoxy)benzene according to the methodology described for **48**. Yield 44%; yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, J = 8.8 Hz, 1H), 7.31 (d, J = 8.5 Hz, 1H), 7.23 (d, J = 2.9 Hz, 1H), 6.85 (dd, J = 8.8, 2.9 Hz, 1H), 6.31 (dd, J = 8.5, 2.3 Hz, 1H), 6.25 (d, J = 2.2 Hz, 1H), 5.17 (s, 2H), 4.52 (s, 2H), 3.79 (s, 3H), 3.48 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.7, 156.3, 150.2, 133.5, 133.0, 126.7, 119.9, 117.8, 116.9, 104.8, 100.2, 99.4, 94.6, 92.5, 91.5, 56.2, 55.3.

3-Methoxy-8-(methoxymethoxy)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (62). This compound was obtained from **61** according to the methodology described for **49**. Yield 30%; brown solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.93 (s, 1H), 7.91 (d, J = 8.6 Hz, 1H), 7.70 (d, J = 8.8 Hz, 1H), 7.65 (s, 1H), 7.11−7.07 (m, 2H), 6.94 (d, J = 8.3 Hz, 1H), 5.26 (s, 2H), 3.83 (s, 3H), 3.42 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.5, 159.3, 159.0, 154.1, 149.8, 140.4, 124.8, 122.6, 115.5, 112.2, 111.4, 107.8, 106.9, 104.7, 99.2, 94.7, 55.6, 55.5.

8-Hydroxy-3-methoxybenzofuro[3,2-*c*]quinolin-6(5*H*)-one (63). Treat a solution of **62** (1.0 mmol) in 5 mL MeOH dropwise with HCl (3 M in aqueous, 8.0 mmol) at rt. Heat the mixture to reflux for 5 h. After cooled to rt, the reaction mixture was evaporated of the solvent, the crude mixture was purified by column chromatography to give products. Yield 30%; brown solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.76 (s, 1H), 9.54 (s, 1H), 7.94 (d, J = 8.7 Hz, 1H), 7.59 (d, J = 8.8 Hz, 1H), 7.41 (d, J = 2.1 Hz, 1H), 7.03 (d, J = 1.6 Hz, 1H), 6.96 (dd, J = 8.9, 1.8 Hz, 1H), 6.87 (dd, J = 8.8, 2.2 Hz, 1H), 3.86 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.3, 159.5, 158.8, 154.6, 148.5, 140.3, 124.8, 122.6, 113.8, 111.9, 111.3107.8, 105.8, 104.8, 99.1, 55.5.

8-Hydroxy-3-methoxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (64). This compound was obtained from **63** by employing method C. Yield 29%; white solid; m.p > 250 °C. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ = 1/3) δ 7.96 (d, J = 8.0 Hz, 1H), 7.39 (dd, J = 8.8, 2.7 Hz, 1H), 6.93−6.90 (m, 2H), 6.85 (d, J = 8.8 Hz, 1H), 4.84 (s, 2H), 3.89 (s, 3H), 2.87−2.56 (m, 4H), 1.66−1.53 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3/CD_3OD$ = 1/3) δ 162.3, 161.0, 160.7, 156.3, 149.4, 139.8, 123.9, 123.4, 115.2, 114.0, 112.5, 111.1, 108.8, 105.9, 98.6, 58.7, 55.7, 53.6 (2C), 25.7 (2C), 23.9. HRMS (ESI) *m/z*: Calcd for $C_{22}H_{23}N_2O_4$ $(M + H)^+$, 379.1658, found 379.1653.

3,8-Dihydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (65). This compound was obtained from **64** demethylation according to the methodology described for **45**. Yield 57%; yellow solid; m.p 226.3−227.5 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 7.86 (d, J = 9.1 Hz, 1H), 7.57 (d, J = 8.9 Hz, 1H), 7.01 (d, J = 8.9 Hz, 1H), 6.87−6.85 (m, 2H), 4.87 (s, 1H), 3.71−3.43 (m, 2H), 3.23−3.01 (m, 2H), 1.93−1.60 (m, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 162.6, 162.5, 162.3, 155.8, 150.6, 141.7, 126.8, 124.3, 115.0, 114.9, 114.4, 109.4, 108.9, 105.6, 101.9, 53.9, 53.6 (2C), 24.2 (2C), 22.9. HRMS (ESI) *m/z*: Calcd for $C_{21}H_{21}N_2O_4$ $(M + H)^+$, 365.1501, found 365.1489.

8-Hydroxy-2-methoxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (66). This compound was obtained from 2-ethynyl-4-methoxyaniline according to the methodology described for **64**. Overall yield 4.0%; white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.66 (d, J = 8.9 Hz, 1H), 7.47 (d, J = 2.2 Hz, 1H), 7.42 (d, J = 9.0 Hz, 1H), 7.25 (dd, J = 9.0, 2.2 Hz, 1H), 7.09 (d, J = 8.9 Hz, 1H), 4.91 (s, 2H), 3.93 (s, 3H), 3.61−3.58 (m, 2H), 3.17−3.12 (m, 2H), 2.01−1.95 (m, 2H), 1.85−1.70 (m, 3H), 1.61−1.58 (m, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 161.7, 161.1, 157.3, 156.0, 151.0, 134.0, 126.6, 122.4, 118.9, 115.9, 115.4, 113.1, 112.2, 109.7, 103.3, 56.3, 53.9, 53.7 (2C), 24.2 (2C), 22.9. HRMS (ESI) *m/z*: Calcd for $C_{22}H_{23}N_2O_4$ $(M + H)^+$, 379.1658, found 379.1653.

2,8-Dihydroxy-7-(piperidin-1-ylmethyl)benzofuro[3,2-*c*]quinolin-6(5*H*)-one (67). This compound was obtained from **66** demethylation according to the methodology described for **45**. Yield 37%; white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.70 (d, J = 9.0 Hz, 1H), 7.45 (d, J = 1.2 Hz, 1H), 7.41 (d, J = 8.9 Hz, 1H), 7.18 (d, J = 8.6 Hz, 1H), 7.11 (d, J = 8.8 Hz, 1H), 4.97 (s, 2H), 3.67−3.52 (m, 2H), 3.17−3.14 (m, 2H), 2.05−1.92 (m, 2H), 1.89−1.73 (m, 3H), 1.66−1.57 (m, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 161.7, 161.2, 156.0, 154.9, 151.0, 133.1, 126.6, 122.4, 118.7, 115.9, 115.3, 113.4, 112.1, 109.8,

106.0, 53.9, 53.6 (2C), 24.2 (2C), 22.9. HRMS (ESI) *m/z*: Calcd for $C_{21}H_{21}N_2O_4$ $(M + H)^+$, 365.1501, found 365.1523.

4.1. General procedures for biological studies

Antitubercular activity of compounds against the *M. tuberculosis* strain $H_{37}Rv$ was determined using the MABA assay as reported in the literature [21]. Cytotoxicity of selected compounds was determined using Vero cells and the MABA assay as reported previously [22].

Growth inhibition of compound **65** against *S. aureus* Newman, *B. subtilis* 168, *E. coli* AB1157, and *E. coli* DH5α was determined using a previously reported protocol [23]. All strains were grown at 37 °C overnight in 10 mL TSB without antibiotics. Overnight cultures diluted 1000-fold were grown at 37 °C for 2–3 h until A_{600} 0.6. Then bacteria were diluted 1:400 into fresh TSB medium. Compound **65** were prepared in DMSO and diluted serially by two-fold to final concentrations in the range of 0.20–25 mg/mL. Equal volume of bacteria and compound were added to 96 well plates and mixed well by shaking. After incubating in 37 °C for 18 h, the MIC of compound **65** was observed. Vancomycin, Tetracycline and Kanamycin was used as positive control. Experiments were performed three times for each condition.

T_m of purified Pks13-TE was determined using nanoDSF assay. As reported in NCBI database, the nucleotide sequence of the *Mtb Pks13* gene (Rv3800c) was obtained. The wild-type Pks13-TE domain construct gene was cloned and inserted into PMCSG-19 plasmid, which was synthesized by Generay Biotechnology (Shanghai, China). The expression of the N-Mbp-His tagged Pks13-TE protein was induced with 0.5 mM IPTG in *E. coli* BL21 (DE3)pLysS strains (Shanghai Institute of Materia Media, China), and the cells were harvested at 20 °C after 18 h of growth. Similar to the procedures reported previously, the Pks13 TE protein was purified with >95% purity monitored by SDS-PAGE, and stored at −80 °C in gel filtration buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM DTT). To a solution of 1 mM of Pks13-TE protein in buffer (100 mM NaCl, 20 mM Tris-HCl pH 8.0) was incubated with different concentrations of tested compounds (1–10 mM in DMSO) in a 30 μL reaction volume for 30 min, and drug-free proteins containing DMSO solution served as a blank control. Approximately 10 μL of the supernatant fraction was loaded to each capillary, which was then placed on the holder in the sample rack. The thermal denaturation curves were determined by the measurement of the protein intrinsic fluorescence on label-free native nanoDSF (NanoTemper, Prometheus NT.48). The temperature was increased from 30 to 90 °C at a rate of 2 °C·min^{-1}. The fluorescence intensity was recorded at 330 and 350 nm, respectively. Changes in the fluorescence ratio (F350/F330) was monitored to determine the apparent melting temperature (T_m).

Enzyme activity of Pks13-TE were assessed based on previously reported protocol [10]. IC_{50} values were measured using 4-methylumbelliferyl heptanoate (4-MUH) as a fluorogenic substrate. The concentrations of the tested compounds were diluted to 20 μM, 10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.625 μM, 0.3125 μM, 0.156 μM, 0.078 μM, 0.039 μM, 0.0195 μM respectively. Pks13-TE (1 μM) in 0.1 M Tris-HCl, pH 7.0 buffer, with 1 μL of each dilution of the compound or DMSO in a total volume of 99 μL was incubated in a 96-well plate format. The reaction was initiated by addition of 1 μL of 2 mM 4-MUH in DMSO (20 μM final concentration) to the reaction mix. The fluorescence of the hydrolyzed product 4-methylumbelliferone was read (excitation at 360 nm and emission at 465 nm) using Spectra Max Flex Station 3 reader (Molecular Device) at 10 min intervals over 60 min. The data points were collected in triplicate and the averaged value was used to generate concentration-response plots for tested compounds. The IC_{50} value for each compound was obtained by nonlinear regression curve fitting of a three-parameter variable slope equation to the dose-response data using Prism software (GraphPad).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113202.

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