Article

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Identification of Novel Coumestan Derivatives as Polyketide Synthase 13 Inhibitors against Mycobacterium Tuberculosis

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ABSTRACT

Inhibition of the mycolic acid pathway has proven a viable strategy in antitubercular drug discovery. The AccA3/AccD4/FadD32/Pks13 complex of *Mycobacterium tuberculosis* constitutes an essential biosynthetic mechanism for mycolic acids. Small molecules targeting the thioesterase domain of Pks13

have been reported, including a benzofuran-based compound, whose X-ray co-crystal structure has been very recently solved. Its initial inactivity in a serum inhibition titration (SIT) assay led us to further probe other structurally-related benzofurans with the aim to improve their potency and bioavailability. Herein we report our preliminary structure-activity relationship studies around this scaffold, highlighting a natural products-inspired cyclization strategy to form coumestans that are shown to be active in SIT. Whole genome deep sequencing of the coumestan-resistant mutants confirmed a single nucleotide polymorphism in the *pks13* gene responsible for the resistance phenotype, demonstrating the druggability of this target for development of new antitubercular agents.

INTRODUCTION

Tuberculosis (TB) is an insidious infectious disease caused by *Mycobacterium tuberculosis (Mtb)*, which has become a serious public health challenge globally.[1, 2] According to the 2016 World Health Organization (WHO) report, there were 10.4 million incident cases of TB worldwide in 2015, accompanied with 1.8 million deaths. This has officially made TB the leading infectious disease killer, surpassing HIV/AIDS. Global control efforts have been hampered by the widespread occurrence of drug-resistant forms of TB, namely multidrug-resistant (MDR), extensively drug-resistant (XDR) and totally drug-resistant (TDR) TB, as well as co-infection with HIV/AIDS in endemic areas. The WHO reported that 400,000 out of the 1.8 million deaths caused by TB in 2015 were caused by TB-HIV co-infection. These alarming figures clearly highlight the global burden caused by this pathogen, necessitating the urgent development of novel chemical therapies. Drug discovery efforts towards this goal have thus far resulted in the accelerated approval of two anti-TB drugs, bedaquiline[3, 4] (**1**) and delamanid[5] (**2**), as part of the treatment regimen for MDR and XDR-TB (**Figure 1**). The diarylquinoline bedaquiline specifically binds to subunit c of the mycobacterial ATP synthase and inhibits the energy metabolism of *Mtb*. Delamanid belongs to the nitroimidazole derivatives that require bioactivation by an

intracellular mycobacterial deazaflavin-dependent nitroreductase, resulting in the inhibition of mycolic acid synthesis.[6] Being the only two new drugs approved in the last five years, their use in MDR-TB needs to be closely monitored for their safety and efficacy as per the WHO interim recommendations, especially when administered in combination with antiretrovirals for HIV. Prolongations of QT interval have been associated[7] with the use of these two drugs in the clinic, urging discovery of more effective agents with less serious side effects.

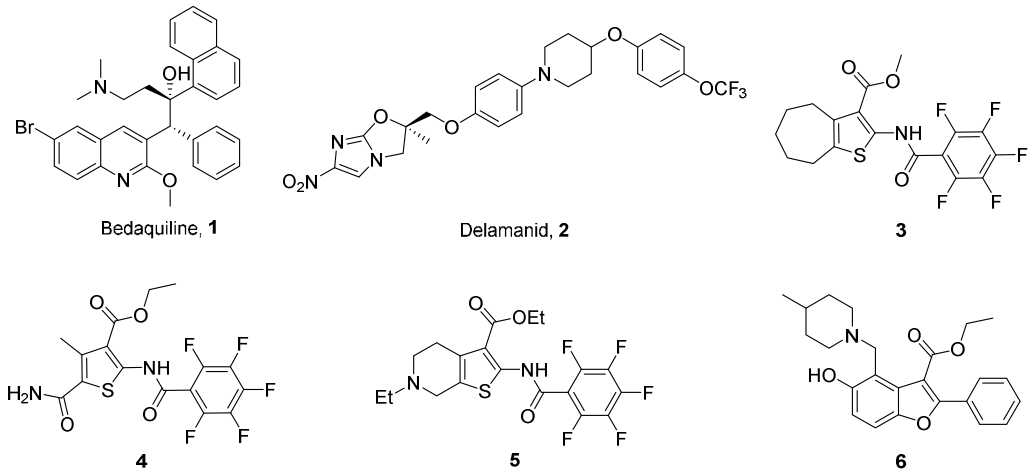


Figure 1. Chemical structures of bedaquiline (**1**), delamanid (**2**), thiophene-based Pks13 inhibitors (**3–5**), and a benzofuran-based Pks13 inhibitor TAM1 (**6**).

Mtb possesses a unique, thick cell wall complex composed of a variety of lipid components that aid in survival and pathogenicity. In particular, mycolic acids are the characteristic, large 3-hydroxy, 2-alkyl long chain fatty acids that form an integral component of the mycomembrane. They influence the cell wall permeability and overall integrity of the cell.[8] The biosynthesis of mycolic acid involves a complex consisting of the acyl-AMP ligase (FadD32) and polyketide synthase 13 (Pks13), encoded by the *fadD32-pks13-accD4* cluster.[9] Pks13 catalyzes a key Claisen condensation step between the long meromycolic acid and saturated α-alkyl chain to form the α-alkyl β-ketoacids, the precursors for mycolic acids biosynthesis.[9] The Pks13 complex belongs to the type I polyketide synthase (PKS) gene

family[10] and is structurally made up of acyl carrier protein (ACP) domains located at the amino terminus (*N*-ACP) and carboxy-terminus (*C*-ACP), a ketoacylsynthase (KS), an acyl transferase (AT) and a thioesterase (TE) domain.[11] The TE domain acts as a transacylase and transfers the ACP-bound mycolates onto trehalose to provide the oxo form of trehalose monomycolate (TMM-o).[12, 13] Further reduction by the *Mtb* homolog of *Corynebacterineae* mycolate reductase A (CmrA) provides trehalose monomycolate (TMM), which are then shuttled to the mycomembrane by the MmpL3 transporter.[14, 15]

Inhibition of pathways related to mycolic acid synthesis has generally been accepted as a viable approach for anti-TB drug discovery, as evidenced by the clinical use of the first-line drug isoniazid, ethionamide, and more recently, preclinical efficacy of reported MmpL3 inhibitors, such as indoleamides and adamantyl ureas.[14, 15] The Alland group demonstrated the intracellular bactericidal efficacy of thiophene-based Pks13 inhibitors, exemplified by compounds **3** and **4** (**Figure 1**), and that sterilizing activity was obtained upon combination with isoniazid.[16] Based on whole genome sequencing of thiophene-resistant mutants, compounds **3** (MIC = 1.0 μM) and **4** (MIC = 0.5 μM) were found to inhibit Pks13 complex by interfering with the function of the *N*-ACP domain, thereby compromising the loading of meromycoloyl-AMP by FadD32.[16] The Sucheck group reported a library of 42 aminothiophenes, with the most active compound **5** (**Figure 1**) having an MIC value of 0.2–0.4 μM against *Mtb* strains resistant to isoniazid, rifampin and fluoroquinolones.[17]

In 2013, compound **6** was discovered as a Pks13 inhibitor from high throughput screening efforts combined with whole-genome sequencing of the resistant isolates.[18] This compound possesses an MIC value of 2.0 μM, and the mutations observed (D1644G and D1607N) in the resistant colonies were found in the carboxy terminus of the thioesterase domain of Pks13. However, our group herein demonstrated that compound **6** was not active in the mouse serum inhibition titration assay, suggesting its poor bioavailability. During the preparation of this manuscript, Sacchettini group reported their

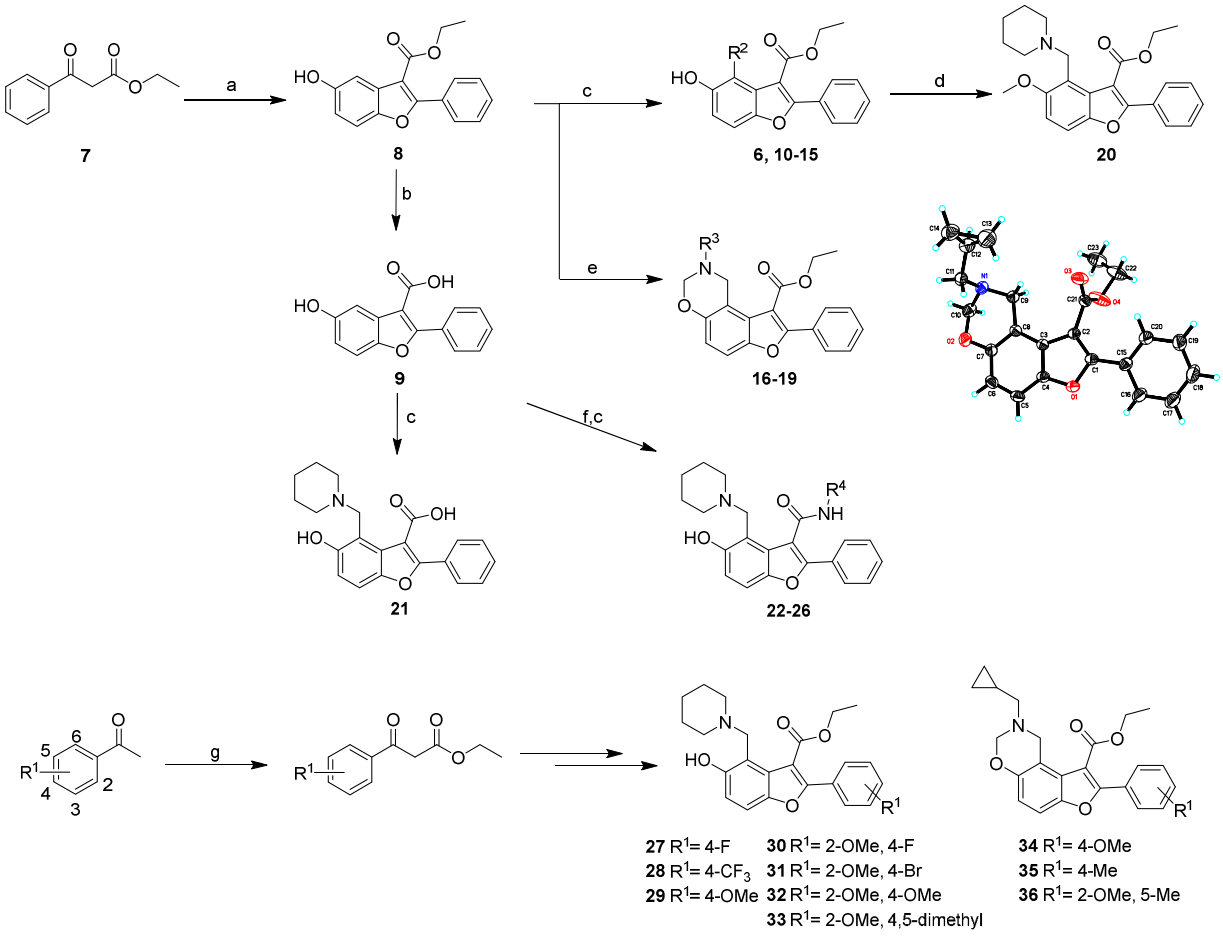
follow-up findings on this compound, culminating in a 2-phenol-3-amidobenzofuran analog TAM-16 that was shown to be efficacious in a murine model of TB, along with an X-ray co-crystal structure of their initial compound **6** bound to the Pks13.[19] In this article, we present our efforts in the SAR optimization of compound **6** that led to the identification of coumestan analogues as potential novel anti-TB compounds in vitro, and more importantly, showing activity in the mouse SIT assays.

RESULTS AND DISCUSSION

Chemistry

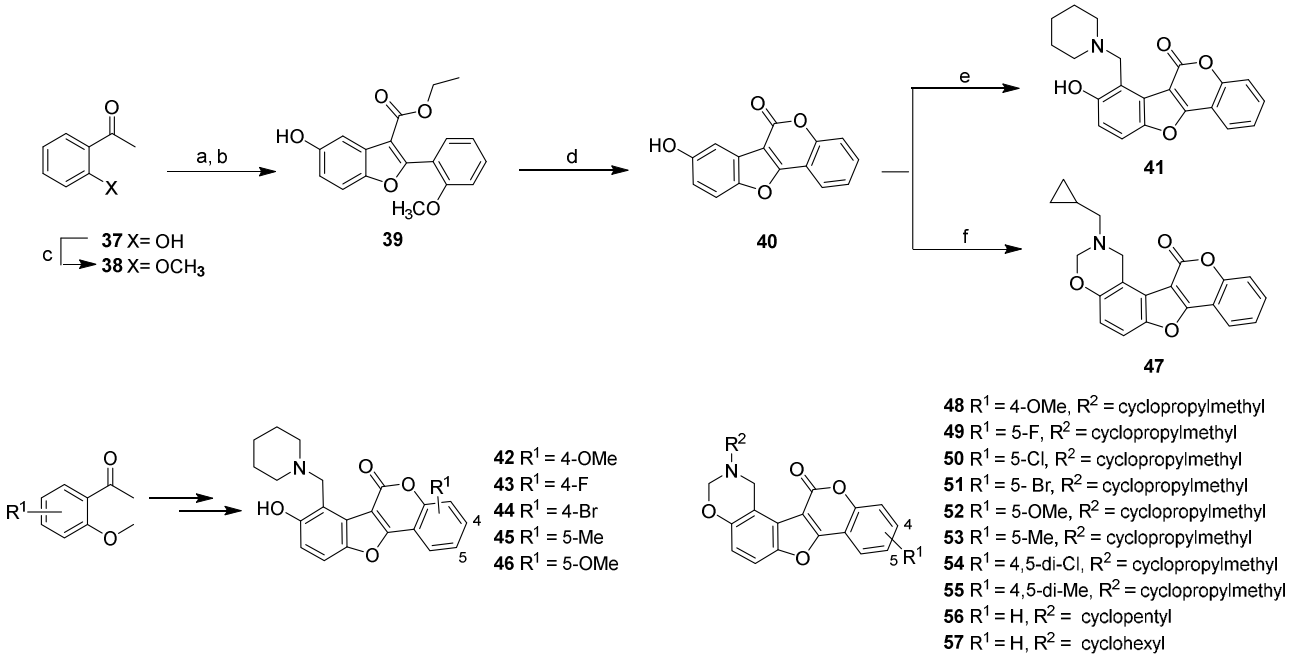
The benzofuran derivatives **6** and **8–36** were synthesized in 2–5 steps utilizing the synthetic routes shown in Scheme 1. Compound **8** was formed via a copper-catalyzed cyclization of 1,4-benzoquinones and ethyl 3-oxo-3-phenylpropanoate, which was prepared as described in the literature from commercially available acetophenone **7**.[20] Compound **8** underwent Mannich reaction with 37% aqueous formaldehyde and appropriate secondary amines to yield compounds **6** and **10–15.** *O*-Methylation of the phenolic group of **10** with methyl iodide afforded the methoxy derivative **20.** When primary amines were used in the Mannich reaction with intermediate **8**, only the 1,3-oxazinanes **16–19** were formed and no ring-opened product was observed. The structure of 1,3-oxazinane **16** was confirmed by X-ray crystallography (Scheme 1). Hydrolysis of the ethyl ester **8** under basic condition afforded the carboxylic acid **9**, which was subjected to subsequent amide coupling and Mannich reaction with piperidine to afford compounds **22–26**. Similarly, compounds **27–33** and **34–36** were synthesized in a similar manner to compounds **10** and **16** from commercially available substituted acetophenones.

Scheme 1[a]



[a] Reagents and conditions: (a) benzoquinone, $Cu(OTf)_2$ (5 mol %), toluene, reflux, 7–12 hr; (b) LiOH, 1,4-dioxane, H_2O, 60 ºC, 24 hr; (c) formaldehyde (37% aq), corresponding secondary amines, EtOH, reflux, 8–12 hr; (d) CH_3I, NaH, DMF, 0 ºC, 1 hr; (e) formaldehyde (37% aq), corresponding primary amines, EtOH, reflux, 8–12 hr; (f) EDC·HCl, HOBt, 3-methylpyridine, corresponding amines, DMF, rt, 5–8 hr; (g) diethyl carbonate, NaH, toluene, reflux, 30 min.

Scheme 2[a]



[a]Reagents and conditions: (a) diethyl carbonate, NaH, toluene, reflux, 30 min; (b) benzoquinone, $Cu(OTf)_2$ (5 mol %), toluene, reflux, 7–12 hr; (c) CH_3I, K_2CO_3, THF, rt, 3–5 hr; (d) BBr_3 in CH_2Cl_2 (1 M), CH_2Cl_2, rt, overnight, then EtOH, reflux, 1 hr; (e) formaldehyde (37% aq), piperidine, EtOH, reflux, 8–12 hr; (f) formaldehyde (37% aq), cyclopropylmethanamine, EtOH, reflux, 8–12 hr.

Following a similar procedure to synthesize compound **8**, ethyl 5-hydroxy-2-(2-methoxyphenyl) benzofuran-3-carboxylate **39** was formed starting from the commercially available 2-acetylphenol **37** (Scheme 2). Coumestan **40** was prepared via cyclization of ester **39** in refluxing ethanol.[21] Subsequent Mannich reaction afforded coumestan derivatives **41** and **47** (Scheme 2). Coumestans **42**–**46** and **48**–**57** were synthesized in a similar manner to compounds **41** and **47** respectively, from commercially available substituted 2-acetylanisoles.

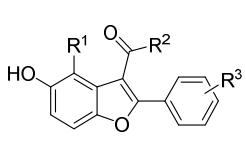
Initial SAR for Benzofurans.

All the final compounds were initially evaluated for their MIC values against *Mtb* strain H37Rv in a microplate alamar blue assay (MABA).[22] The original hit compound **6** (TAM1) had an MIC value of 1 μg/mL, corresponding to 2.5 μM, which was consistent with the reported activity.[18] As shown in Table 1, we first examined the substitutions at position 4 of the benzofuran ring (the R^1 group). Deletion of the (methylpiperidin-1-yl)methyl to give compound **8** resulted in at least a 64-fold reduction of activity, indicating the importance of R^1 substitution, which is consistent with the SAR trend reported previously.[19] Piperidin-1-yl-methyl and pyrrolidin-1-yl-methyl (**10** and **11**) substituents at R^1 generally maintained activity, but the ring-open diisopropylamine analog **12** had an 8-fold reduction in activity. Addition of polar atoms to the piperidine ring (**13**–**15**) were found to be deleterious for the activity, with MIC values ranging from 16 to >64 μg/mL. The SAR trends observed herein are consistent with those reported by Sacchettini group. The X-ray co-crystal structure demonstrated the binding mode of these benzofuran derivatives, with the piperidine and 5-hydroxy groups binding deeper into the pocket of the Pks13 thioesterase domain's active site, leaving the 2-phenyl and 3-ester groups more exposed to solvents.[19] The cyclization of 4-aminomethyl substituents with 5-hydroxyl group of the benzofurans resulted in compounds **16**–**19** with MIC values ranging from 1–16 μg/mL. Among this series, the cycloaliphatic derivatives **16** and **18** were found to be more potent, with the cyclopropylmethyl analog **16** having an MIC value of 1 μg/mL. Methylation of the 5-hydroxyl group of compound **10** to give the methoxy analog **20** also resulted in an 8-fold decrease of activity (MIC = 16 μg/mL), indicating that the 5-hydroxy group may provide an optimal hydrogen bond to the carboxylate group of D1644.[19] Next, we assessed the effects of substituting the benzene ring at 2-position of the benzofurans with various electron-donating groups (EDG; OMe, Me) and electron-withdrawing groups (EWG; F, Br, CF_3). At the *para* position, a fluoro group (compound **27**) resulted in a 4-fold increase of activity with MIC value of

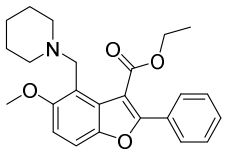
0.5 μg/mL, while a trifluoromethyl or methoxy group (**28** or **29**) resulted in compounds with MIC values of 8 and 16 μg/mL respectively. The most potent benzofuran derivative reported by Sacchettini group was TAM16, which has a 4-hydroxyl substituent on the benzene ring.[19] Various di- or tri-substitutions on this benzene ring (**30**–**33**) generally resulted in compounds with reduced antitubercular activity (MIC values of 4 to >64 μg/mL), particularly with a 2-methoxy group being deleterious for activity. Within the oxazine series (**34**–**36**), EDG substitutions were generally less tolerated with activities ranging from 2 to 8 μg/mL.

Compounds containing an ester group are prone to hydrolysis by various endogenous esterases. Carboxylic acid derivative **21** was completely inactive (MIC value > 64 μg/mL), suggesting that these 2-phenylbenzofuran-3-carboxylates may be prone to metabolic liability, which was also reported by Sacchettini group. Subsequently, we investigated the replacement of ethyl ester with substituted amides **22**–**26**. The replacement of ethyl ester with an ethyl amide (**22**) resulted in a slight drop in activity (MIC = 4 μg/mL). The MIC values further dropped with increasing size of the amide substituents, ranging from cyclopropyl to adamantyl (**23**–**26**). This is consistent with the obtained X-ray co-crystal structure showing the ester group is partially solvent exposed.

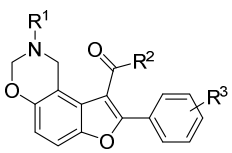
Table 1. Antitubercular activity of benzofuran derivatives against the *M. tuberculosis* strain H37Rv.[a]



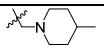
6, 8,10-15, 21-33

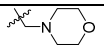
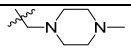
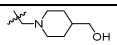
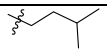


20



16-19, 34-36

Compound	R^1	R^2	R^3	MIC[a], μg/mL	cLogP[b]
6		OCH_2CH_3	H	1	6.53
8	H	OCH_2CH_3	H	> 64	5.04

10		OCH_2CH_3	H	2	6.01
11		OCH_2CH_3	H	2	5.45
12		OCH_2CH_3	H	8	6.94
13		OCH_2CH_3	H	> 64	4.73
14		OCH_2CH_3	H	≥ 64	5.18
15		OCH_2CH_3	H	16	4.55
16		OCH_2CH_3	H	1	6.15
17		OCH_2CH_3	H	16	5.67
18		OCH_2CH_3	H	2	6.56
19		OCH_2CH_3	H	4	7.16
20	-	-	-	16	6.44
21		OH	H	> 64	2.04
22		*N*-CH_2CH_3	H	4	4.15
23		*N*-cyclopropyl	H	8	4.21
24		*N*-1-adamantyl	H	> 64	6.28
25		*N*-Bn	H	16	5.59
26		*N*-Ph	H	8	5.41
27		OCH_2CH_3	4-F	0.5	6.16
28		OCH_2CH_3	4-CF_3	8	6.90
29		OCH_2CH_3	4-OCH_3	16	5.95
30		OCH_2CH_3	2-OCH_3, 4-F	8	5.66

31		OCH_2CH_3	2-OCH_3, 4-Br	>64	6.38
32		OCH_2CH_3	2,4-di-OCH_3	16	5.47
33		OCH_2CH_3	2-OCH_3, 4,5-di-CH_3	4	6.34
34		OCH_2CH_3	4-OCH_3	2	6.09
35		OCH_2CH_3	4-CH_3	4	6.65
36		OCH_2CH_3	2-OCH_3-5-CH_3	8	6.02

[a]See Experimental Section. The lowest concentration of drug leading to at least 90% inhibition of bacterial growth signal by the MABA. MIC values are reported as an average of three individual measurements. CLogP was calculated using ChemBioDraw Ultra 13.0.

Identification of the Coumestan Analogues.

Natural products have been long recognized as a rich source of lead compounds for drug development.[23] More than half of the clinically available therapeutic agents were derived originally from natural products and their related structures. Coumestan is a δ-lactone containing a tetracycle, which constitutes the central core of various bioactive natural products such as coumestrol, wedelolactone, and psoralidin, among others. A broad range of pharmacological activities of coumestans has been reported, including anticancer, antioxidant, antibacterial and antidepressant effects.[24-26] We envisaged that the ester oxygen of the previously described benzofuran series could be connected to the adjacent *ortho*-position of the 2-phenyl ring to form a coumestan framework (**Figure 2**). This approach is expected to circumvent the ester liability issue that may arise in the original benzofuran series. Therefore, in the next iteration of SAR studies, we employed the initial SAR obtained from the benzofuran series to the coumestans.

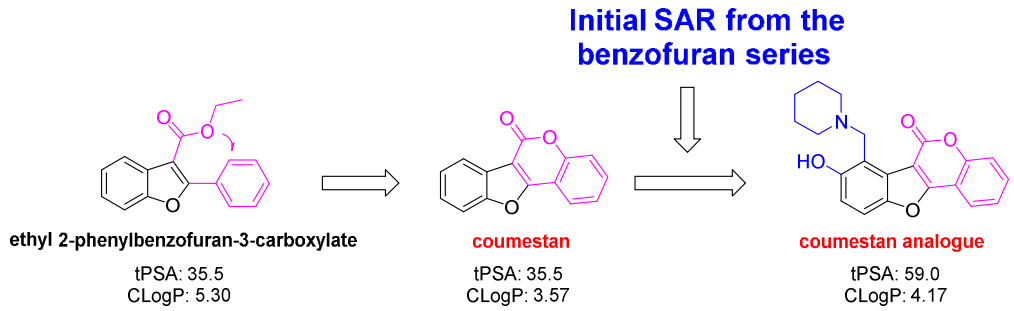
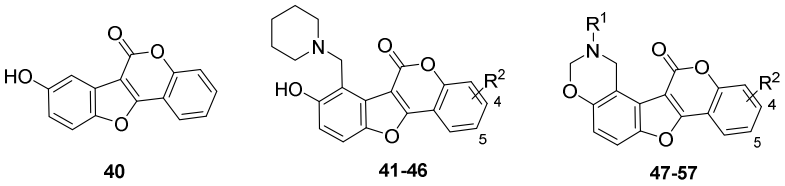


Figure 2. From the benzofurans to the coumestans.

Gratifyingly, the coumestan derivatives **41**–**57** generally exhibited improved antitubercular activity against the *Mtb* strain H37Rv as shown in Table 2. Compound **41**, which is the coumestan derivative of compound **10**, had an 8- to 16-fold increase in activity with an MIC value of 0.125–0.25 μg/mL. Similarly, the antitubercular activity of the coumestans were either improved or maintained when compared to the corresponding ethyl 2-phenylbenzofuran-3-carboxylates, e.g. **41** vs **10**, **42** vs **29**, and **43** vs **27**. A bulkier 4-bromo derivative **44** and the smaller 5-methyl analog **45** also maintained activities between 0.5 and 1 μg/mL. Monosubstitutions on the right hand side benzene ring of the coumestans with various EDG and EWG were generally tolerated, with the exception of 5-methoxy analog **46** (MIC value of 4 μg/mL). Among the oxazine-coumestans **47**–**57**, the unsubstituted and the 4-methoxy analogs **47** and **48** were of similar activities compared to their direct benzofuran analogs **16** and **34**, respectively. However, EDG (methyl or methoxy) and EWG (halogen) substitutions at the 4- or 5-position were generally less tolerated, with MIC values ranging from 4 μg/mL to inactive (> 64 μg/mL). Within the oxazine-coumestans, the pyrrolidine analog **56** emerged as the most potent analog with an MIC value of 0.5 μg/mL.

Table 2. Antitubercular activity of coumestan derivatives against the *M. tuberculosis* strain H37Rv [a]



Compound	R^1	R^2	MIC[a], μg/mL	CLogP[b]
40	-	-	> 64	3.19
41	H	H	0.125–0.25	4.17
42	H	4-OCH_3	0.25	4.24
43	H	4-F	1	4.31
44	H	4-Br	1	5.03
45	H	5-CH_3	0.5	4.67
46	H	5-OCH_3	4	4.24
47		H	2	4.37
48		4-OCH_3	2	4.57
49		5-F	16	4.51
50		5-Cl	16	5.08
51		5-Br	8	5.23
52		5-OCH_3	16	4.57
53		5-CH_3	4	4.86
54		4,5-di-Cl	≥ 64	5.67
55		4,5-di-CH_3	8	5.31
56		H	0.5	4.77

57		H	16	5.33

[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 13.0.

Toxicity to Mammalian Cells.

All compounds showing MIC values lower than 1 μg/mL were next evaluated for their toxicity to Vero cells, which are derived from African green monkey's kidney. Comparison of the Vero cells toxicity (IC_{50} values) and the MIC values for *Mtb* growth inhibition gives a measure of general toxicity profile of the compound in interest. The selectivity index (SI) values were calculated from the ratio of $IC_{50\ (Vero)}$ /MIC $_{(Mtb)}$ as shown in Table 3. Most selected compounds were found to possess low to moderate SI values of between 8–32 when compared to Vero cells, with the exception of compound **56**, which exhibited equal values of IC_{50} and MIC.

Table 3. Vero toxicity and Selectivity index (SI) of selected compounds.

Compound	Toxicity to Vero cells IC_{50}, μg/mL	MIC H37Rv, μg/mL	SI [a]
6	16	1	16
16	8	1	8
27	16	0.5	32
41	4	0.125–0.25	16–32
42	4	0.25	16
43	8	1	8

44	8	1	8
45	8	0.5	16
56	0.5	0.5	1

[a] SI = IC_{50} (Vero cells) / MIC (H37Rv)

In addition to the Vero cells, the two most potent coumestans **41** and **42** were evaluated for their cytotoxicity profiles against four human-derived cell lines: MRC-5 and HFL1 (human lung fibroblast cells), QSG-7701 (human liver cell), and HEK-293 (human embryonic kidney cell). As shown in Table 4, both coumestans **41** and **42** were generally much less toxic to the human-derived cell lines, with SI values between 155 and 400. The methoxy-substituted coumestan **42** was slightly more toxic to the HFL-1 and QSG-7701 cells with IC_{50} values of 50.2 and 38.8 μM, respectively. The observed trend among the four human cell lines is consistent with that seen in the Vero cells, with compound **41** possessing slightly higher selectivity index compared to compound **42**. Collectively, the coumestans are shown to be non-cytotoxic to these human-derived cell lines at concentrations of more than 100-fold their MIC values for *M.tb*.

Table 4. Toxicity of **41** and **42** in human-derived cell lines.

Compound	MRC-5 cells (IC_{50}, μg/mL)[a]	HFL-1 cells (IC_{50}, μg/mL)	QSG-7701 cells (IC_{50}, μg/mL)	HEK-293 cells (IC_{50}, μg/mL)
41	>50	>50	>50	>50
42	>50	50.2±5.3	38.8±2.6	>50

[a] IC_{50} values were obtained as an average from three independent experiments and are expressed in μg/mL

Serum Inhibition Titration (SIT) Assay.

The most potent benzofuran **27** and the coumestans **41** and **42**, as well as the hit compound **6,** were evaluated in the SIT assay.[22] Carboxymethylcellulose (0.5%) was used as vehicle and isoniazid included as a positive control. In this assay, BALB/c mice were orally gavaged with 100 mg/kg of compounds **6**, **27**, **41**, **42,** or 10 mg/kg of INH, with blood collected at 30, 60 and 120 min. The sera were then separated and prepared in 2-fold dilutions and incubated with a bacterial suspension for 7 days. Growth inhibition of serially diluted serum on H37Rv was determined using the microplate Alamar Blue assay. As shown in **Figure 3**, the original hit benzofuran **6** was inactive in the SIT assay, most likely due to the hydrolysis of its ethyl ester into inactive carboxylic acid derivative. A similar trend was observed for the most potent benzofuran derivative **27**, supporting evidence for the ester metabolic liability. The coumestans **41** and **42**, however, showed activity in the SIT assay up to 1:32 / 1:64 dilution (**Figure 3**). While the blood samples from 30, 60 and 120 min gave very comparable inhibitory effects for coumestan **42**, the blood sample from 30-min cohort gave the most potent inhibition for coumestan **41**. This result is consistent with the human microsomal stability data obtained for coumestans **41** and **42**, in which 14.5% and 45.5% remained non-metabolized, respectively, after incubation for 60 min at 10 μM concentration (see Supplementary Information).

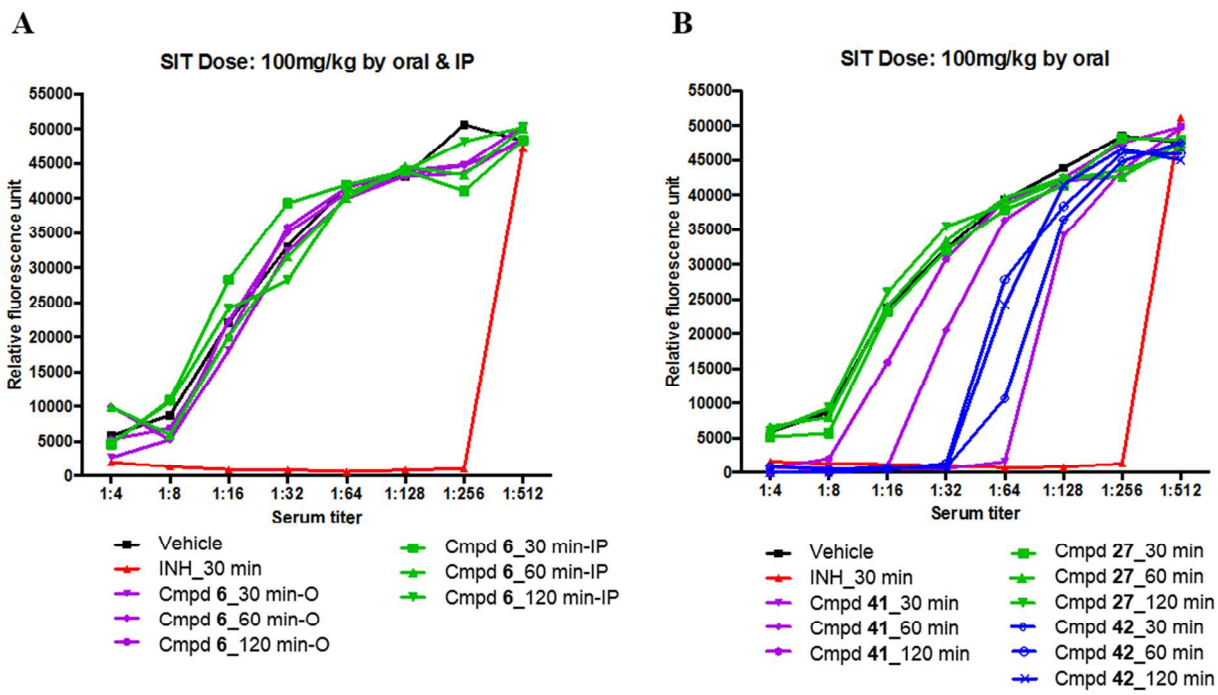


Figure 3. Serum inhibition titration data for compounds **6**, **27**, **41**, and **42.**

Target Verification.

Nine resistant mutants of *M. tuberculosis* were selected from 7H11 agar plates containing compound **41**, compound **42** and compound **27**, respectively (Table 5). MIC shifts of 8- to 128-fold were observed between the mutated and wild-type strains. Again, the coumestan-resistant mutants showed more dramatic MIC shifts (64- to 128-fold, for **41** and **42**) compared to the benzofuran-resistant mutants (8-fold, for **27**). Genomic DNA was subsequently isolated from each mutant and the non-mutated parent strain, and subjected to whole genome sequencing and comparative analysis (see Methods). As shown in Table 6, all nine clones contained one of four distinct non-synonymous mutations in the *pks13* gene. Strikingly, six out of the nine clones contained only a *pks13* gene mutation (A1667V, D1644G, N1640K or N1640S). The other three resistant mutants contained a single additional non-synonymous mutation within one of three genes encoding distinct hypothetical proteins. Given that *pks13* was the only consistently altered gene, we do not suspect a role for these other mutations in the resistant phenotype.

Interestingly, the D1644G mutation observed for the coumestan **41**-resistant mutant also conferred resistance[18] to the benzofuran **6** and is proximal to the more frequently observed N1640K in this study. All the observed mutation sites (A1667V, D1644G, N1640K or N1640S) are co-localized within the active site of the thioesterase domain of Pks13, consistent with the disruption of their binding as seen in the obtained X-ray co-crystal structure.[14]

Table 5. Selection of resistant mutants.

Compound	WT MIC (μg/mL)	Selection concentration (μg/mL)	Clones selected	Mutant MIC (μg/mL)	MIC shift (fold)
41	0.125-0.25	3	3	16	64-128
42	0.25	4	3	16	64
27	0.5	2	3	4	8

Table 6. Single nucleotide polymorphisms identified in resistant mutants.[a]

ORF	Gene	AA change	# of clones with SNP	Compound **41**			Compound **42**			Compound **27**		
				1	2	3	1	2	3	1	2	3
Rv1765c	Conserved hypothetical	M95I	1								X	
Rv2015c	Conserved hypothetical	R58L	1					X				
Rv3800c	*pks13*	A1667V	2							X		X
Rv3800c	*pks13*	D1644G	1		X							

Rv3800c	*pks13*	N1640K	5	X		X	X	X	X			
Rv3800c	*pks13*	N1640S	1								X	
Rv3921c	Conserved membrane	A312V	1							X		

[a]See Experimental Section.

Conclusions

Herein we reported the identification of coumestan analogs as novel antitubercular compounds targeting Pks13, resulting from natural products-inspired structural modifications of the initial hit compound benzofuran **6**. These coumestans represent a natural scaffold that showed reasonably potent anti-TB activity in vitro, as well as oral bioavailability in mouse. As Pks13 is intimately involved in the biosynthesis of mycolic acids, which is also the target of well-known anti-tuberculosis drugs such as isoniazid and ethionamide, this study and others support the notion that biochemical reactions on the pathway to synthesis and processing of mycolic acids are viable targets for new anti-TB drug discovery (target validation). In the present study, whole genome deep sequencing of the wild-type and resistant mutants confirmed that these coumestans indeed inhibit *pks13*, since single SNPs in *pks13* led to resistance phenotypes, thus demonstrating the druggability for *pks13*. The mutation sites observed in our resistant mutants correspond to the active site of the thioesterase domain of Pks13, consistent with the reported X-ray co-crystal structure reported by Sacchettini group. Further investigation of the potential use of these coumestans as anti-TB therapeutics is warranted and work is underway to improve their general toxicity profile.

Experimental Section

General Methods: Starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification, unless otherwise stated. Anhydrous THF 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 TLC on SiO_2 and LCMS. Silica gel for column chromatography (CC) was of 200–300 mesh particle size, and an EtOAc/petroleum ether mixture or gradient was used unless stated otherwise. ^{1}H NMR spectra were recorded at a spectrometer frequency of 400 MHz, and ^{13}C NMR spectra were recorded at 101 MHz. Chemical shifts were reported in δ (ppm) using the δ 0 signal of tetramethylsilane (TMS) 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 an Agilent 1260 HPLC system or a Waters HPLC system using either a Phenomenex Luna-C18 column (5 micron, 250 x 4.6 mm) or InertSustain-C18 column (5 micron, 250 x 4.6 mm) with detection at 280, 254 and 220 nm on a variable wavelength detector G1314F. Method A: Phenomenex Luna C18 column, flow rate = 1.0 mL/min, gradient elution of 30–90% acetonitrile in water (containing 0.05 vol% of HCO_2H) over 20 min or Method B: InertSustain C18 column, flow rate = 1.0 mL/min, gradient of 30–90% acetonitrile in water (containing 0.05 vol% of HCO_2H) over 25 min. See Supplementary Information for analytical HPLC purities and the melting points for all final compounds.

General Procedure for the Synthesis of 5-Hydroxy-2-phenyl Benzofurans from ethyl **Benzoylacetates. (Method A)**. To a solution of ethyl benzoylacetate (1.3 mmol) and $Cu(OTf)_2$ (5 mol%) in toluene (5 mL) was added a solution of benzoquinone (1.0 mmol) in toluene (1.5 mL) under N_2. The reaction mixture was allowed to reflux for 8–12 hrs and then cooled to rt. The reaction mixture was quenched with saturated NH_4Cl solution and extracted with EtOAc (2×20 mL). The combined organic phases were washed with water (3×10 mL), dried over anhydrous Na_2SO_4 and evaporated. The residue was purified by flash chromatography to give the 5-hydroxy-2-phenyl benzofuran.

General Procedure for the Mannich Reaction of 5-Hydroxy-2-phenylbenzofuran or Coumestan with Amine and Formaldehyde (Method B). To a solution of 5-hydroxy-2-phenylbenzofuran or coumestan derivative (1 mmol) in ethanol (3 mL) were added formaldehyde (37% in water, 4 mmol) and the appropriate amine (4 mmol) at rt under N_2. The reaction mixture was allowed to reflux for 5–12 hr 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 Amidation of 5-Hydroxy-2-phenylbenzofuran-3-carboxylic Acid (Method C). To the solution of compound **9** (1 mmol) in anhydrous DMF (5 mL), EDC·HCl (1.3 mmol), HOBt (1.3 mmol), 3-methylpyridine (2.1 mmol) and appropriate amine (1.2 mmol) under N_2 were added at rt. After stirring overnight at rt, 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, brine, dried over anhydrous Na_2SO_4 and evaporated. The residue was purified by flash chromatography to give the amide product.

General Procedure for the Preparation of Coumestans from Substituted Ethyl 5-hydroxy-2-(2-methoxyphenyl)benzofuran-3-carboxylates (Method D). To a solution of ethyl 5-hydroxy-2-(2-methoxyphenyl)benzofuran-3-carboxylate (1.0 mmol) in anhydrous CH_2Cl_2 (10 ml)BBr_3 (1M in CH_2Cl_2, 2.0 mmol) was added at rt under N_2. After stirring overnight, the reaction mixture was quenched with EtOH. The resulting mixture was allowed to reflux for 1 hr and evaporated. The residue was purified by flash chromatography or by recrystallization in EtOH to obtain coumestans.

General Procedure for the Synthesis of Ethyl Benzoylacetates from Acetophenones. (Method E). To a stirred solution of diethyl carbonate (2.0 mmol) in toluene (4 mL), NaH (60% dispersion in oil, 2.5 mmol) was added at rt. After stirring for 10 min, a solution of substituted acetophenone (1 mmol) in 1

mL of toluene was added. The reaction mixture was allowed to reflux for 30 min and then cooled to rt. The reaction was quenched with water under ice cooling, acidified with glacial AcOH and extracted with EtOAc (3×10 mL). The combined organic phases were washed with water (3×10 mL), dried over Na_2SO_4 and evaporated. The residue was purified by flash chromatography to give the substituted ethyl benzoylacetate.

Ethyl 5-hydroxy-4-((4-methylpiperidin-1-yl)methyl)-2-phenylbenzofuran-3-carboxylate (6). This compound was obtained from **8** and 4-methylpiperidine employing Method B. Yield 82%; pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (dd, *J* = 7.7, 1.7 Hz, 2H), 7.48–7.40 (m, 3H), 7.31 (d, *J* = 8.8 Hz, 1H), 6.86 (d, *J* = 8.8 Hz, 1H), 4.35 (q, *J* = 7.1 Hz, 2H), 3.97 (s, 2H), 3.03 (d, *J* = 10.9 Hz, 2H), 2.16 (t, *J* = 11.0 Hz, 2H), 1.69 (d, *J* = 12.8 Hz, 2H), 1.45 (br s, 1H), 1.37–1.23 (m, 5H), 0.95 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.5, 156.9, 155.6, 148.3, 130.1, 129.7, 128.5, 128.0, 125.5, 115.4, 112.4, 110.8, 110.2, 61.6, 57.7, 53.5, 34.3, 30.6, 21.8, 14.1. HRMS (ESI) m/z: Calcd for $C_{24}H_{28}NO_4$ $(M+H)^+$ 394.2013, found 394.2014.

Ethyl 5-hydroxy-2-phenylbenzofuran-3-carboxylate (8). This compound was obtained from **7** employing Method A. Yield 46%; pale yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.12–7.75 (m, 2H), 7.52–7.41 (m, 4H), 7.35 (d, *J* = 8.8 Hz, 1H), 6.85 (dd, *J* = 8.8, 2.6 Hz, 1H), 4.35 (q, *J* = 7.1 Hz, 2H), 1.36 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 165.5, 162.5, 155.5, 149.7, 131.2, 131.2, 130.4, 129.2, 129.0, 115.2, 112.4, 109.8, 108.1, 61.7, 14.5. HRMS (ESI) m/z: Calcd for $C_{17}H_{14}NaO_4$ $(M+Na)^+$ 305.0784, found 305.0786.

5-Hydroxy-2-phenylbenzofuran-3-carboxylic Acid (9). To a solution of **8** (1.0 mmol) in 1,4-dioxane (5 mL), LiOH (6.0 mmol) in H_2O (1.0 mL) was added at rt. After stirring at 60 ºC for 24 hr the reaction mixture was acidified with 2 M aqueous HCl to pH 5–6. The resultant solid was filtered off to give a white solid with a 87.4% yield. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 12.95 (s, 1H), 9.38 (s, 1H), 7.98–7.92

(m, 2H), 7.56–7.49 (m, 3H), 7.47 (d, *J* = 8.8 Hz, 1H), 7.40 (d, *J* = 2.3 Hz, 1H), 6.83 (dd, *J* = 8.8, 2.5 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.7, 159.7, 154.3, 147.4, 130.0, 129.5, 129.1, 128.1, 127.9, 114.2, 111.6, 109.4, 106.7.

Ethyl 5-hydroxy-2-phenyl-4-(piperidin-1-ylmethyl)benzofuran-3-carboxylate (10). This compound was obtained from **8** and piperidine employing Method B. Yield 89%; pale yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.73–7.66 (m, 2H), 7.47–7.43 (m, 3H), 7.35 (d, *J* = 8.8 Hz, 1H), 6.83 (d, *J* = 8.9 Hz, 1H), 4.33 (q, *J* = 7.1 Hz, 2H), 4.04 (s, 2H), 2.67 (br s, 4H), 1.71–1.60 (m, 4H), 1.54 (brs, 2H), 1.24 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.7, 158.6, 156.3, 149.6, 131.2, 130.9, 129.5, 129.0, 127.0, 116.0, 113.0, 112.3, 111.5, 62.8, 57.6, 54.8, 26.5, 24.7, 14.3. HRMS (ESI) m/z: Calcd for $C_{23}H_{26}NO_4$ $(M+H)^+$ 380.1856, found 380.1860.

Ethyl 5-hydroxy-2-phenyl-4-(pyrrolidin-1-ylmethyl)benzofuran-3-carboxylate (11). This compound was obtained from **8** and pyrrolidine employing Method B. Yield 67%; white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.76–7.66 (m, 2H), 7.50–7.41 (m, 3H), 7.32 (d, *J* = 8.8 Hz, 1H), 6.82 (d, *J* = 8.8 Hz, 1H), 4.34 (q, *J* = 7.1 Hz, 2H), 4.10 (s, 2H), 2.82–2.47 (m, 4H), 2.01–1.74 (m, 4H), 1.26 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.7, 157.8, 156.4, 149.4, 131.2, 130.7, 129.6, 128.8, 126.4, 116.1, 114.7, 111.8, 111.5, 62.7, 54.7, 54.3, 24.6, 14.3. HRMS (ESI) m/z: Calcd for $C_{22}H_{24}NO_4$ $(M+H)^+$ 366.1700, found 366.1688.

Ethyl 4-((dipropylamino)methyl)-5-hydroxy-2-phenylbenzofuran-3-carboxylate (12). This compound was obtained from **8** and dipropylamine employing Method B. Yield 53%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78–7.72 (m, 2H), 7.48–7.40 (m, 3H), 7.31 (d, *J* = 8.8 Hz, 1H), 6.85 (d, *J* = 8.8 Hz, 1H), 4.35 (q, *J* = 7.1 Hz, 2H), 4.06 (s, 2H), 2.54 (t, *J* = 7.5 Hz, 4H), 1.66–1.56 (m, 4H), 1.29 (t, *J* = 7.2 Hz, 3H), 0.91 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.4, 156.6, 155.6, 148.1,

130.0, 129.5, 128.3, 127.8, 125.3, 115.2, 112.9, 110.6, 110.0, 61.4, 55.7, 54.2, 19.6, 13.9, 11.8. HRMS (ESI) m/z: Calcd for $C_{24}H_{30}NO_4$ $(M+H)^+$ 396.2169, found 396.2168.

Ethyl 5-hydroxy-4-(morpholinomethyl)-2-phenylbenzofuran-3-carboxylate (13). This compound was obtained from **8** and morpholine employing Method B. Yield 89%; pale yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.79–7.59 (m, 2H), 7.52–7.39 (m, 3H), 7.32 (d, *J* = 8.8 Hz, 1H), 6.84 (d, *J* = 8.8 Hz, 1H), 4.33 (q, *J* = 7.1 Hz, 2H), 3.92 (s, 2H), 3.66 (t, *J* = 4.6 Hz, 4H), 2.50 (t, *J* = 4.0 Hz, 4H), 1.26 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.6 , 157.8, 155.2, 149.7, 131.1, 130.7, 129.5, 128.8, 127.1, 115.8, 114.3, 111.9, 111.9, 67.9, 62.7, 56.5, 54.0, 14.3. HRMS (ESI) m/z: Calcd for $C_{22}H_{24}NO_5$ $(M+H)^+$ 382.1649, found 382.1629.

Ethyl 5-hydroxy-4-((4-methylpiperazin-1-yl)methyl)-2-phenylbenzofuran-3-carboxylate (14). This compound was obtained from **8** and 1-methylpiperazine employing Method B. Yield 68%; pale yellow oil. ^{1}H NMR (400 MHz, CD_3OD) δ 7.72–7.70 (m, 2H), 7.47–7.45 (m, 3H), 7.33 (d, *J* = 8.8 Hz, 1H), 6.84 (d, *J* = 8.8 Hz, 1H), 4.34 (q, *J* = 7.1 Hz, 2H), 3.95 (s, 2H), 2.57 (br, 8H), 2.28 (s, 3H), 1.27 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.6, 157.8, 155.4, 149.7, 131.1, 130.8, 129.6, 128.7, 127.0, 115.8, 114.3, 111.9, 111.8, 62.7, 56.2, 55.8, 53.0, 45.9, 14.3. HRMS (ESI) m/z: Calcd for $C_{23}H_{27}N_2O_4$ $(M+H)^+$ 395.1965, found 395.1938.

Ethyl 5-hydroxy-4-((4-(hydroxymethyl)piperidin-1-yl)methyl)-2-phenylbenzo-furan-3- carboxylate (15). This compound was obtained from **8** and piperidin-4-ylmethanol employing Method B. Yield 56%; pale yellow oil. ^{1}H NMR (400 MHz, CD_3OD) δ 7.73–7.67 (m, 2H), 7.51–7.45 (m, 3H), 7.39 (d, *J* = 8.9 Hz, 1H), 6.88 (d, *J* = 8.9 Hz, 1H), 4.33 (q, *J* = 7.1 Hz, 2H), 4.16 (s, 2H), 3.43 (d, *J* = 6.3 Hz, 2H), 3.33–3.30 (m, 1H), 3.19 (d, *J* = 11.9 Hz, 2H), 2.45 (t, *J* = 11.3 Hz, 2H), 1.84 (d, *J* = 13.0 Hz, 2H), 1.63 (brs, 1H), 1.45–1.30 (m, 2H), 1.23 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.6, 159.3, 156.1,

149.7, 131.2, 130.9, 129.5, 129.2, 127.2, 115.8, 112.7, 112.5, 111.4, 67.3, 62.7, 56.5, 53.8, 38.9, 29.1, 14.2. HRMS (ESI) m/z: Calcd for $C_{24}H_{28}NO_5$ $(M+H)^+$ 410.1962, found 410.1980.

Ethyl 2-(cyclopropylmethyl)-8-phenyl-2,3-dihydro-1*H*-benzofuro[4,5-*e*][1,3]oxaz-ine-9-carboxylate (16). This compound was obtained from **8** and cyclopropylmethanamine employing Method B. Yield 87%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD=4/1) δ 7.75–7.70 (m, 2H), 7.49–7.43 (m, 3H), 7.31 (d, *J* = 8.9 Hz, 1H), 6.81 (d, *J* = 8.9 Hz, 1H), 4.94 (s, 2H), 4.43– 4.29 (m, 4H), 2.64 (d, *J* = 6.7 Hz, 2H), 1.28 (t, *J* = 7.1 Hz, 3H), 1.03–0.92 (m, 1H), 0.60–0.51 (m, 2H), 0.15 (q, *J* = 4.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$/CD_3OD = 4/1) δ 166.5, 159.1, 151.5, 149.8, 130.8, 130.7, 129.2, 129.0, 125.4, 116.1, 112.8, 111.3, 111.0, 82.0, 62.3, 57.1, 49.9, 14.2, 9.9, 4.1. HRMS (ESI) m/z: Calcd for $C_{22}H_{24}NO_4$ $(M+H)^+$ 366.1700, found 366.1707.

Ethyl 2-cyclopropyl-8-phenyl-2,3-dihydro-1*H*-benzofuro[4,5-*e*][1,3]oxazine-9-carboxylate (17). This compound was obtained from **8** and cyclopropylamine employing Method B. Yield 79%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (dd, *J* = 6.3, 1.6 Hz, 2H), 7.50–7.43 (m, 3H), 7.31 (d, *J* = 8.9 Hz, 1H), 6.87 (d, *J* = 8.9 Hz, 1H), 4.91 (s, 2H), 4.44–4.31 (m, 4H), 2.42 (t, *J* = 4.7 Hz, 1H), 1.31 (t, *J* = 7.1 Hz, 3H), 0.58 (d, *J* = 4.8 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.3, 158.0, 150.7, 148.7, 129.9, 129.7, 128.3, 128.3, 124.5, 115.4, 112.9, 110.3, 110.2, 82.0, 61.3, 49.8, 32.9, 13.9, 7.0. HRMS (ESI) m/z: Calcd for $C_{22}H_{22}NO_4$ $(M+H)^+$ 364.1543, found 364.1555.

Ethyl 2-cyclopentyl-8-phenyl-2,3-dihydro-1*H*-benzofuro[4,5-*e*][1,3]oxazine-9-carboxylate (18). This compound was obtained from **8** and cyclopentylamine employing Method B. Yield 65%; pale yellow solid. 1H NMR (400 MHz, $CDCl_3$) δ 7.84–7.72 (m, 2H), 7.50–7.42 (m, 3H), 7.29 (d, *J* = 8.9 Hz, 1H), 6.82 (d, *J* = 8.9 Hz, 1H), 4.96 (s, 2H), 4.49–4.22 (m, 4H), 3.37–3.12 (m, 1H), 2.11–1.84 (m, 2H), 1.84–1.69 (m, 2H), 1.65–1.46 (m, 4H), 1.31 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.4,

157.8, 151.2, 148.6, 129.9, 129.7, 128.3, 128.2, 124.4, 115.1, 112.7, 110.2, 110.2, 81.1, 61.3, 59.8, 48.0, 31.5, 23.7, 14.0. HRMS (ESI) m/z: Calcd for $C_{24}H_{25}NNaO_4$ $(M+Na)^+$ 414.1676, found 414.1679.

Ethyl 2-isopentyl-8-phenyl-2,3-dihydro-1*H*-benzofuro[4,5-*e*][1,3]oxazine-9-carboxylate (19). This compound was obtained from **8** and 3-methylbutan-1-amine employing Method B. Yield 91%; pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71–7.66 (m, 2H), 7.39–7.32 (m, 3H), 7.20 (d, *J* = 8.9 Hz, 1H), 6.74 (d, *J* = 8.9 Hz, 1H), 4.80 (s, 2H), 4.27 (q, *J* = 7.1 Hz, 2H), 4.19 (s, 2H), 2.68 (t, *J* = 7.6 Hz, 2H), 1.59–1.52 (m, 1H), 1.40 (dd, *J* = 14.9, 7.1 Hz, 2H), 1.21 (t, *J* = 7.1 Hz, 3H), 0.82 (d, *J* = 6.6 Hz, 6H).^{13}C NMR (101 MHz, $CDCl_3$) δ 165.3, 157.9, 150.7, 148.6, 129.9, 129.6, 128.2, 128.2, 124.5, 115.2, 112.6, 110.2, 110.2, 81.7, 61.2, 49.7, 49.2, 37.1, 26.1, 22.6, 13.9. HRMS (ESI) m/z: Calcd for $C_{24}H_{28}NO_4$ $(M+H)^+$ 394.2013, found 394.2014.

Ethyl 5-methoxy-2-phenyl-4-(piperidin-1-ylmethyl)benzofuran-3-carboxylate (20). To a solution of **10** (1 mmol) in anhydrous DMF (5 mL), NaH (60% dispersion in oil, 1.2 mmol) and CH_3I were added at 0 ºC under N_2. After stirring for 1 hr at rt, the reaction was quenched with water and extracted with EtOAc (2×30 mL). The combined organic phases were washed with water (3×15 mL), dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography to give methyl ether. Yield 33%; pale yellow oil. ^{1}H NMR (400 MHz, CD_3OD) δ 7.80–7.66 (m, 2H), 7.49–7.42 (m, 3H), 7.40 (d, *J* = 9.0 Hz, 1H), 7.04 (d, *J* = 9.0 Hz, 1H), 4.31 (q, *J* = 7.2 Hz, 2H), 3.83 (s, 3H), 3.79 (s, 2H), 2.25 (brs, 4H), 1.50–1.31 (m, 6H), 1.22 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.2, 158.0, 156.2, 150.7, 131.2, 130.7, 129.5, 129.0, 127.9, 121.1, 113.2, 111.7, 111.2, 62.4, 57.6, 55.0, 53.1, 26.8, 25.6, 14.2. HRMS (ESI) m/z: Calcd for $C_{24}H_{28}NO_4$ $(M+H)^+$ 394.2013, found 394.2004.

5-Hydroxy-2-phenyl-4-(piperidin-1-ylmethyl)benzofuran-3-carboxylic acid (21). This compound was obtained from **9** and piperidine employing Method B. Yield 44%; pale yellow solid. ^{1}H NMR (400

MHz, CD_3OD) δ 7.81–7.74 (m, 2H), 7.57 (d, *J* = 8.9 Hz, 1H), 7.53–7.46 (m, 3H), 7.03 (d, *J* = 8.9 Hz, 1H), 4.71 (s, 2H), 3.56 (d, *J* = 12.0 Hz, 2H), 3.16 (t, *J* = 11.0 Hz, 2H), 1.97 (d, *J* = 14.2 Hz, 2H), 1.86–1.71 (m, 3H), 1.65–1.54 (m, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 169.0, 162.8, 155.8, 150.0, 131.4, 131.2, 130.3, 129.2, 128.6, 115.3, 115.1, 111.6, 108.7, 54.1, 53.3, 24.1, 22.8.

***N*-Ethyl-5-hydroxy-2-phenyl-4-(piperidin-1-ylmethyl)benzofuran-3-carboxamide (22).** This compound was obtained from **9**, ethylamine, and piperidine employing Methods C and B. Yields 92% and 38%; pale yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.81 (d, *J* = 8.0 Hz, 2H), 7.58–7.29 (m, 4H), 6.86 (d, *J* = 8.8 Hz, 1H), 4.10 (s, 2H), 3.42 (q, *J* = 7.3 Hz, 2H), 2.83 (br s, 4H), 1.83–1.68 (m, 4H), 1.59 (br s, 2H), 1.20 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 168.7, 155.7, 154.8, 149.5, 130.8, 130.6, 129.9, 127.9, 127.8, 115.5, 114.4, 112.9, 111.3, 54.8, 54.6, 36.0, 26.1, 24.3, 14.2. HRMS (ESI) m/z: Calcd for $C_{23}H_{27}N_2O_3$ $(M+H)^+$ 379.2025, found 379.2016.

***N*-Cyclopropyl-5-hydroxy-2-phenyl-4-(piperidin-1-ylmethyl)benzofuran-3-carboxamide (23).** This compound was obtained from **9**, cyclopropylamine, and piperidine employing Methods C and B. Yields 74% and 56%; yellow oil. ^{1}H NMR (400 MHz, CD_3OD) δ 7.79–7.65 (m, 2H), 7.56–7.40 (m, 4H), 6.96 (d, *J* = 8.9 Hz, 1H), 4.32 (s, 2H), 3.12 (br s, 4H), 3.05–2.89 (m, 1H), 1.91–1.75 (m, 4H), 1.67 (br s, 2H), 0.90–0.68 (m, 2H), 0.59–0.45 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 169.9, 156.2, 155.5, 149.6, 131.0, 130.5, 129.8, 128.3, 128.3, 115.2, 114.3, 113.9, 109.1, 54.3, 54.1, 25.1, 24.2, 23.4, 5.8. HRMS (ESI) m/z: Calcd for $C_{24}H_{27}N_2O_3$ $(M+H)^+$ 391.2016, found 391.2011.

***N*-((3*S*,5*S*,7*S*)-Adamantan-1-yl)-5-hydroxy-2-phenyl-4-(piperidin-1-ylmethyl)benzofuran-3-carboxamide (24).** This compound was obtained from **9**, amantadine, and piperidine employing Methods C and B. Yields 86% and 40%; pale yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.84 (d, *J* = 7.3 Hz, 2H), 7.53–7.44 (m, 3H), 7.40 (d, *J* = 8.8 Hz, 1H), 6.86 (d, *J* = 8.8 Hz, 1H), 4.20 (s, 2H), 2.88 (br

s, 4H), 2.15–2.11 (m, 11H), 1.66 (s, 10H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.4, 153.9, 151.1, 146.9, 128.6, 128.1, 127.6, 125.6, 125.1, 114.0, 113.5, 111.1, 109.5, 56.0, 53.0, 51.7, 40.2, 35.3, 28.4, 24.7, 22.9. HRMS (ESI) m/z: Calcd for $C_{31}H_{37}N_2O_3$ $(M+H)^+$ 485.2799, found 485.2805.

***N*-Benzyl-5-hydroxy-2-phenyl-4-(piperidin-1-ylmethyl)benzofuran-3-carboxamide (25).** This compound was obtained from **9**, benzylamine, and piperidine employing Methods C and B. Yields 96% and 87%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (d, J = 3.5 Hz, 2H), 7.34–7.10 (m, 8H), 6.71 (d, J = 8.8 Hz, 1H), 6.26 (t, J = 6.4 Hz, 1H), 4.51 (d, J = 6.0 Hz, 2H), 3.75 (s, 2H), 2.92–1.85 (br s, 4H), 1.63–1.26 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.2, 154.1, 151.7, 146.9, 136.3, 128.4, 128.1, 127.8, 127.7, 127.4, 126.7, 125.6, 124.9, 114.0, 112.0, 111.1, 109.5, 56.1, 52.6, 43.3, 24.8, 22.8. HRMS (ESI) m/z: Calcd for $C_{28}H_{29}N_2O_3$ $(M+H)^+$ 441.2173, found 441.2188.

5-Hydroxy-*N*,2-diphenyl-4-(piperidin-1-ylmethyl)benzofuran-3-carboxamide (26). This compound was obtained from **9**, aniline, and piperidine employing Methods C and B. Yields 74% and 79%; brown solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (s, 1H), 7.73 (d, J = 6.5 Hz, 2H), 7.49 (d, J = 7.4 Hz, 2H), 7.36–7.30 (m, 4H), 7.19 (s, 1H), 7.12 (t, J = 6.3 Hz, 1H), 6.78 (d, J = 8.3 Hz, 1H), 3.89 (s, 2H), 2.79–2.25 (br s, 4H), 1.55 (br s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.4, 155.2, 153.2, 148.0, 137.7, 129.4, 129.4, 129.2, 128.9, 126.7, 125.9, 125.0, 120.1, 115.3, 113.5, 111.9, 110.8, 57.0, 53.8, 25.6, 23.7. HRMS (ESI) m/z: Calcd for $C_{27}H_{27}N_2O_3$ $(M+H)^+$ 427.2016, found 427.2029.

Ethyl 2-(4-fluorophenyl)-5-hydroxy-4-(piperidin-1-ylmethyl)benzofuran-3-carboxylate (27). This compound was obtained from ethyl 4-fluorobenzoylacetate and piperidine employing Methods A and B. Yields 56% and 84%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD = 5/1) δ 7.75 (dd, J = 8.6, 5.4 Hz, 2H), 7.33 (d, J = 8.8 Hz, 1H), 7.19 (t, J = 8.7 Hz, 2H), 6.83 (d, J = 8.8 Hz, 1H), 4.35 (q, J = 7.1 Hz, 2H), 4.02 (s, 2H), 2.64 (br s, 4H), 1.73–1.63 (m, 4H), 1.54 (br s, 2H), 1.29 (t, J = 7.2 Hz, 3H). ^{13}C

NMR (101 MHz, $CDCl_3/CD_3OD$ = 5/1) δ 167.2, 164.7 (d, J_{C-F} = 249.8 Hz), 157.4, 156.2, 149.3, 131.1 (d, J_{C-F} = 8.5 Hz), 127.3 (d, J_{C-F} = 3.3 Hz), 126.5, 116.3 (d, J_{C-F} = 22.2 Hz), 116.0, 113.0, 111.9, 111.1, 62.6, 58.1, 54.6, 26.5, 24.6, 14.3. HRMS (ESI) m/z: Calcd for $C_{23}H_{25}FNO_4$ $(M+H)^+$ 398.1762, found 398.1778.

Ethyl 5-hydroxy-4-(piperidin-1-ylmethyl)-2-(4-(trifluoromethyl)phenyl)benzo-furan-3-carboxylate (28). This compound was obtained from ethyl 4-trifluoromethylbenzoylacetate and piperidine employing Methods A and B. Yields 46% and 79%; white solid. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ = 3/1) δ 7.87 (d, *J* = 8.2 Hz, 2H), 7.73 (d, *J* = 8.3 Hz, 2H), 7.33 (d, *J* = 8.9 Hz, 1H), 6.84 (d, *J* = 8.9 Hz, 1H), 4.37 (q, *J* = 7.1 Hz, 2H), 3.95 (s, 2H), 2.58 (br s, 4H), 1.70–1.61 (m, 4H), 1.52 (br s, 2H), 1.29 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3/CD_3OD$ = 3/1) δ 167.4, 156.6, 155.9, 149.8, 134.7, 134.7, 133.3 (q, J_{C-F} = 32.8 Hz), 129.3, 126.5 (q, J_{C-F} = 3.8 Hz), 125.4 (q, J_{C-F} = 272.6 Hz), 117.0, 113.7, 112.9, 112.2, 63.1, 58.6, 55.0, 26.9, 25.0, 14.6. HRMS (ESI) m/z: Calcd for $C_{24}H_{25}F_3NO_4$ $(M+H)^+$ 448.1730, found 448.1748.

Ethyl 5-hydroxy-2-(4-methoxyphenyl)-4-(piperidin-1-ylmethyl)benzofuran-3-carboxylate (29). This compound was obtained from ethyl 4-methoxybenzoylacetate and piperidine employing Methods A and B. Yields 67% and 86%; white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.62 (d, *J* = 8.7 Hz, 2H), 7.25 (d, *J* = 8.8 Hz, 1H), 6.95 (d, *J* = 8.7 Hz, 2H), 6.75 (d, *J* = 8.8 Hz, 1H), 4.31 (q, *J* = 7.1 Hz, 2H), 3.91 (s, 2H), 3.80 (s, 3H), 2.53 (br s, 4H), 1.67–1.55 (m, 4H), 1.48 (br s, 2H), 1.26 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.8, 162.3, 158.2, 156.2, 149.2, 130.4, 126.9, 123.4, 115.5, 114.9, 113.3, 111.7, 110.1, 62.6, 58.3, 55.8, 54.8, 26.8, 24.9, 14.4. HRMS (ESI) m/z: Calcd for $C_{24}H_{28}NO_5$ $(M+H)^+$ 410.1962, found 410.1973.

Ethyl 2-(4-fluoro-2-methoxyphenyl)-5-hydroxy-4-(piperidin-1-ylmethyl)benzofuran-3-carboxylate (30). This compound was obtained from 2-methoxy-4-fluoroacetophenone and piperidine employing Methods E, A, and B. Yields 56%, 28%, and 84%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (t, *J* = 7.2 Hz, 1H), 7.21 (d, *J* = 8.8 Hz, 1H), 6.77 (d, *J* = 8.7 Hz, 1H), 6.70 (t, *J* = 7.8 Hz, 1H), 6.61 (d, *J* = 10.4 Hz, 1H), 4.13 (q, *J* = 6.7 Hz, 2H), 4.03 (s, 2H), 3.72 (s, 3H), 2.58 (br s, 4H), 1.71–1.31 (m, 6H), 1.07 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.7, 164.5 (d, J_{C-F} = 250.5 Hz), 158.3 (d, J_{C-F} = 10.2 Hz), 155.7, 154.8, 148.4, 131.6 (d, J_{C-F} = 10.4 Hz), 124.8, 116.1, 114.9, 112.6, 112.3, 110.5, 107.3 (d, J_{C-F} = 21.8 Hz), 99.2 (d, J_{C-F} = 26.1 Hz), 60.7, 57.9, 55.7, 53.8, 25.9, 23.9, 13.9. HRMS (ESI) m/z: Calcd for $C_{24}H_{27}FNO_5$ $(M+H)^+$ 428.1868, found 428.1876.

Ethyl 2-(4-bromo-2-methoxyphenyl)-5-hydroxy-4-(piperidin-1-ylmethyl)benzofuran-3-carboxylate (31). This compound was obtained from 2-methoxy-4-bromoacetophenone and piperidine employing Methods E, A, and B. Yields 56%, 49%, and 65%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$/ CD_3OD = 4/1) δ 7.46 (d, *J* = 8.1 Hz, 1H), 7.31 (d, *J* = 8.8 Hz, 1H), 7.28–7.19 (m, 2H), 6.82 (d, *J* = 8.8 Hz, 1H), 4.22 (q, *J* = 7.1 Hz, 2H), 4.12 (s, 2H), 3.82 (s, 3H), 2.63 (br s, 4H), 1.73–1.64 (m, 4H), 1.55 (br s, 2H), 1.17 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$/CD_3OD = 4/1) δ 166.9, 158.2, 156.1, 155.4, 149.4, 131.9, 125.7, 125.5, 124.4, 119.8, 115.6, 115.4, 113.4, 113.3, 111.4, 61.8, 58.3, 56.2, 54.4, 26.5, 24.5, 14.1. HRMS (ESI) m/z: Calcd for $C_{24}H_{27}BrNO_5$ $(M+H)^+$ 488.1067, found 488.1087.

Ethyl 2-(2,4-dimethoxyphenyl)-5-hydroxy-4-(piperidin-1-ylmethyl)benzofuran-3-carboxylate (32). This compound was obtained from 2,4-dimethoxyacetophenone and piperidine employing Methods E, A, and B. Yields 32%, 65%, and 69%; white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.47 (d, *J* = 9.0 Hz, 1H), 7.28 (d, *J* = 8.8 Hz, 1H), 6.76 (d, *J* = 8.8 Hz, 1H), 6.66–6.59 (m, 2H), 4.18 (q, *J* = 7.1 Hz, 2H), 4.11 (s, 2H), 3.85 (s, 3H), 3.77 (s, 3H), 2.61 (br s, 4H), 1.72–1.62 (m, 4H), 1.58–1.49 (m, 2H), 1.12 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.8, 164.0, 159.6, 157.4, 156.4, 149.5, 132.0, 126.6, 115.2,

113.7, 113.4, 112.6, 111.8, 106.2, 99.3, 62.0, 58.1, 56.0, 55.9, 54.7, 26.8, 24.9, 14.3. HRMS (ESI) m/z: Calcd for $C_{25}H_{30}NO_6$ $(M+H)^+$ 440.2068, found 440.2079.

Ethyl 5-hydroxy-2-(2-methoxy-4,5-dimethylphenyl)-4-(piperidin-1-ylmethyl)benzofuran-3-carboxylate (33). This compound was obtained from 2-methoxy-4,5-dimethylacetophenone and piperidine employing Methods E, A, and B. Yields 43%, 28%, and 57%; gray solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (s, 1H), 7.28 (d, *J* = 8.9 Hz, 1H), 6.82 (d, *J* = 8.8 Hz, 1H), 6.74 (s, 1H), 4.22 (q, *J* = 7.1 Hz, 2H), 4.09 (s, 2H), 3.76 (s, 3H), 3.03–2.00 (m, 10H), 1.74–1.46 (m, 6H), 1.17 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.2, 155.5, 155.5, 154.8, 148.3, 139.8, 131.0, 128.5, 125.1, 116.9, 114.5, 112.5, 112.4, 111.8, 110.5, 60.7, 57.8, 55.5, 53.8, 25.9, 24.0, 20.3, 18.7, 13.9. HRMS (ESI) m/z: Calcd for $C_{26}H_{32}NO_5$ $(M+H)^+$ 438.2275, found 438.2282.

Ethyl 2-(cyclopropylmethyl)-8-(4-methoxyphenyl)-2,3-dihydro-1*H*-benzofuro[4,5-*e*][1,3] oxazine-9-carboxylate (34). This compound was obtained from 4-methoxyacetophenone and cyclopropylmethanamine employing Methods A and B. Yields 67% and 89%; colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 8.8 Hz, 2H), 7.26 (d, *J* = 8.8 Hz, 1H), 6.97 (d, *J* = 8.8 Hz, 2H), 6.77 (d, *J* = 8.9 Hz, 1H), 4.99 (s, 2H), 4.51–4.24 (m, 4H), 3.86 (s, 3H), 2.65 (d, *J* = 6.7 Hz, 2H), 1.32 (t, *J* = 7.1 Hz, 3H), 1.04–0.91 (m, 1H), 0.56–0.51 (m, 2H), 0.15 (q, *J* = 5.0 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.4, 160.7, 158.1, 150.5, 148.3, 129.8, 124.7, 122.3, 114.7, 113.7, 112.1, 110.1, 108.9, 81.3, 61.2, 56.2, 55.3, 49.2, 14.0, 9.5, 3.5. HRMS (ESI) m/z: Calcd for $C_{24}H_{26}NO_5$ $(M+H)^+$ 408.1805, found 408.1808.

Ethyl 2-(cyclopropylmethyl)-8-(*p*-tolyl)-2,3-dihydro-1*H*-benzofuro[4,5-*e*][1,3]oxazine-9-carboxylate (35). This compound was obtained from 4-methylacetophenone and cyclopropylmethanamine employing Methods E, A, and B. Yields 37%, 34%, and 67%; brown solid. ^{1}H

NMR (400 MHz, $CDCl_3$) δ 7.67 (d, *J* = 7.6 Hz, 2H), 7.30–7.23 (m, 3H), 6.79 (d, *J* = 8.8 Hz, 1H), 4.99 (s, 2H), 4.43–4.29 (m, 4H), 2.65 (d, *J* = 6.4 Hz, 2H), 2.41 (s, 3H), 1.32 (t, *J* = 6.9 Hz, 3H), 1.04–0.92 (m, 1H), 0.54 (d, *J* = 7.3 Hz, 2H), 0.15 (d, *J* = 4.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.6, 158.2, 150.7, 148.6, 140.0, 129.1, 128.2, 127.1, 124.8, 115.0, 112.4, 110.3, 109.7, 81.6, 61.4, 56.4, 49.2, 21.5, 14.2, 9.7, 3.6. HRMS (ESI) m/z: Calcd for $C_{24}H_{26}NO_4$ $(M+H)^+$ 392.1856, found 392.1847.

Ethyl 2-(cyclopropylmethyl)-8-(2-methoxy-5-methylphenyl)-2,3-dihydro-1*H*-benzofuro[4,5-*e*][1,3] oxazine-9-carboxylate (36). This compound was obtained from 2-methoxy-5-methylacetophenone and cyclopropylmethanamine employing Methods E, A, and B. Yield 56%, 33%, and 56%; brown solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (s, 1H), 7.19 (d, *J* = 6.4 Hz, 1H), 7.13 (d, *J* = 7.9 Hz, 1H), 6.78 (d, *J* = 6.3 Hz, 1H), 6.70 (d, *J* = 8.6 Hz, 1H), 4.91 (s, 2H), 4.36 (s, 2H), 4.15 (q, *J* = 6.9 Hz, 2H), 3.69 (s, 3H), 2.59 (d, *J* = 6.4 Hz, 2H), 2.27 (s, 3H), 1.18 (br s, 1H), 1.09 (t, *J* = 5.9 Hz, 3H), 0.92 (br s, 1H), 0.78 (br s, 1H), 0.46 (d, *J* = 6.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.2, 155.7, 154.8, 150.6, 148.8, 131.6, 130.6, 129.8, 124.3, 119.3, 114.7, 112.5, 112.5, 110.9, 110.1, 81.4, 60.7, 56.4, 55.5, 49.4, 20.4, 13.9, 9.6, 3.6. HRMS (ESI) m/z: Calcd for $C_{25}H_{28}NO_5$ $(M+H)^+$ 422.1962, found 422.1956.

Ethyl 5-hydroxy-2-(2-methoxyphenyl)benzofuran-3-carboxylate (39). This compound was obtained from 2-methoxyacetophenone employing Method A. Yield 64%; pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.41 (s, 1H), 7.50 (d, *J* = 7.6 Hz, 2H), 7.45 (d, *J* = 8.8 Hz, 1H), 7.31 (d, *J* = 2.4 Hz, 1H), 7.16 (d, *J* = 8.1 Hz, 1H), 7.07 (t, *J* = 7.4 Hz, 1H), 6.82 (dd, *J* = 8.8, 2.5 Hz, 1H), 4.17 (q, *J* = 7.1 Hz, 2H), 3.75 (s, 3H), 1.15 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.9, 157.9, 157.2, 154.2, 147.7, 131.8, 130.8, 126.8, 120.0, 118.8, 113.9, 111.7, 111.4, 110.4, 105.8, 59.8, 55.5, 13.9.

8-Hydroxy-6*H*-benzofuro[3,2-*c*]chromen-6-one (40). This compound was obtained from **39** employing Method D. Yield 86%; pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.73 (s, 1H),

7.96 (d, *J* = 8.0 Hz, 1H), 7.67 (t, *J* = 7.7 Hz, 1H), 7.63 (d, *J* = 9.0 Hz, 1H), 7.53 (d, *J* = 8.2 Hz, 1H), 7.45 (t, *J* = 7.6 Hz, 1H), 7.27 (d, *J* = 2.4 Hz, 1H), 6.93 (dd, *J* = 9.0, 2.4 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.8, 157.2, 155.3, 153.0, 148.9, 132.2, 124.9, 123.7, 121.6, 117.1, 115.3, 112.6, 112.1, 105.3, 105.0. HRMS (ESI) m/z: Calcd for $C_{15}H_8NaO_4$ $(M+Na)^+$ 275.0315, found 275.0335.

8-Hydroxy-7-(piperidin-1-ylmethyl)-6*H*-benzofuro[3,2-*c*]chromen-6-one (41). This compound was obtained from **40** and piperidine employing Method B. Yield 89%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ = 4/1) δ 7.92 (d, *J* = 7.6 Hz, 1H), 7.61 (t, *J* = 7.7 Hz, 1H), 7.45 (d, *J* = 8.9 Hz, 1H), 7.42–7.36 (m, 2H), 6.94 (d, *J* = 8.9 Hz, 1H), 4.71 (s, 2H), 2.96 (br s, 4H), 1.83–1.70 (m, 4H), 1.61 (br s, 2H). ^{13}C NMR (101 MHz, $CDCl_3/CD_3OD$ = 4/1) δ 161.9, 159.9, 157.5, 154.1, 150.2, 133.2, 125.8, 124.2, 122.6, 117.7, 116.9, 113.5, 112.9, 112.3, 106.6, 56.6, 54.0, 25.1, 23.6. HRMS (ESI) m/z: Calcd for $C_{21}H_{20}NO_4$ $(M+H)^+$ 350.1387, found 350.1389.

8-Hydroxy-3-methoxy-7-(piperidin-1-ylmethyl)-6*H*-benzofuro[3,2-*c*]chromen-6-one (42). This compound was obtained from 2,4-dimethoxyacetophenone and piperidine employing method E, A, D, and B. Yields 32%, 46%, 73%, and 71%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ = 4/1) δ 7.86 (d, *J* = 8.8 Hz, 1H), 7.45 (d, *J* = 8.9 Hz, 1H), 6.98 (d, *J* = 8.8 Hz, 1H), 6.93 (d, *J* = 8.8 Hz, 2H), 4.75 (s, 2H), 3.90 (s, 3H), 3.00 (br s, 4H), 1.81–1.73 (m, 4H), 1.62 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3/CD_3OD$ = 4/1) δ 164.3, 162.7, 160.3, 157.0, 156.0, 149.9, 124.5, 123.7, 115.9, 114.2, 113.4, 111.8, 106.0, 104.0, 101.6, 56.3, 53.9, 49.6, 24.9, 23.4. HRMS (ESI) m/z: Calcd for $C_{22}H_{22}NO_5$ $(M+H)^+$ 380.1492, found 380.1494.

3-Fluoro-8-hydroxy-7-(piperidin-1-ylmethyl)-6*H*-benzofuro[3,2-*c*]chromen-6-one (43). This compound was obtained from 2-methoxy-4-fluoroacetophenone and piperidine employing Methods E, A, D, and B. Yields 56%, 28%, 75%, and 61%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ =

4/1) δ 8.04 (dd, *J* = 8.4, 6.1 Hz, 1H), 7.44 (d, *J* = 8.9 Hz, 1H), 7.25–7.14 (m, 2H), 6.90 (d, *J* = 8.9 Hz, 1H), 4.65 (s, 2H), 2.68 (br s, 4H), 1.71–1.61 (m, 4H), 1.55 (br s, 2H). ^{13}C NMR (101 MHz, $CDCl_3/CD_3OD$ = 4/1) δ 165.4 (d, J_{C-F} = 253.6 Hz), 161.3, 159.2, 158.1, 155.2 (d, J_{C-F} = 13.0 Hz), 150.1, 124.4 (d, J_{C-F} = 10.4 Hz), 123.1, 117.1, 114.9, 113.7 (d, J_{C-F} = 23.3 Hz), 112.0, 110.0, 105.5, 105.1 (d, J_{C-F} = 26.2 Hz), 58.8, 54.1, 26.2, 24.3. HRMS (ESI) m/z: Calcd for $C_{21}H_{19}FNO_4$ $(M+H)^+$ 368.1293, found 368.1307.

3-Bromo-8-hydroxy-7-(piperidin-1-ylmethyl)-6*H*-benzofuro[3,2-*c*]chromen-6-one (44). This compound was obtained from 2-methoxy-4-bromoacetophenone and piperidine employing Methods E, A, D, and B. Yields 56%, 49%, 60%, and 72%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, *J* = 8.4 Hz, 1H), 7.66 (s, 1H), 7.52 (d, *J* = 8.3 Hz, 1H), 7.43 (d, *J* = 8.9 Hz, 1H), 6.97 (d, *J* = 8.9 Hz, 1H), 4.66 (s, 2H), 2.51 (br, 4H), 1.67–1.49 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.0, 157.7, 157.5, 153.5, 149.5, 128.0, 125.7, 122.8, 122.4, 120.3, 116.8, 114.9, 111.6, 111.1, 106.2, 58.4, 53.6, 25.8, 23.9. HRMS (ESI) m/z: Calcd for $C_{21}H_{19}BrNO_4$ $(M+H)^+$ 428.0492, found 428.0464.

8-Hydroxy-2-methyl-7-(piperidin-1-ylmethyl)-6*H*-benzofuro[3,2-*c*]chromen-6-one (45). This compound was obtain from 2-methoxy-3-methylacetophenone and piperidine by employing Methods E, A, D, and B. Yields 56%, 33%, 82%, and 76%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.47 (br s, 1H), 7.78 (s, 1H), 7.47–7.30 (m, 3H), 6.94 (d, *J* = 8.8 Hz, 1H), 4.68 (s, 2H), 3.18–2.29 (m, 7H), 1.90–1.20 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.7, 158.5, 157.3, 151.7, 149.4, 134.4, 132.9, 122.7, 121.5, 116.7, 116.3, 114.9, 112.2, 110.9, 106.0, 58.5, 53.6, 25.9, 23.9, 21.0. HRMS (ESI) m/z: Calcd for $C_{22}H_{22}NO_4$ $(M+H)^+$ 364.1543, found 364.1532.

8-Hydroxy-2-methoxy-7-(piperidin-1-ylmethyl)-6*H*-benzofuro[3,2-*c*]chromen-6-one (46). This compound was obtained from 2,3-dimethoxyacetophenone and piperidine by employing Methods E, A,

D, and B. Yields 30%, 34%, 53%, and 77%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (br s, 1H), 7.57–7.34 (m, 3H), 7.17–7.13 (m, 1H), 6.89–6.94 (m, 1H), 4.70 (d, *J* = 2.8 Hz, 2H), 3.93 (d, *J* = 3.0 Hz, 3H), 2.75 (m, 4H), 1.85–1.42 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.6, 158.4, 157.3, 156.3, 149.4, 148.0, 122.7, 120.3, 118.3, 116.4, 114.9, 112.7, 110.9, 106.3, 103.2, 58.4, 55.9, 53.6, 25.9, 23.9. HRMS (ESI) m/z: Calcd for $C_{22}H_{22}NO_5$ $(M+H)^+$ 380.1492, found 380.1507.

2-(Cyclopropylmethyl)-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (47). This compound was obtained from **40** and cyclopropylmethanamine by employing Method B. Yield 68%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 7.8 Hz, 1H), 7.59 (t, *J* = 7.7 Hz, 1H), 7.47 (d, *J* = 8.3 Hz, 1H), 7.43–7.36 (m, 2H), 6.90 (d, *J* = 8.9 Hz, 1H), 5.03 (s, 2H), 4.86 (s, 2H), 2.70 (d, *J* = 6.6 Hz, 2H), 1.09–0.97 (m, 1H), 0.56 (d, *J* = 7.7 Hz, 2H), 0.16 (d, *J* = 4.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.4, 158.0, 153.6, 151.9, 150.1, 131.8, 124.6, 121.8, 121.7, 117.2, 116.6, 114.4, 112.6, 110.6, 106.5, 82.0, 56.5, 50.0, 9.6, 3.6. HRMS (ESI) m/z: Calcd for $C_{21}H_{18}NO_4$ $(M+H)^+$ 348.1230, found 348.1252.

2-(Cyclopropylmethyl)-10-methoxy-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (48). This compound was obtained from 2,4-dimethoxyacetophenone and cyclopropylmethanamine employing Methods E, A, D, and B. Yields 32%, 46%, 73%, and 87%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, *J* = 8.7 Hz, 1H), 7.34 (d, *J* = 8.9 Hz, 1H), 7.03–6.91 (m, 2H), 6.85 (d, *J* = 8.9 Hz, 1H), 5.02 (s, 2H), 4.84 (s, 2H), 3.91 (s, 3H), 2.69 (d, *J* = 6.6 Hz, 2H), 1.11–0.96 (m, 1H), 0.56 (d, *J* = 7.5 Hz, 2H), 0.16 (d, *J* = 4.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.0, 161.1, 158.4, 155.4, 151.8, 149.7, 122.8, 121.8, 115.8, 114.2, 113.1, 110.3, 105.8, 104.0, 101.0, 81.9, 56.4, 55.8, 50.0, 9.6, 3.6. HRMS (ESI) m/z: Calcd for $C_{22}H_{20}NO_5$ $(M+H)^+$ 378.1336, found 378.1333.

2-(Cyclopropylmethyl)-9-fluoro-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (49). This compound was obtained from 2-methoxy-3-fluoroacetophenone and cyclopropylmethanamine by employing Methods E, A, D, and B. Yields 34%, 36%, 73%, and 71%; pale gray solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (d, *J* = 7.6 Hz, 1H), 7.39 (dd, *J* = 9.0, 4.1 Hz, 1H), 7.33 (d, *J* = 8.9 Hz, 1H), 7.23 (d, *J* = 9.9 Hz, 1H), 6.86 (d, *J* = 8.9 Hz, 1H), 4.97 (s, 2H), 4.78 (s, 2H), 2.64 (d, *J* = 6.6 Hz, 2H), 1.01–0.94 (m, 1H), 0.51 (d, *J* = 7.8 Hz, 2H), 0.10 (d, *J* = 4.8 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.1, 158.4, 156.7, 151.0, 149.2, 148.6, 120.4, 118.3 (d, J_{C-F} = 24.8 Hz), 117.9 (d, J_{C-F} = 8.5 Hz), 116.1, 113.5, 112.2 (d, J_{C-F} = 9.7 Hz), 109.6, 106.5 (d, J_{C-F} = 25.8 Hz), 106.2, 81.0, 55.4, 48.9, 8.5, 2.6. HRMS (ESI) m/z: Calcd for $C_{21}H_{17}FNO_4$ $(M+H)^+$ 366.1136, found 366.1166.

9-Chloro-2-(cyclopropylmethyl)-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (50). This compound was obtained from 2-methoxy-3-chloroacetophenone and cyclopropylmethanamine by employing Methods E, A, D, and B. Yields 42%, 36%, 66%, and 78%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (s, 1H), 7.52 (d, *J* = 8.4 Hz, 1H), 7.40 (m, 2H), 6.92 (d, *J* = 8.8 Hz, 1H), 5.03 (s, 2H), 4.83 (s, 2H), 2.69 (d, *J* = 6.0 Hz, 2H), 1.03 (s, 1H), 0.56 (d, *J* = 6.3 Hz, 2H), 0.15 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.0, 156.5, 151.0, 150.7, 149.2, 130.7, 129.1, 120.3, 120.2, 117.6, 116.1, 113.5, 112.6, 109.6, 106.2, 81.0, 55.4, 48.9, 8.5, 2.6. HRMS (ESI) m/z: Calcd for $C_{21}H_{17}ClNO_4$ $(M+H)^+$ 382.0841, found 382.0862.

9-Bromo-2-(cyclopropylmethyl)-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (51). This compound was obtained from 2-methoxy-3-bromoacetophenone and cyclopropylmethanamine employing by Methods E, A, D, and B. Yields 34%, 21%, 55%, and 69%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (s, 1H), 7.65 (d, *J* = 8.3 Hz, 1H), 7.38 (d, *J* = 8.9 Hz, 1H), 7.31 (d, *J* = 8.8 Hz, 1H), 6.91 (d, *J* = 8.9 Hz, 1H), 5.02 (s, 2H), 4.82 (s, 2H), 2.69 (d, *J* = 6.6 Hz, 2H), 1.02 (br s, 1H), 0.56 (d, *J* = 7.2 Hz, 2H), 0.15 (d, *J* = 4.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ

158.9, 157.5, 152.2, 152.1, 150.2, 134.5, 124.3, 121.3, 118.9, 117.4, 117.2, 114.5, 114.1, 110.7, 107.2, 82.0, 56.5, 49.9, 9.6, 3.6. HRMS (ESI) m/z: Calcd for $C_{21}H_{17}BrNO_4$ $(M+H)^+$ 426.0335, found 426.0321.

2-(Cyclopropylmethyl)-9-methoxy-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (52). This compound was obtained from 2,3-dimethoxyacetophenone and cyclopropylmethanamine by employing Methods E, A, D, and B. Yields 30%, 34%, 53% and 68%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 (m, 7.38–7.36, 3H), 7.13 (d, *J* = 9.2 Hz, 1H), 6.89 (d, *J* = 8.9 Hz, 1H), 5.02 (s, 2H), 4.84 (s, 2H), 3.92 (s, 3H), 2.69 (d, *J* = 6.7 Hz, 2H), 1.13–0.95 (m, 1H), 0.55 (d, *J* = 7.6 Hz, 2H), 0.15 (d, *J* = 4.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.3, 157.2, 155.2, 150.8, 149.0, 147.1, 120.7, 119.2, 117.4, 115.6, 113.5, 111.7, 109.4, 105.7, 102.2, 80.9, 55.4, 54.9, 49.0, 8.6, 2.6. HRMS (ESI) m/z: Calcd for $C_{22}H_{20}NO_5$ $(M+H)^+$ 378.1336, found 378.1333.

2-(Cyclopropylmethyl)-9-methyl-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (53). This compound was obtained from 2-methoxy-3-methylacetophenone and cyclopropylmethanamine by employing Methods E, A, D, and B. Yields 56%, 33%, 82%, and 78%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (s, 1H), 7.42–7.31 (m, 3H), 6.88 (d, *J* = 8.9 Hz, 1H), 5.02 (s, 2H), 4.84 (s, 2H), 2.69 (d, *J* = 6.6 Hz, 2H), 2.47 (s, 3H), 1.02 (s, 1H), 0.55 (d, *J* = 7.6 Hz, 2H), 0.15 (d, *J* = 4.6 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.5, 157.3, 150.8, 150.7, 149.0, 133.4, 131.9, 120.7, 120.4, 115.9, 115.4, 113.4, 111.1, 109.4, 105.4, 80.9, 55.4, 49.0, 19.9, 8.6, 2.6. HRMS (ESI) m/z: Calcd for $C_{22}H_{19}NNaO_4$ $(M+Na)^+$ 384.1206, found 384.1214.

9,10-Dichloro-2-(cyclopropylmethyl)-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (54). This compound was obtained from 2-methoxy-3,4-dichloroacetophenone and cyclopropylmethanamine by employing Methods E, A, D, and B. Yields 30%, 34%, 65%, and 61%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (s, 1H), 7.59 (s, 1H), 7.39 (d, *J* = 9.0 Hz, 1H),

6.93 (d, *J* = 8.9 Hz, 1H), 5.02 (s, 2H), 4.81 (s, 2H), 2.68 (d, *J* = 6.6 Hz, 2H), 1.07–0.97 (m, 1H), 0.56 (d, *J* = 7.6 Hz, 2H), 0.15 (d, *J* = 4.6 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.4, 157.1, 152.2, 151.6, 150.3, 135.9, 129.0, 122.6, 121.3, 119.2, 117.4, 114.5, 112.2, 110.7, 107.2, 82.0, 56.5, 49.9, 9.6, 3.6. HRMS (ESI) m/z: Calcd for $C_{21}H_{16}Cl_2NO_4$ $(M+H)^+$ 416.0451, found 416.0446.

2-(Cyclopropylmethyl)-9,10-dimethyl-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (55). This compound was obtained from 2-methoxy-3,4-dimethylacetophenone and cyclopropylmethanamine by employing Methods E, A, D, and B. Yields 43%, 28%, 82%, and 63%; white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (s, 1H), 7.35 (d, *J* = 8.8 Hz, 1H), 7.23 (s, 1H), 6.86 (d, *J* = 8.8 Hz, 1H), 5.01 (s, 2H), 4.84 (s, 2H), 2.69 (d, *J* = 6.6 Hz, 2H), 2.37 (d, *J* = 5.5 Hz, 6H), 1.02 (d, *J* = 5.6 Hz, 1H), 0.56 (d, *J* = 7.5 Hz, 2H), 0.16 (d, *J* = 4.2 Hz, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 159.8, 157.5, 151.1, 150.8, 148.8, 141.0, 132.5, 120.8, 120.7, 116.6, 115.1, 113.3, 109.4, 109.0, 104.7, 80.9, 55.4, 49.1, 19.5, 18.3, 8.6, 2.6. HRMS (ESI) m/z: Calcd for $C_{23}H_{22}NO_4$ $(M+H)^+$ 376.1543, found 376.1545.

2-Cyclopentyl-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (56). This compound was obtained from **40** and cyclopentylamine by employing Method B. Yield 58%; pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, *J* = 7.6 Hz, 1H), 7.58 (t, *J* = 7.5 Hz, 1H), 7.47 (d, *J* = 8.2 Hz, 1H), 7.39 (d, *J* = 8.1 Hz, 2H), 6.91 (d, *J* = 8.9 Hz, 1H), 4.98 (s, 2H), 4.83 (s, 2H), 3.22 (s, 1H), 1.99 (s, 2H), 1.74 (s, 2H), 1.56 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.4, 158.1, 153.5, 152.4, 150.1, 131.8, 124.5, 121.8, 121.5, 117.2, 116.5, 114.8, 112.5, 110.4, 106.6, 81.7, 60.1, 49.0, 31.4, 23.7. HRMS (ESI) m/z: Calcd for $C_{22}H_{20}NO_4$ $(M+H)^+$ 362.1387, found 362.1400.

2-Cyclohexyl-2,3-dihydro-1*H*,13*H*-chromeno[4',3':2,3]benzofuro[4,5-*e*][1,3]oxazin-13-one (57). This compound was obtained from **40** and cyclohexylamine by employing Method B. Yield 59%; pale

yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, *J* = 7.7 Hz, 1H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.46 (d, *J* = 8.3 Hz, 1H), 7.38 (t, *J* = 8.3 Hz, 2H), 6.88 (d, *J* = 8.8 Hz, 1H), 5.04 (s, 2H), 4.84 (s, 2H), 2.75 (t, *J* = 10.2 Hz, 1H), 2.01 (d, *J* = 11.2 Hz, 2H), 1.76 (d, *J* = 12.0 Hz, 2H), 1.43–1.08 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.4, 158.2, 153.5, 152.8, 150.0, 131.8, 124.5, 121.8, 121.2, 117.2, 116.8, 116.0, 112.6, 110.3, 106.6, 80.4, 59.2, 47.1, 31.6, 25.9, 25.6. HRMS (ESI) m/z: Calcd for $C_{23}H_{22}NO_4$ $(M+H)^+$ 376.1543, found 376.1562.

Statistical Analysis. Data are expressed as the mean ± SEM. Multiple group comparisons for formalin test were performed using unpaired, two-tailed t-test. Multiple group comparisons for rotarod test were performed by two-way ANOVA followed by Bonferroni posttest. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

General Procedures for Biological Studies. As reported in the literature, MIC was determined by using MABA assay.[27] Cytotoxicity of selected compounds, expressed as IC_{50}, was determined using Vero cells and MABA assay as reported previously.[14] Oral bioavailability was analyzed by using serum inhibition titration assay. Compounds were ground to homogenate suspension in 0.5% carboxymethyl cellulose. Six-week female BALB/c mice (20 g) were administered orally with 100 mg/kg dose for each experimental compound. Isoniazid at 10 mg/kg was used as positive control, and 0.5% carboxymethyl cellulose treatment was used as vehicle control. At 30, 60, and 120 min after administration, cardiac blood was collected and serum was separated. Two-fold serial titration was carried out using 96-well plates, and 10^4 colony forming units of *M. tuberculosis* H37Rv were added to testing wells. Plates were then incubated and processed as regular MABA. The Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine approved all animal procedures in this study.

For the cytotoxicity testing of compounds **41** and **42** at four human-derived cell lines, the QSG-7701 (normal liver cell), MRC-5 (lung fibroblast cell), HFL-1 (lung fibroblast cell), and HEK-293 (human embryonic kidney cell) were obtained from the American Type Culture Collection (Manassas, VA, USA). QSG-7701and HEK293 cells were cultured in DMEM medium, whereas MRC-5 and HFL-1 were cultured in DMEM/F12 medium. Both of the media contained 10% fetal bovine serum (FBS, HyClone Laboratories, UT, USA) and 100 unit/mL penicillin and 100 μg/mL streptomycin. All cells were cultured at 37°C in a humidified incubator with 5% CO_2. To measure the cytotoxicity, cells were plated in 96-well plates at a density of 5,000 cells per well. After incubating overnight, the cells were directly treated with the indicated drug concentrations for 48 hours. 20μl CellTiter 96® AQueous One Solution Cell Proliferation kit (Promega, WI, USA) were added to each well of the 96-well plate, followed by incubation for 1 hour. The absorbance values at 490 nm were measured with a FlexStation3 microplate reader (Molecular Devices, CA, USA). All experiments were performed independently three times. The IC_{50} values were calculated by GraphPad Prism software (GraphPad Software, CA, USA). Data was shown as mean ± SD.

Genome Sequencing and Analysis of Resistant Mutants.

Whole genome shotgun (WGS) libraries were prepared as follows. DNA samples were quantified by Quant-iT PicoGreen dsDNA Assay (Life Technologies) and normalized to a concentration of 250pg/uL. Illumina sequencing libraries were prepared from 250pg of DNA using the Nextera XT DNA Library Preparation kit (Illumina) according to the manufacturer's recommended protocol, with reaction volumes scaled accordingly. Libraries were pooled by transferring equal volumes of each library using a Labcyte Echo 550 liquid handler. Insert sizes and concentrations for pooled libraries were determined using an Agilent Bioanalyzer DNA 1000 kit (Agilent Technologies). Each sample was sequenced on Illumina HiSeq2500 at the Broad Institute to produce 101-bp paired-end reads.

Nine mutant strains, as well as the reference CSU.1 and the parental strain JHU.1 were sequenced. For all sequenced strains, reads were mapped onto the finished reference genome *M. tuberculosis* H37Rv (Genbank accession NC_000962) using BWA mem version 0.7.12.[28] Variants were identified using Pilon version 1.22.[29] Variants shared between a mutant strain and the non-mutated parent were removed from the analysis; only variants differing between the mutant strain and the parent strain were analyzed. All sequences have been deposited at NCBI.

Supporting Information. ^{1}H and ^{13}C NMR spectra and analytical HPLC purities of all final compounds **;** melting points for all solid compounds; microsomal stability data for compounds **41** and **42**; crystallographic data of compound **16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED:

Serum inhibition titration, SIT; polyketide synthase, Pks; tuberculosis, TB; *Mycobacterium tuberculosis, Mtb*; world health organization, WHO; multidrug-resistant, MDR; extensively drug-resistant, XDR; totally drug-resistant, TDR; acyl carrier protein, ACP; ketoacylsynthase, KS; acyl transferase, AT; thioesterase, TE; *corynebacterineae* mycolate reductase A, CmrA; trehalose monomycolate, TMM; microplate alamar blue assay, MABA; electron-donating groups, EDG; electron-withdrawing groups, EWG; column chromatography, CC; tetramethylsilane, TMS; high resolution mass spectra, HRMS; whole genome shotgun, WGS.

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