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Expanding the scaffold for bacterial RNA polymerase inhibitors: design, synthesis and structure–activity relationships of ureido-heterocyclic-carboxylic acids

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The emergence of bacterial resistance requires the development of new antibiotics with an alternative mode of action. Based on class I, developed in our previous study, a new series of RNA polymerase (RNAP) inhibitors targeting the switch region was designed. Feasible synthetic procedures for the aryl-ureido-heterocyclic-carboxylic acids were developed including three regioisomeric thiophene classes (II–IV), as well as three isosteric furan (V, VI) and thiazole (VII) classes. Biological evaluation using a RNAP transcription inhibition assay revealed that class II compounds possess the same activity as the parent class I, whereas classes III, V–VII were active, however with lower potency. Structure–activity relationship (SAR) studies, supported by molecular modeling, elucidated the structural requirements necessary for interaction with the binding site. Beside the RNAP inhibitory effects, the new compounds displayed good antibacterial activities against Gram positive bacteria and the Gram negative *E. coli* TolC strain. Moreover, they showed no cross resistance with the clinically used RNAP inhibitor rifampicin (Rif) and a lower rate of resistance compared to Rif.

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Introduction

The eternal battle against pathogenic bacteria demands the discovery and development of new weapons aimed at vital targets, since the prevalence of antibiotic resistance poses a real threat to human health.^{1,2} Bacterial RNAP is a multisubunit enzyme responsible for transcription.³ It is necessary for cell survival allowing efficacy, and structurally distinguished from eukaryotic counterparts affording therapeutic selectivity.⁴ However, the only clinically used drugs targeting RNAP are the rifamycins which are applied to treat *Mycobacterium tuberculosis* infections, and the recently FDA approved Fidaxomicin for *Clostridium difficile* infections.⁵ Hence, while proven as a drug target, RNAP is still underexploited. Our mission is to discover and optimize RNAP inhibitors with an alternative binding site compared to rifamycins, and consequently, with low probability of cross resistance. Recently the “switch region”, a binding pocket distinct from the rifamycins binding site, was discovered and proved to be a promising target site for antibacterial drug discovery.^{6–8} The RNAP inhibitors myxopyronin B (Myx B), a natural α -pyrone antibiotic isolated from the myxobacterium

Myxococcus fulvus,⁹ and its synthetic derivative desmethyl myxopyronin B (dMyx B),¹⁰ have been identified to bind to the “switch region”.^{6,7} Although the myxopyronins are highly active *in vitro* and show no cross resistance to rifamycins,^{6,11,12} their clinical application is hampered by inadequate physicochemical properties.¹³

These facts motivated us and other research groups to develop novel “switch region” inhibitors. McPhillie *et al.* used a structure based *de novo* design based on the crystal structure of the dMyx B binding site. Although the compounds inhibited RNAP, they displayed no antibacterial activity.¹⁴ Buurman *et al.* applied a high throughput screening, identified RNAP inhibitors and confirmed the switch region as their target site. However, they showed only weak antibacterial activity.¹⁵ Yakushiji *et al.* pursued a hybrid strategy, combining the core α -pyrone of Myx with holothin. The resulting RNAP inhibitor was active against Gram positive bacteria.¹⁶

In a previous work of our group, based on a hit candidate discovered by virtual screening, a series of 5-aryl-3-ureidothiophene-2-carboxylic acids (class I) was synthesized and optimized based on SAR studies. Moreover, the binding mode was experimentally validated. The compounds showed good antibacterial activities accompanied by a low resistance frequency (Fig. 1).¹⁷

In this work, we focused on finding new chemical scaffolds inspired from class I with better or at least retained biological

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Fig. 1 Development of second generation bacterial RNAP inhibitors of the ureido-heterocyclic-carboxylic acid type.

activities. To achieve this goal, we followed an analog design strategy accompanied by SAR exploration (Fig. 1). The study was supported by molecular modeling to gain deeper insights into the structural features necessary for activity.

Results and discussion

Design strategy

Analog design was accomplished through two approaches: *via* design of regioisomers of the parent class I, and *via* bioisosteric exchange of the heterocyclic core. By reversing the positions of ureido and carboxyl substituents of class I (class II), shifting the aryl position in class I (class IV), or shifting the aryl position in class II (class III), three classes of regioisomers were initially investigated to identify the optimum configuration of the aryl-ureido-thiophene-carboxylic acids for interaction with the target enzyme (Fig. 2). Based on our previous results,¹⁷ as aryl motif phenyl rings bearing substituents with high π and σ values, namely 4-chlorophenyl and 3,4-dichlorophenyl, were chosen. It was also shown that at the ureido motif hydrophobic and bulky substituents are preferred, therefore *n*-hexyl, benzyl and *N*-ethylbenzyl amine were employed. In the next step, the biological results of classes I–IV were taken into consideration. Based on classes I and II, displaying the highest RNAP inhibitory activity, the classical isosteric ring equivalents –O– for –S–

(classes V and VI) or –N= for –CH= (class VII) were investigated (Fig. 2).

Chemistry

The synthesis of compounds 6–11 (class I) started by reacting the acetophenones 1a,b (Scheme 1) with POCl₃ in DMF followed by NH₂OH·HCl according to a modified Vilsmeier–Haack–Arnold reaction¹⁸ to give the β -chlorocinnamionitriles 2a,b which were cyclized using methyl thioglycolate under basic condition (NaOMe) to afford the methyl 5-aryl-3-aminothiophene-2-carboxylates 3a,b.^{17,19} Esters were saponified to the corresponding acids 4a,b which were treated with triphosgene to form the thiasatoic anhydrides 5a,b. The latter reacted with the appropriate amines in water followed by acidic workup to yield the desired 5-aryl-3-ureidothiophene-2-carboxylic acids 6–11.^{17,20,21} The compounds of classes II and III were synthesized by a straightforward procedures *via* Gewald reaction of the arylacetaldehydes 12a,b or the acetophenones 1a,b (Scheme 1) with ethyl cyanoacetate and elemental sulfur under basic conditions in a one-pot reaction to afford the ethyl esters 13a,b and 22a,b^{22,23} respectively. After saponification, synthesis of both the 5- and 4-aryl-2-ureidothiophene-3-carboxylic acids 16–21 and 25–30 *via* the thiasatoic anhydrides 15a,b and 24a,b was also successfully employed as described for the class I derivatives. The synthesis of compounds of class IV was achieved by treating the arylacetonitriles 31a,b (Scheme 1) with ethyl formate in presence of NaOMe, followed by acidic workup to furnish the 2-aryl-3-hydroxyacrylonitriles 32a,b.^{24–26} Ring closure was accomplished by activation of 32a,b using benzenesulfonyl chloride to yield the sulfonates 33a,b which reacted with methyl thioglycolate under basic condition to produce the methyl 3-amino-4-arylthiophene-2-carboxylates 34a,b.²⁶ Further synthetic steps *via* the thiasatoic anhydrides 36a,b proceeded smoothly to deliver the desired 4-aryl-3-ureidothiophene-2-carboxylic acids 37–42.

For the synthesis of compounds 51 and 52 (class V), acetophenone 1b (Scheme 2) was converted to the β -ketonitrile 44 *via* bromination²⁷ and subsequent nucleophilic substitution using KCN.^{28,29} Compound 44 was further reacted under Mitsunobu conditions³⁰ with ethyl glycolate to the intermediate vinyl ether 45 that was cyclized under basic condition (NaH) to yield the ethyl 3-aminofuran-2-carboxylate 46. We initially attempted to adopt the “isatoic anhydride strategy” for the furan class as described for synthesis of the ureidothiophene analogs. Unfortunately, the required 3-aminofuran-2-carboxylic acid could not be obtained. Various conditions for alkaline hydrolysis of the ester 46 led to decomposition of the furan ring, and a ring opening product was isolated. This observation is attributed to the unusual instability and weak aromatic properties characterizing the aminofurans.^{31–33} Therefore, we decided to postpone the hydrolysis to the end of the synthesis as the ureido-furan derivatives are less electron rich and should be less prone to decomposition.³⁴ To prepare the urea derivatives, compound 46 was treated directly with the carbamoylimidazoles³⁵ or isocyanate³⁶ but no conversion was observed. Finally, an alternative route *via* the phenyl carbamate 47 followed by

Fig. 2 Analog design strategies based on the parent class I.

nucleophilic substitution with the appropriate amines³⁷ gave the desired ureido-furan-carboxylic esters **48** and **49**. The hydrolysis to the free acids proceeded smoothly under basic condition for the *N*-benzyl-*N*-ethylurea derivative **49** to afford **52**, but for the *N*-benzyl derivative **48** cyclization yielding the uracil derivative **50** occurred. Therefore, non-saponificative, mild dealkylation was conducted using AlCl_3 in tetrahydrothiophene (THT)³⁸ affording the desired carboxylic acid **51**. The synthesis of the regioisomeric furan system class VI proceeded *via*

coupling of the phenacyl bromide **43** (Scheme 2) with ethyl cyanoacetate to give the intermediate **53** that was cyclized under acidic condition (TFA) to deliver the 2-aminofuran-carboxylic ester **54**. Interestingly, reaction of **54** with phenyl chloroformate afforded only the diacylated product **55** even when a stoichiometric amount of reagent was used. This is consistent with previous findings regarding the reactivity of 2-aminofurans.³⁹ The subsequent transformation into the urea derivatives **57** and **58** required an excess of the amine to eliminate the second

Scheme 1 Synthesis of compound classes I–IV.

Scheme 2 Synthesis of compound classes V–VII.

carbamoyl group from the intermediate **56**. Finally, the acids **59** and **60** were obtained by dealkylation using AlCl_3 in THF. The thiazole class VII was prepared starting from the benzyl chloride **61** (Scheme 2) that was reacted with elemental sulfur and alkylated with methyl iodide to give the carbodithioate **62**. For the ring closure, **62** was first reacted with cyanamide in basic medium (NaOMe) and further *S*-alkylated with methyl bromoacetate to give the intermediate **63** that was cyclized under basic conditions affording the 4-aminothiazole ester **64**. After alkaline hydrolysis, the acid **65** was converted to the thiazoloi-satoic anhydride **66**. This intermediate reacted in the same manner as described for the thiophene derivatives to the ureidothiazole carboxylic acids **67** and **68**.

In vitro RNA polymerase inhibitory activity

Compounds of classes I–VII were tested for their inhibitory activity against *E. coli* RNAP and the results are shown in Table 1. Generally compounds with 3,4-dichloro substituents exhibited higher activity than 4-chlorophenyl derivatives in the same class.

This finding is in accordance with our previous study of the parent class **1**.¹⁷ An increase of activity was also observed with substituents having larger hydrophobic volume at the ureido motif with the general trend benzylethyl $\sim$ *n*-hexyl > benzyl. Compounds of class II showed RNAP inhibitory activities as good as the parent class **1**. Interestingly, the 3,4-dichloro derivatives **19–21** (IC_{50} : 18, 43, and 21 μM respectively) showed identical activities to their analogous compounds **9–11** of class I (IC_{50} : 18, 46, and 22 μM respectively). The class III analogs **28–30** displayed moderate activities (IC_{50} : 75, 84, and 57 μM respectively, about 2–4 fold decrease), while class IV derivatives **40–42** possessed weaker activities (IC_{50} : 74 to >100 μM , >4 fold decrease).

The outstanding role of classes I and II regarding RNAP inhibition can be explained on the basis of molecular similarity, *i.e.* similar molecules exhibit similar activities.^{40–42} The similarity of classes I–IV was analyzed *in silico* by using molecular fingerprint method, where a graph 3-point pharmacophore (GpiDAPH3) was applied as 2D fingerprint system. As similarity metric the Tanimoto coefficient (T_C) was used.⁴³ Class II showed maximum similarity to I ($T_C = 1.00$), followed by III ($T_C = 0.93$),

Table 1 *In vitro* inhibitory activity against *E. coli* RNA polymerase.

<div> <div> </div> <div> </div> <div> </div> <div> </div> </div> <div> <div>class I 6–11</div> <div>class II 16–21</div> <div>class III 25–30</div> <div>class IV 37–42</div> </div>			
Compd	Ar	R ¹ R ²	Inhibition of <i>E. coli</i> RNAP ^d
6		H, <i>n</i> -Hex	68 μM
7		H, Bn	31%
8 ^b		Et, Bn	75 μM
9 ^b		H, <i>n</i> -Hex	18 μM
10 ^b		H, Bn	46 μM
11 ^b		Et, Bn	22 μM
16		H, <i>n</i> -Hex	14%
17		H, Bn	84 μM
18		Et, Bn	54 μM
19		H, <i>n</i> -Hex	18 μM
20		H, Bn	43 μM
21		Et, Bn	21 μM
25		H, <i>n</i> -Hex	11%
26		H, Bn	n.i.
27		Et, Bn	14%
28		H, <i>n</i> -Hex	75 μM
29		H, Bn	84 μM
30		Et, Bn	57 μM
37		H, <i>n</i> -Hex	n.i.
38		H, Bn	n.i.
39		Et, Bn	n.i.
40		H, <i>n</i> -Hex	74 μM
41		H, Bn	8%
42		Et, Bn	100 μM
<div> <div> </div> <div> </div> <div> </div> </div> <div> <div>class V 51, 52</div> <div>class VI 59, 60</div> <div>class VII 67, 68</div> </div>			
51		H, Bn	116 μM
52		Et, Bn	61 μM
59		H, Bn	26%
60		Et, Bn	60 μM
67		H, Bn	48 μM
68		Et, Bn	51 μM
Myx B			0.35 μM
Rif			0.03 μM

^a IC₅₀ values (μM) or % inhibition at 100 μM of *E. coli* RNAP; n.i. = inhibition ≤5% at 100 μM. ^b Previously reported.¹⁷

while IV exhibited low similarity ($T_C = 0.65$). Another similarity assessment *via* flexible alignment of classes I–IV revealed that the aryl, ureido, and carboxyl substituents as well as the thiophene core of I and II are coincided (Fig. 3A). Class III also matches except that the carboxyl group is located in the

opposite position to that of I and II, whereas neither the ureido nor the carboxyl substituents of IV fit to the configuration of I and II (Fig. 3A). Hence class I and II are similar with respect to their configuration in space. Consequently they can assume the same orientation and binding mode, which results in the same inhibitory activities. These results were confirmed by docking of compounds 11, 21, and 30 representing classes I–III respectively, in the dMyx B binding site of *T. Thermophilus* RNA polymerase (PDB code 3EQL).⁷ Both 11 and 21 bind to the crescent shape pocket in the same manner (Fig. 3B and C). The thiophene core is located at the top of the cavity opening, anchored by hydrogen bond or ion pair interaction of the carboxyl group with the β'Lys610 residue. The 3,4-dichlorophenyl moiety occupies the lower part of the enecarbamate binding pocket of dMyx B. The ureido group carrying the lipophilic benzyl and ethyl substituents is located deeply in the hydrophobic pocket occupied by the dMyx B dienone side chain, and stabilized by CH–π interaction with βLeu1088 as well as an intramolecular hydrogen bond with the carboxyl group. On the other hand compound 30 (class III) binds mainly through CH–π interaction between the lipophilic substituted ureido moiety and βLeu1088, but lacks the interaction with β'Lys610, as the carboxyl group is oriented in the opposite direction (Fig. 3D). Accordingly, a lower inhibitory activity of 30 (IC₅₀: 57 μM) in comparison with compounds 11 or 21 (IC₅₀: 22 μM, 21 μM) was observed.

The effect of exchanging the heterocyclic core on the RNAP inhibitory activity was studied for compounds of classes V–VII. The results revealed that both of the furan classes V and VI displayed about a threefold decrease in activity compared to the corresponding thiophene analogs (class I and II), whereas the thiazole class VII exhibited only a slightly lowered potency (Table 1). By replacement or introduction of hetero atoms the electronic properties as well as the size of the ring is influenced and both effects can have an impact on the affinity to target. The latter is more likely to be responsible for the observed differences in activity. The ring size influences interatomic distances, bond angles, and determines the overall shape of the ligand.⁴⁴ According to the observed activities, thiophene is obviously most appropriate to keep the aryl, ureido, and carboxyl functionalities in the optimal geometry necessary for binding to the target enzyme. This is reflected by the relationship of the angle (α) between the aryl and ureido substituents and RNAP activity (pIC₅₀) for classes I–VII (Fig. 4). A parabolic curve was obtained, with the optimum range for α between 150 and 159° (classes I, II, and VII located at the maximum). The observed exception for class III ($\alpha = 156^\circ$) can be explained by the different localization of the carboxyl group leading to a reduced binding affinity as discussed above.

Antibacterial activity

To explore the antibacterial spectrum of our RNAP inhibitors, eight compounds representing the most active classes were selected. Compounds with *n*-hexyl substituents were excluded due to solubility problems. The antibacterial activities were evaluated in the Gram positive *B. subtilis* and *S. aureus*, as well as in the Gram negative strains *E. coli* K12, *P. aeruginosa*, *E. coli*

Fig. 3 (A) Flexible alignment of compounds **10** (white), **20** (magenta), **29** (orange), and **41** (turquoise). (B) Docking pose of compound **11** (violet) in the dMyx B (turquoise) binding site: hydrophobic surface (green), polar surface (pink), β chain (yellow), β' chain (red). (C) Docking pose of **21** (violet). (D) Docking pose of **30** (violet).

TolC, a mutant deficient in the AcrAB-TolC efflux system, and two Rif-resistant *E. coli* TolC mutants and are expressed as minimal inhibitory concentrations (MIC) values. As reference compounds Myx B and Rif were used (Table 2). It was found that the compounds possess antibacterial activities against the Gram positive strains. Regarding the Gram negative bacteria

with the exception of *E. coli* TolC, compounds were not active similarly to Myx B. It is noteworthy that the antibacterial activity against *S. aureus* is well correlating with the RNAP inhibitory activity, whereas in case of *B. subtilis* and *E. coli* TolC the correlation was less pronounced. Similar discrepancies between RNAP inhibition and MIC values are also observed for Myx B

Fig. 4 Relationship between RNAP inhibitory activity (pIC₅₀) and angle α .

Table 2 Antibacterial activities of selected aryl-ureido-heterocyclic-carboxylic acids

Compd	IC ₅₀ RNAP (μM)	MIC ₉₅ ^a (μg mL ⁻¹)						
		<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i> TolC	<i>E. coli</i> TolC β Q513L ^b	<i>E. coli</i> TolC β H526Y ^b	<i>E. coli</i> K12	<i>P. aeruginosa</i>
11	22	8	11	14	14	16	>25	>25
18	54	20	5	11	—	—	>25	>25
21	21	10	2	10	7	9	>25	>25
29	84	80	15	10	—	—	>100	>100
30	57	23	6	7	7	7	>100	>100
52	61	33	12	30	25	25	>50	>50
60	60	28	14	>50	—	—	>50	>50
68	51	36	45	47	50	50	>50	>50
Myx B	0.35	0.5	1	1	1	1	>25	>25
Rif	0.03	0.02	5	6	>100	>100	7	13

^a > MIC determination was limited due to insufficient solubility of the test compound. ^b Rif-resistant *E. coli* TolC strains with mutations in the rpoB gene encoding for the RNAP β subunit.

and Rif (Table 2), and have been frequently reported.^{17,45,46} In the experiments with the Rif-resistant strains all of the five tested compounds representing different classes showed no reduction of antibacterial activity compared to the normal *E. coli* TolC strain. This demonstrates that there is no cross resistance with Rif as it has been expected due to the different binding sites.

Role of cell wall penetration and drug efflux for antibacterial activity in *E. coli* strains

Considering the facts that RNAP is highly conserved in bacteria,³ and our compounds were active against Gram positive strains as well as *E. coli* TolC, but not against Gram negative bacteria, the most likely conclusion to be drawn is that they are unable to accumulate in the cytoplasm to inhibit RNAP. This could be due to cell wall impermeability, *i.e.* slow diffusion through porins or the outer membrane (OM) lipid bilayer, efflux mechanisms or both. The observed activity in the TolC mutant lacking the OM part of the tripartite efflux machinery gives a strong hint that efflux plays a prominent role for our compounds. To verify this hypothesis and to get a better understanding of their uptake pathway, the antibacterial effect for selected compounds was determined in presence of the OM permeability enhancer polymyxin B nonapeptide (PMBN)⁴⁷ or the efflux pumps inhibitor PAβN.^{48,49} MIC values were determined against *E. coli* TolC, *E. coli*

D22 (LPS mutant with increased OM permeability) and *E. coli* K12 (intact cell wall system) and are shown in Table 3.

PMBN produced only a slight decrease in *E. coli* TolC MIC values (factors 1.4–2.7) and no effect was observed in the K12 strain. Moreover, the compounds showed no enhanced activity against the *E. coli* D22 strain. It cannot be excluded that an increased membrane permeability may be counteracted by efflux.⁵⁰ It is known that PMBN enhances penetration of antibiotics which diffuse across the OM (10–300 fold decrease in MIC values against *E. coli*), but it has only a slight effect when antibiotics traverse through porins.⁴⁷ Hence, the uptake pathway of our compounds into Gram negative bacteria appears to be mainly permeation through the porins. On the other hand, PAβN increased the susceptibility to the compounds in *E. coli* TolC by factors of 5–14 and by factors ≥3 in the K12 and D22 strains. These results indicate that both the OM barrier and efflux pumps contribute to the inactivity of our compounds in Gram negative bacteria, however, efflux mechanisms play the major role. Obviously, beside AcrAB-TolC other efflux systems are involved in *E. coli* drug efflux.

Spontaneous resistance rate

Low propensity of resistance development is a criterion for an effective antibacterial agent. Spontaneous resistance rate towards Myx B in *S. aureus* was found to be 4 to 7 × 10⁻⁸,

Table 3 Effect of PMBN and PAβN on antibacterial activities of selected aryl-ureido-heterocyclic-carboxylic acids

Compd	<i>E. coli</i> TolC			<i>E. coli</i> K12			<i>E. coli</i> D22	
	MIC ₉₅ ^a	MIC ₉₅ + PMBN (1 μg mL ⁻¹)	MIC ₉₅ + PAβN (10 μg mL ⁻¹)	MIC ₅₀	MIC ₅₀ + PMBN (1 μg mL ⁻¹)	MIC ₅₀ + PAβN (20 μg mL ⁻¹)	MIC ₅₀	MIC ₅₀ + PAβN (20 μg mL ⁻¹)
11	14	7	1	>25	>25	>25	>50	>25
21	10	4	1	>25	>25	4	>25	12
30	7	5	1	>100	>100	18	40	15
52	30	11	6	>50	>50	>50	>100	49
68	47	22	4	>50	>50	>50	>50	>50

^a MIC values in μg mL⁻¹.

similar to that of Rif.¹² However, Myx B-resistant mutants possessed higher fitness costs compared to Rif-resistant mutants, giving an advantage to Myx B, and other RNAP “switch region” inhibitors, of having a lower clinical prevalence of resistance than Rif.¹² Determination of *in vitro* resistance rate for **30** in *E. coli* TolC at $2 \times \text{MIC}$ revealed a significant lower rate ($<4.2 \times 10^{-11}$) compared to Rif (8.3×10^{-8}) and Myx B (7.1×10^{-8}) as previously observed for the class I derivatives.¹⁷ This observation indicates that the probability of resistance development is reduced with the ureido-thiophene-carboxylic acids compared to MyxB and Rif. An explanation for this finding could be that our compounds occupy only a part of the “switch region” whereas Myx B fills a larger space including the enecarbamate binding pocket. Mutations in this part, responsible for Myx resistance,^{6,12} should not prevent our compounds from binding and inhibiting the enzyme.¹⁷ Another explanation for a reduced resistance rate could be an additional effect on another target.

Cytotoxicity

The *in vitro* toxicity of selected compounds was evaluated by monitoring the cytotoxicity in HEK 293 cells at different time points using doxorubicin (LD_{50} : 0.3 μM) and Rif (LD_{50} : 80 μM) as positive and negative controls respectively. After 72 h, the compounds displayed LD_{50} values in the range of 25 to $>100 \mu\text{M}$ comparable to Rif (Table 4).

Table 4 Cytotoxicity of selected aryl-ureido-heterocyclic-carboxylic acids

Compd	LD_{50} (μM)		
	24 h	48 h	72 h
8	95	78	91
10	>100	>100	>100
11	67	50	46
18	84	82	75
21	61	57	62
30	40	17	25
52	61	61	57
Doxorubicin	5	0.7	0.3
Rif	24% ^a	38% ^a	80

^a At 100 μM .

Conclusion

Following an analog design strategy novel chemical scaffolds as bacterial RNAP inhibitors were developed. Derived from the parent class I, a series of regioisomeric ureido-thiophene-carboxylic acid derivatives and bioisosteric heterocyclic classes were designed and studied. The synthetic route *via* the “isatoic anhydrides” for the thiophene and thiazole derivatives was robust and feasible. For the synthesis of the furan derivatives the established strategy had to be modified due to the instability of the furan system. Thereby, class II possessing the same RNAP

inhibitory activity as the parent class I, as well as classes III, and V–VII with only slightly lowered potency were discovered. The detailed investigation of the SAR, including molecular alignment, docking studies and angle analysis contributed to a deeper understanding of the structural requirements for interaction with the protein target. The compounds were active against Gram positive bacteria including the pathogen *S. aureus* but ineffective against Gram negative bacteria. The non-susceptibility can be attributed to drug efflux. Nevertheless, the observed low mammalian cytotoxicity, the reduced resistance frequency and the activity against Rif-resistant strains make these novel scaffolds promising for further optimization as antibacterial agents against Gram positive pathogens.

Experimental

Materials and methods

Starting materials and solvents were purchased from commercial suppliers, and used without further purification. All chemical yields refer to purified compounds, and were not optimized. Reaction progress was monitored using TLC silica gel 60 F₂₅₄ aluminium sheets, and visualization was accomplished by UV at 254 nm. Flash chromatography was performed using silica gel 60 Å (40–63 μm). Preparative RP-HPLC was carried out on a Waters Corporation setup contains a 2767 sample manager, a 2545 binary gradient module, a 2998 PDA detector and a 3100 electron spray mass spectrometer. Purification was performed using a Waters XBridge column (C18, $150 \times 19 \text{ mm}$, 5 μm), a binary solvent system A and B (A = water with 0.1% formic acid; B = MeCN with 0.1% formic acid) as eluent, a flow rate of 20 mL min^{-1} and a gradient of 60% to 95% B in 8 min were applied. Melting points were determined on a Stuart Scientific melting point apparatus SMP3 (Bibby Sterilin, UK), and are uncorrected. NMR spectra were recorded either on Bruker DRX-500 (^1H , 500 MHz; ^{13}C , 126 MHz), or Bruker Fourier 300 (^1H , 300 MHz; ^{13}C , 75 MHz) spectrometer at 300 K. Chemical shifts are recorded as δ values in ppm units by reference to the hydrogenated residues of deuterated solvent as internal standard (CDCl_3 : $\delta = 7.26$, 77.02; $\text{DMSO}-d_6$: $\delta = 2.50$, 39.99). Splitting patterns describe apparent multiplicities and are designated as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), m (multiplet). Coupling constants (J) are given in Hertz (Hz). Purity of all compounds used in biological assays was $\geq 95\%$ as measured by LC/MS Finnigan Surveyor MSQ Plus (Thermo Fisher Scientific, Dreieich, Germany). The system consists of LC pump, autosampler, PDA detector, and single-quadrupole MS detector, as well as the standard software Xcalibur for operation. RP C18 Nucleodur 100-5 ($125 \times 3 \text{ mm}$) column (Macherey-Nagel GmbH, Dühren, Germany) was used as stationary phase, and a binary solvent system A and B (A = water with 0.1% TFA; B = MeCN with 0.1% TFA) was used as mobile phase. In a gradient run the percentage of B was increased from an initial concentration of 0% at 0 min to 100% at 15 min and kept at 100% for 5 min. The injection volume was 10 μL and flow rate was set to 800 $\mu\text{L min}^{-1}$. MS (ESI) analysis was carried out at a spray voltage of 3800 V, a

capillary temperature of 350 °C and a source CID of 10 V. Spectra were acquired in positive mode from 100 to 1000 *m/z* and at 254 nm for UV tracing.

Chemistry

Synthesis of 5-(aryl)-3-[3-(substituted)ureido]thiophene-2-carboxylic acids **6–11** was previously described,¹⁷ as well as the experimental data of compounds **8–11**.¹⁷

5-(4'-Chlorophenyl)-3-(3-hexylureido)thiophene-2-carboxylic acid **6.**²¹ Beige crystals; mp 198–199 °C; δ_{H} (300 MHz, DMSO-*d*₆) 0.86 (3H, t, *J* = 4.8 Hz, Me), 1.19–1.49 (8H, m, Me(CH₂)₄CH₂NH), 3.08 (2H, m, CH₂CH₂NH), 7.50 (2H, d, *J* = 7.8 Hz, 3',5'-Ar-H), 7.63 (1H, t, *J* = 4.8 Hz, NHCH₂), 7.68 (2H, d, *J* = 7.8 Hz, 2',6'-Ar-H), 8.28 (1H, s, C4-H), 9.33 (1H, br s, NHCO), 13.13 (1H, br s, COOH); δ_{C} (75 MHz, DMSO-*d*₆) 14.39 (C6''), 22.54 (C5''), 26.56 (C3''), 29.85 (C2''), 31.47 (C4''), 39.78 (C1''), 107.40 (C2), 118.89 (C4), 127.81 (C2', C6'), 129.77 (C3', C5'), 132.23 (C1'), 134.07 (C4'), 145.64 (C5), 146.74 (C3), 154.25 (NHCO), 165.23 (COOH); *m/z* (ESI+) 381 (17%, (M + H)⁺), 761 (33, 2M + H), 295 (40, M – C₆H₁₃), 236 (100, M – C₆H₁₃, NH, CO₂); *t*_R = 14.86 min.

3-(3-Benzylureido)-5-(4'-chlorophenyl)thiophene-2-carboxylic acid **7.**²¹ White crystals; mp 216–217 °C; δ_{H} (300 MHz, DMSO-*d*₆) 4.31 (2H, d, *J* = 5.7 Hz, CH₂), 7.21–7.38 (5H, m, Ph), 7.51 (2H, d, *J* = 8.6 Hz, 3',5'-Ar-H), 7.70 (2H, d, *J* = 8.6 Hz, 2',6'-Ar-H), 8.22 (1H, t, *J* = 5.7 Hz, NHCH₂), 8.30 (1H, s, C4-H), 9.43 (1H, br s, NHCO), 13.15 (1H, br s, COOH); δ_{C} (75 MHz, DMSO-*d*₆) 43.46 (CH₂), 107.42 (C2), 118.91 (C4), 127.30 (C4''), 127.76 (C2'', C6''), 127.87 (C2', C6'), 128.79 (C3'', C5''), 129.79 (C3', C5'), 132.15 (C1'), 134.15 (C4'), 140.26 (C1''), 145.90 (C5), 146.65 (C3), 154.36 (NHCO), 165.16 (COOH); *m/z* (ESI+) 387 (19%, (M + H)⁺), 772 (10, 2M), 295 (10, M – C₇H₇), 236 (100, M – C₇H₇, NH, CO₂); *t*_R = 13.38 min.

General procedures for synthesis of 2-(aryl)acetaldehydes **12a** and **12b**

To a stirred ice-cooled suspension of pyridinium chlorochromate (12.9 g, 60.0 mmol) in anhydrous DCM (80 mL), the appropriate 2-(aryl)ethanol^{51,52} (40.0 mmol) in DCM (10 mL) was added in one portion. The reaction mixture was allowed to warm to rt, and stirred for 2 h, then anhydrous Et₂O (100 mL) was added, and the supernatant was decanted from the black gum. The insoluble residue was washed thoroughly with anhydrous Et₂O (2 × 50 mL), the combined organic solution was passed through a short pad of silica, and the solvent was removed by vacuum distillation. The crude product was used directly in the next step without further purification. Purity was determined to be 70–80% as indicated from ¹H-NMR spectra.

2-(4'-Chlorophenyl)acetaldehyde **12a.**⁵³ Yellow oil; δ_{H} (300 MHz, CDCl₃) 3.67 (2H, d, *J* = 1.0 Hz, CH₂), 7.15 (2H, d, *J* = 8.1 Hz, 3',5'-Ar-H), 7.33 (2H, d, *J* = 8.1 Hz, 2',6'-Ar-H), 9.74 (1H, t, *J* = 1.0 Hz, CHO).

2-(3',4'-Dichlorophenyl)acetaldehyde **12b.**⁵⁴ Yellow oil; δ_{H} (300 MHz, CDCl₃) 3.68 (2H, d, *J* = 1.9 Hz, CH₂), 7.05 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.32 (1H, d, *J* = 2.0 Hz, 2'-Ar-H), 7.37 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 9.75 (1H, t, *J* = 1.9 Hz, CHO).

General procedures for synthesis of ethyl 2-amino-5-(aryl)thiophene-3-carboxylates **13a** and **13b**

To a stirred suspension of the appropriate aldehyde **12a** or **12b** (30.0 mmol), ethyl cyanoacetate (3.39 g, 30.0 mmol), and sulfur (0.96 g, 30.0 mmol) in EtOH (30 mL), a solution of NEt₃ (4.04 g, 30.0 mmol) in EtOH (5 mL) was added slowly. The reaction mixture was stirred at 70 °C for 12 h, then solvent was removed by vacuum distillation. The obtained residue was dissolved in DCM (50 mL) and washed with water (2 × 50 mL). The organic layer was dried (MgSO₄) and concentrated. The crude material was purified by flash chromatography (SiO₂, *n*-hexane–EtOAc = 6 : 1).

Ethyl 2-amino-5-(4'-chlorophenyl)thiophene-3-carboxylate **13a** (5.49 g, 65%). Pale yellow solid; mp 100–101 °C; δ_{H} (300 MHz, CDCl₃) 1.36 (3H, t, *J* = 7.1 Hz, Me), 4.30 (2H, q, *J* = 7.1 Hz, CH₂O), 6.03 (2H, br s, NH₂), 7.21 (1H, s, C4-H), 7.27 (2H, d, *J* = 8.9 Hz, 3',5'-Ar-H), 7.35 (2H, d, *J* = 8.9 Hz, 2',6'-Ar-H); δ_{C} (75 MHz, CDCl₃) 14.54 (Me), 59.94 (CH₂), 108.04 (C3), 121.79 (C4), 123.48 (C5), 125.81 (C2', C6'), 128.91 (C3', C5'), 132.16 (C4'), 132.56 (C1'), 162.17 (C2), 165.29 (C=O); *m/z* (ESI+) 281 (8%, M⁺), 236 (100, M – EtO); *t*_R = 14.05 min.

Ethyl 2-amino-5-(3',4'-dichlorophenyl)thiophene-3-carboxylate **13b** (6.05 g, 64%). Pale yellow solid; mp 130–131 °C; δ_{H} (300 MHz, CDCl₃) 1.37 (3H, t, *J* = 7 Hz, Me), 4.30 (2H, q, *J* = 7.0 Hz, CH₂O), 6.08 (2H, br s, NH₂), 7.22 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.23 (1H, s, C4-H), 7.36 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 7.48 (1H, d, *J* = 2.0 Hz, 2'-Ar-H); δ_{C} (75 MHz, CDCl₃) 14.53 (Me), 60.03 (CH₂), 108.11 (C3), 121.90 (C4), 122.78 (C6'), 123.74 (C5), 126.11 (C2'), 130.04 (C4'), 130.62 (C5'), 132.91 (C3'), 134.12 (C1'), 162.48 (C2), 165.20 (C=O); *m/z* (ESI+) 315 (18%, M⁺), 270 (100, M – EtO); *t*_R = 15.14 min.

General procedures for synthesis of 2-amino-5-(aryl)thiophene-3-carboxylic acids **14a** and **14b**

To a stirred solution of the appropriate ester **13a** or **13b** (25.0 mmol) in MeOH (100 mL), KOH (6.17 g, 110 mmol) in water (100 mL) was added. The reaction mixture was stirred at reflux for 3–5 h (TLC monitoring), then MeOH was evaporated by vacuum distillation. The residue was diluted with water (50 mL) and washed with EtOAc (2 × 50 mL). The aqueous layer was cooled in an ice bath and acidified by KHSO₄ (saturated aqueous solution) to pH 3–4. The precipitated solid was collected by filtration, washed with cold water (2 × 30 mL), *n*-hexane (2 × 30 mL), and dried over CaCl₂ in amber glass vacuum desiccator.

2-Amino-5-(4'-chlorophenyl)thiophene-3-carboxylic acid **14a** (4.11 g, 65%). Beige solid; mp 195–197 °C; δ_{H} (300 MHz, DMSO-*d*₆) 7.26 (1H, s, C4-H), 7.36 (2H, d, *J* = 8.6 Hz, 3',5'-Ar-H), 7.45 (2H, d, *J* = 8.6 Hz, 2',6'-Ar-H), 7.47 (2H, br s, NH₂), 12.10 (1H, br s, COOH); δ_{C} (75 MHz, DMSO-*d*₆) 106.36 (C3), 120.76 (C4), 123.19 (C5), 125.94 (C2', C6'), 129.30 (C3', C5'), 130.69 (C4'), 133.31 (C1'), 163.93 (C2), 166.48 (C=O); *m/z* (ESI+) 253 (72%, M⁺), 255 (28, [M + 2]⁺), 236 (100, M – OH), 209 (14, M – CO₂); *t*_R = 10.83 min.

2-Amino-5-(3',4'-dichlorophenyl)thiophene-3-carboxylic acid **14b** (5.88 g, 82%). Beige solid; mp 229–231 °C; δ_{H} (300 MHz, DMSO-*d*₆) 7.38 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.39 (1H, s, C4-H), 7.52 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 7.55 (2H, br s, NH₂), 7.69 (1H,

d, $J = 2.0$ Hz, 2'Ar-H), 12.15 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 106.57 (C3), 119.11 (C4), 124.37 (C6'), 124.77 (C5), 125.60 (C2'), 128.26 (C4'), 131.33 (C5'), 132.16 (C3'), 135.18 (C1'), 164.37 (C2), 166.43 (C=O); m/z (ESI+) 287 (88%, M^+), 289 (56, $[M + 2]^+$), 270 (100, $M - OH$), 243 (8, $M - CO_2$), 227 (25, $M - CO_2$, NH_2); $t_R = 11.87$ min.

General procedures for synthesis of 6-(aryl)-1H-thieno[2,3-d]-[1,3]oxazine-2,4-diones 15a and 15b

To a stirred solution of the appropriate acid **14a** or **14b** (6.00 mmol) in THF (60 mL), triphosgene (1.29 g, 4.36 mmol) was added portionwise over 30 min. The reaction mixture was stirred at rt for 2 h, then $NaHCO_3$ (saturated aqueous solution, 30 mL) was added cautiously, and the resulting mixture was extracted with EtOAc-THF (1 : 1, 2 $\times$ 50 mL). The combined organic layer was washed with brine (50 mL), dried ($MgSO_4$), and the solvent was removed by vacuum distillation. The obtained crude material was suspended in *n*-hexane-EtOAc (4 : 1, 50 mL), stirred in a water bath at 40 °C for 10 min, cooled, and collected by filtration.

6-(4'-Chlorophenyl)-1H-thieno[2,3-d][1,3]oxazine-2,4-dione 15a (1.2 g, 74%). Beige solid; mp 254–256 °C; δ_{H} (300 MHz, DMSO- d_6) 7.40 (2H, d, $J = 8.7$ Hz, 3',5'-Ar-H), 7.48 (1H, s, C5-H), 7.60 (2H, d, $J = 8.7$ Hz, 2',6'-Ar-H), 11.73 (1H, br s, NH); δ_{C} (75 MHz, DMSO- d_6) 109.70 (C4a), 119.10 (C5), 126.74 (C2', C6'), 128.28 (C6), 129.39 (C3', C5'), 131.75 (C4'), 133.00 (C1'), 150.22 (C7a), 152.83 (C2), 159.67 (C4); m/z (ESI+) 279 (20%, M^+), 235 (100, $M - CO_2$); $t_R = 10.40$ min.

6-(3',4'-Dichlorophenyl)-1H-thieno[2,3-d][1,3]oxazine-2,4-dione 15b (1.5 g, 81%). Beige solid; mp > 300 °C; δ_{H} (300 MHz, DMSO- d_6) 7.61 (2H, m, 5',6'-Ar-H), 7.79 (1H, s, C5-H), 7.96 (1H, s, 2'-Ar-H), 12.76 (1H, br s, NH); δ_{C} (75 MHz, DMSO- d_6) 111.46 (C4a), 120.60 (C5), 125.72 (C6'), 127.17 (C2'), 130.87 (C4'), 131.57 (C5'), 131.83 (C6), 132.50 (C3'), 133.21 (C1'), 147.81 (C7a), 155.32 (C2), 155.82 (C4); m/z (ESI+) 313 (14%, M^+), 355 (100, $M + H$, MeCN), 627 (28, 2M + H), 296 (35, $M - OH$); $t_R = 11.44$ min.

General procedures for synthesis of 5-(aryl)-2-[3-(substituted)-ureido]thiophene-3-carboxylic acids 16–21

To a stirred suspension of thiaisatoic anhydride **15a** or **15b** (0.64 mmol) in water (8 mL), the appropriate amine (1.40 mmol) was added. The reaction mixture was stirred at rt for 2 h, then poured on ice-cooled 2 M HCl (40 mL), and extracted with EtOAc-THF (1 : 1, 40 mL). The organic layer was washed with 2 M HCl (40 mL), brine (40 mL), dried ($MgSO_4$), and concentrated *in vacuo*. The obtained crude material was suspended in *n*-hexane-EtOAc (4 : 1, 50 mL), stirred in a water bath at 40 °C for 10 min, cooled, and collected by filtration.

5-(4'-Chlorophenyl)-2-(3-hexylureido)thiophene-3-carboxylic acid 16 (132 mg, 54%). Pale brown crystals; mp 230–232 °C; δ_{H} (300 MHz, DMSO- d_6) 0.87 (3H, t, $J = 6.8$ Hz, Me), 1.03–1.57 (8H, m, $Me(CH_2)_4CH_2NH$), 3.11 (2H, m, CH_2CH_2NH), 7.40 (2H, d, $J = 8.6$ Hz, 3',5'-Ar-H), 7.43 (1H, s, C4-H), 7.59 (2H, d, $J = 8.6$ Hz, 2',6'-Ar-H), 7.94 (1H, t, $J = 4.2$ Hz, $NHCH_2$), 10.24 (1H, br s, NHCO), 12.82 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 14.39 (C6''), 22.54 (C5''), 26.50 (C3''), 29.71 (C2''), 31.43 (C4''), 39.86

(C1''), 111.55 (C3), 121.27 (C4), 126.63 (C2', C6'), 128.77 (C5), 129.45 (C3', C5'), 131.62 (C4'), 133.17 (C1'), 151.41 (C2), 153.88 (NHCO), 166.35 (COOH); m/z (ESI+) 380 (33%, M^+), 761 (100, 2M + H), 295 (18, $M - C_6H_{13}$); $t_R = 14.38$ min.

2-(3-Benzylureido)-5-(4'-chlorophenyl)thiophene-3-carboxylic acid 17 (136 mg, 55%). Pale brown crystals; mp 250–252 °C; δ_{H} (300 MHz, DMSO- d_6) 4.34 (2H, d, $J = 5.6$ Hz, CH_2), 7.15–7.39 (5H, m, Ph), 7.41 (2H, d, $J = 8.6$ Hz, 3',5'-Ar-H), 7.45 (1H, s, C4-H), 7.59 (2H, d, $J = 8.6$ Hz, 2',6'-Ar-H), 8.49 (1H, t, $J = 5.3$ Hz, $NHCH_2$), 10.35 (1H, br s, NHCO), 12.87 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 43.67 (CH_2), 111.79 (C3), 121.29 (C4), 126.68 (C2', C6'), 127.45 (C4''), 127.81 (C2'', C6''), 128.86 (C3'', C5''), 129.02 (C5), 129.46 (C3', C5'), 131.70 (C4'), 133.09 (C1'), 139.75 (C1''), 151.20 (C2), 154.01 (NHCO), 166.29 (COOH); m/z (ESI+) 387 (30%, $[M + H]^+$), 773 (22, 2M + H), 295 (26, $M - C_7H_7$), 170 (100); $t_R = 13.18$ min.

2-(3-Benzyl-3-ethylureido)-5-(4'-chlorophenyl)thiophene-3-carboxylic acid 18 (136 mg, 51%). Pale brown crystals; mp 215–216 °C; δ_{H} (300 MHz, DMSO- d_6) 1.16 (3H, t, $J = 7.1$ Hz, Me), 3.42 (2H, q, $J = 7.1$ Hz, $MeCH_2N$), 4.60 (2H, s, $PhCH_2N$), 7.24–7.39 (5H, m, Ph), 7.41 (2H, d, $J = 8.6$ Hz, 3',5'-Ar-H), 7.48 (1H, s, C4-H), 7.62 (2H, d, $J = 8.6$ Hz, 2',6'-Ar-H), 10.96 (1H, br s, NHCO), 13.15 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 13.52 (Me), 42.42 ($MeCH_2N$), 49.96 ($PhCH_2N$), 112.30 (C3), 120.98 (C4), 126.79 (C2', C6'), 127.64 (C4''), 127.75 (C2'', C6''), 129.04 (C3'', C5''), 129.48 (C3', C5'), 129.68 (C5), 131.87 (C4'), 132.97 (C1'), 138.06 (C1''), 151.67 (C2), 153.10 (NHCO), 167.43 (COOH); m/z (ESI+) 415 (100%, $[M + H]^+$), 829 (90, 2M + H); $t_R = 14.08$ min.

5-(3',4'-Dichlorophenyl)-2-(3-hexylureido)thiophene-3-carboxylic acid 19 (165 mg, 62%). Beige crystals; mp 245–247 °C; δ_{H} (300 MHz, DMSO- d_6) 0.86 (3H, t, $J = 6.8$ Hz, Me), 1.16–1.56 (8H, m, $Me(CH_2)_4CH_2NH$), 3.11 (2H, m, CH_2CH_2NH), 7.53 (1H, dd, $J = 8.0$, 2.0 Hz, 6'-Ar-H), 7.56 (1H, s, C4-H), 7.59 (1H, d, $J = 8.0$ Hz, 5'-Ar-H), 7.83 (1H, d, $J = 2.0$ Hz, 2'-Ar-H), 7.97 (1H, t, $J = 4.7$ Hz, $NHCH_2$), 10.26 (1H, br s, NHCO), 12.89 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 14.39 (C6''), 22.53 (C5''), 26.49 (C3''), 29.69 (C2''), 31.43 (C4''), 39.85 (C1''), 111.65 (C3), 122.63 (C4), 125.03 (C6'), 126.41 (C2'), 127.27 (C5), 129.29 (C4'), 131.51 (C5'), 132.28 (C3'), 135.00 (C1'), 151.88 (C2), 153.85 (NHCO), 166.27 (COOH); m/z (ESI+) 414 (100%, M^+), 416 (69, $[M + 2]^+$), 829 (67, 2M + H), 329 (42, $M - C_6H_{13}$); $t_R = 15.40$ min.

2-(3-Benzylureido)-5-(3',4'-dichlorophenyl)thiophene-3-carboxylic acid 20 (194 mg, 72%). White crystals; mp 256–258 °C; δ_{H} (300 MHz, DMSO- d_6) 4.34 (2H, d, $J = 5.6$ Hz, CH_2), 7.22–7.39 (5H, m, Ph), 7.53 (1H, dd, $J = 8.0$, 2.0 Hz, 6'-Ar-H), 7.58 (1H, s, C4-H), 7.60 (1H, d, $J = 8.0$ Hz, 5'-Ar-H), 7.84 (1H, d, $J = 2.0$ Hz, 2'-Ar-H), 8.51 (1H, t, $J = 5.6$ Hz, $NHCH_2$), 10.38 (1H, br s, NHCO), 12.93 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 43.68 (CH_2), 111.97 (C3), 122.68 (C4), 125.08 (C6'), 126.47 (C2'), 127.46 (C4''), 127.49 (C5), 127.82 (C2'', C6''), 128.86 (C3'', C5''), 129.37 (C4'), 131.52 (C5'), 132.30 (C3'), 134.94 (C1'), 139.71 (C1''), 151.63 (C2), 153.99 (NHCO), 166.24 (COOH); m/z (ESI+) 421 (47%, $[M + H]^+$), 843 (100, 2M + 3H), 329 (11, $M - C_7H_7$); $t_R = 13.94$ min.

2-(3-Benzyl-3-ethylureido)-5-(3',4'-dichlorophenyl)thiophene-3-carboxylic acid 21 (210 mg, 73%). Beige crystals; mp 228–230 °C; δ_{H} (300 MHz, DMSO- d_6) 1.16 (3H, t, $J = 7.1$ Hz, Me), 3.42 (2H, q, $J = 7.1$ Hz, $MeCH_2N$), 4.60 (2H, s, $PhCH_2N$), 7.24–7.40 (5H, m, Ph),

7.55 (1H, dd, $J = 8.0, 2.0$ Hz, 6'Ar-H), 7.59 (1H, d, $J = 8.0$ Hz, 5'Ar-H), 7.60 (1H, s, C4-H), 7.86 (1H, d, $J = 2.0$ Hz, 2'Ar-H), 10.98 (1H, br s, NHCO), 13.22 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 13.52 (Me), 42.47 (MeCH₂N), 49.99 (PhCH₂N), 112.45 (C3), 122.35 (C4), 125.17 (C6'), 126.58 (C2'), 127.66 (C4''), 127.76 (C5), 128.15 (C2'', C6''), 129.04 (C3'', C5''), 129.54 (C4'), 131.53 (C5'), 132.33 (C3'), 134.80 (C1'), 138.03 (C1''), 152.11 (C2), 153.07 (NHCO), 167.38 (COOH); m/z (ESI+) 448 (100%, M⁺); $t_{\text{R}} = 14.99$ min.

Synthesis and characterization of ethyl 2-amino-4-(aryl)thiophene-3-carboxylates **22a**²² and **22b**²³ were previously described.

Synthesis of 2-amino-4-(aryl)thiophene-3-carboxylic acids **23a** and **23b** were performed as described for **14a** and **14b**.

2-Amino-4-(4'-chlorophenyl)thiophene-3-carboxylic acid 23a (3.48 g, 55%). Pale brown solid; mp 137–139 °C; δ_{H} (300 MHz, DMSO- d_6) 6.17 (1H, s, C5-H), 7.26 (2H, d, $J = 8.6$ Hz, 3',5'Ar-H), 7.34 (2H, d, $J = 8.6$ Hz, 2',6'Ar-H), 7.40 (2H, br s, NH₂), 11.76 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 103.60 (C3), 105.90 (C5), 127.63 (C2', C6'), 130.87 (C3', C5'), 131.61 (C4'), 137.69 (C4), 139.95 (C1'), 165.71 (C2), 166.66 (C=O); m/z (ESI+) 254 (48%, (M + H)⁺), 507 (4, 2M + H), 236 (100, M – OH); $t_{\text{R}} = 10.16$ min.

2-Amino-4-(3',4'-dichlorophenyl)thiophene-3-carboxylic acid 23b (3.59 g, 50%). Pale brown solid; mp 142–144 °C; δ_{H} (300 MHz, DMSO- d_6) 6.27 (1H, s, C5-H), 7.24 (1H, dd, $J = 8.0, 2.0$ Hz, 6'Ar-H), 7.41 (2H, br s, NH₂), 7.47 (1H, d, $J = 2.0$ Hz, 2'Ar-H), 7.53 (1H, d, $J = 8.0$ Hz, 5'Ar-H), 11.96 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 103.37 (C3), 106.79 (C5), 129.53 (C6'), 129.75 (C5'), 130.25 (C4'), 130.32 (C2'), 130.86 (C3'), 138.52 (C4), 139.41 (C1'), 165.78 (C2), 166.44 (C=O); m/z (ESI+) 288 (100%, (M + H)⁺), 575 (19, 2M + H), 270 (77, M – OH); $t_{\text{R}} = 11.04$ min.

Synthesis of 5-(aryl)-1H-thieno[2,3-*d*][1,3]oxazine-2,4-diones **24a** and **24b** were performed as described for preparation of **15a** and **15b**.

5-(4'-Chlorophenyl)-1H-thieno[2,3-*d*][1,3]oxazine-2,4-dione 24a (1.2 g, 71%). Pale grey solid; mp 235–237 °C; δ_{H} (300 MHz, DMSO- d_6) 7.16 (1H, s, C6-H), 7.46 (2H, d, $J = 8.9$ Hz, 3',5'Ar-H), 7.51 (2H, d, $J = 8.9$ Hz, 2',6'Ar-H), 12.68 (1H, br s, NH); δ_{C} (75 MHz, DMSO- d_6) 107.59 (C4a), 116.09 (C6), 128.36 (C2', C6'), 131.12 (C3', C5'), 131.35 (C4'), 133.21 (C5), 137.56 (C1'), 147.93 (C7a), 154.98 (C2), 157.82 (C4); m/z (ESI+) 279 (100%, M⁺), 559 (6, 2M + H); $t_{\text{R}} = 10.23$ min.

5-(3',4'-Dichlorophenyl)-1H-thieno[2,3-*d*][1,3]oxazine-2,4-dione 24b (1.5 g, 80%). Beige solid; mp 236–238 °C; δ_{H} (300 MHz, DMSO- d_6) 7.27 (1H, s, C6-H), 7.48 (1H, dd, $J = 8.0, 2.0$ Hz, 6'Ar-H), 7.68 (1H, d, $J = 8.0$ Hz, 5'Ar-H), 7.75 (1H, d, $J = 2.0$ Hz, 2'Ar-H), 12.70 (1H, br s, NH); δ_{C} (75 MHz, DMSO- d_6) 107.58 (C4a), 117.07 (C6), 129.59 (C6'), 130.51 (C5'), 131.01 (C4'), 131.13 (C2'), 131.15 (C3'), 134.83 (C5), 136.08 (C1'), 147.87 (C7a), 155.07 (C2), 157.85 (C4); m/z (ESI+) 313 (8%, M⁺), 355 (100, M + H, MeCN), 626 (12, 2M), 296 (20, M – OH); $t_{\text{R}} = 10.77$ min.

Synthesis of 4-(aryl)-2-[3-(substituted)ureido]thiophene-3-carboxylic acids **25–30** were performed as described for preparation of **16–21**.

4-(4'-Chlorophenyl)-2-(3-hexylureido)thiophene-3-carboxylic acid 25 (180 mg, 74%). Pale grey crystals; mp 191–193 °C; δ_{H} (300 MHz, DMSO- d_6) 0.87 (3H, t, $J = 6.7$ Hz, Me), 1.21–1.51 (8H, m, Me(CH₂)₄CH₂NH), 3.10 (2H, m, CH₂CH₂NH), 6.63 (1H, s, C5-H), 7.29 (2H, d, $J = 8.6$ Hz, 3',5'Ar-H), 7.36 (2H, d, $J = 8.6$ Hz,

2',6'Ar-H), 7.87 (1H, t, $J = 5.1$ Hz, NHCH₂), 10.39 (1H, br s, NHCO), 12.50 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 14.39 (C6''), 22.55 (C5''), 26.52 (C3''), 29.78 (C2''), 31.45 (C4''), 39.84 (C1''), 108.92 (C3), 114.32 (C5), 127.73 (C2', C6'), 131.06 (C3', C5'), 131.79 (C4'), 137.31 (C4), 138.31 (C1'), 153.29 (C2), 154.16 (NHCO), 166.76 (COOH); m/z (ESI+) 381 (100%, (M + H)⁺), 761 (51, 2M + H), 295 (77, M – C₆H₁₃), 236 (58, M – C₆H₁₃, NH, CO₂); $t_{\text{R}} = 13.37$ min.

2-(3-Benzylureido)-4-(4'-chlorophenyl)thiophene-3-carboxylic acid 26 (200 mg, 81%). Pale brown crystals; mp 197–199 °C; δ_{H} (300 MHz, DMSO- d_6) 4.33 (2H, d, $J = 5.7$ Hz, CH₂), 6.66 (1H, s, C5-H), 7.24–7.37 (9H, m, 4'-ClC₆H₄, Ph), 8.40 (1H, t, $J = 4.8$ Hz, NHCH₂), 10.60 (1H, br s, NHCO), 12.56 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 43.57 (CH₂), 109.64 (C3), 114.39 (C5), 127.39 (C4''), 127.74 (C2'', C6''), 127.75 (C2', C6'), 128.84 (C3'', C5''), 131.08 (C3', C5'), 131.78 (C4'), 137.30 (C4), 138.44 (C1'), 139.96 (C1''), 152.79 (C2), 154.30 (NHCO), 166.85 (COOH); m/z (ESI+) 387 (100%, (M + H)⁺), 773 (37, 2M + H), 295 (44, M – C₇H₇), 236 (50, M – C₇H₇, NH, CO₂); $t_{\text{R}} = 12.21$ min.

2-(3-Benzyl-3-ethylureido)-4-(4'-chlorophenyl)thiophene-3-carboxylic acid 27 (240 mg, 90%). Pale grey crystals; mp 175–177 °C; δ_{H} (300 MHz, DMSO- d_6) 1.15 (3H, t, $J = 6.2$ Hz, Me), 3.41 (2H, q, $J = 6.2$ Hz, MeCH₂N), 4.60 (2H, s, PhCH₂N), 6.71 (1H, s, C5-H), 7.22–7.50 (9H, m, 4'-ClC₆H₄, Ph), 11.38 (1H, br s, NHCO), 12.84 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 13.54 (Me), 42.28 (MeCH₂N), 49.84 (PhCH₂N), 109.67 (C3), 114.80 (C5), 127.58 (C4''), 127.69 (C2'', C6''), 127.74 (C2', C6'), 129.03 (C3'', C5''), 131.22 (C3', C5'), 131.97 (C4'), 137.03 (C4), 138.22 (C1'), 138.46 (C1''), 153.45 (C2), 153.80 (NHCO), 167.93 (COOH); m/z (ESI+) 415 (100%, (M + H)⁺), 829 (6, 2M + H), 236 (12, M – C₇H₇, EtN, CO₂); $t_{\text{R}} = 13.18$ min.

4-(3',4'-Dichlorophenyl)-2-(3-hexylureido)thiophene-3-carboxylic acid 28 (172 mg, 65%). Pale brown crystals; mp 178–180 °C; δ_{H} (300 MHz, DMSO- d_6) 0.87 (3H, t, $J = 6.8$ Hz, Me), 1.21–1.50 (8H, m, Me(CH₂)₄CH₂NH), 3.10 (2H, m, CH₂CH₂NH), 6.72 (1H, s, C5-H), 7.27 (1H, dd, $J = 8.0, 2.0$ Hz, 6'Ar-H), 7.53 (1H, d, $J = 2.0$ Hz, 2'Ar-H), 7.56 (1H, d, $J = 8.0$ Hz, 5'Ar-H), 7.86 (1H, t, $J = 5.0$ Hz, NHCH₂), 10.49 (1H, br s, NHCO), 12.62 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 14.40 (C6''), 22.54 (C5''), 26.51 (C3''), 29.77 (C2''), 31.44 (C4''), 39.82 (C1''), 109.21 (C3), 115.00 (C5), 129.67 (C6'), 129.74 (C5'), 129.82 (C4'), 130.33 (C2'), 131.10 (C3'), 136.99 (C4), 139.16 (C1'), 153.12 (C2), 154.16 (NHCO), 166.70 (COOH); m/z (ESI+) 415 (100%, (M + H)⁺), 829 (30, 2M + H), 329 (92, M – C₆H₁₃); $t_{\text{R}} = 14.26$ min.

2-(3-Benzylureido)-4-(3',4'-dichlorophenyl)thiophene-3-carboxylic acid 29 (210 mg, 78%). Beige crystals; mp 188–190 °C; δ_{H} (300 MHz, DMSO- d_6) 4.34 (2H, d, $J = 5.6$ Hz, CH₂), 6.77 (1H, s, C5-H), 7.24–7.39 (6H, m, 6'Ar-H, Ph), 7.53 (1H, d, $J = 2.0$ Hz, 2'Ar-H), 7.56 (1H, d, $J = 8.0$ Hz, 5'Ar-H), 8.45 (1H, t, $J = 5.0$ Hz, NHCH₂), 10.51 (1H, br s, NHCO), 12.69 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 43.59 (CH₂), 109.06 (C3), 115.30 (C5), 127.42 (C4''), 127.76 (C2'', C6''), 128.85 (C3'', C5''), 129.75 (C6'), 129.78 (C5'), 129.86 (C4'), 130.38 (C2'), 131.11 (C3'), 136.97 (C4), 139.01 (C1'), 139.88 (C1''), 153.19 (C2), 154.28 (NHCO), 166.48 (COOH); m/z (ESI+) 421 (100%, (M + H)⁺), 841 (23, 2M + H), 329 (25, M – C₇H₇); $t_{\text{R}} = 12.98$ min.

2-(3-Benzyl-3-ethylureido)-4-(3',4'-dichlorophenyl)thiophene-3-carboxylic acid 30 (172 mg, 60%). Beige crystals; mp 193–195

$^{\circ}\text{C}$; δ_{H} (300 MHz, DMSO- d_6) 1.15 (3H, t, J = 7.0 Hz, Me), 3.41 (2H, q, J = 7.0 Hz, MeCH_2N), 4.60 (2H, s, PhCH_2N), 6.80 (1H, s, C5-H), 7.24–7.39 (6H, m, 6'Ar-H, Ph), 7.55 (1H, d, J = 2.0 Hz, 2'Ar-H), 7.56 (1H, d, J = 8.0 Hz, 5'Ar-H), 11.43 (1H, br s, NHCO), 12.92 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 13.54 (Me), 42.27 (MeCH_2N), 49.82 (PhCH_2N), 109.72 (C3), 115.50 (C5), 127.59 (C4'), 127.70 (C2'', C6''), 129.03 (C3'', C5''), 129.84 (C6'), 129.88 (C4', C5'), 130.35 (C2'), 131.26 (C3'), 137.08 (C4), 138.23 (C1'), 138.83 (C1''), 153.46 (C2), 153.78 (NHCO), 167.77 (COOH); m/z (ESI+) 449 (100%, (M + H) $^{+}$), 897 (7, 2M + H), 270 (13, M – C $_7\text{H}_7$, EtN, CO $_2$); t_{R} = 14.00 min.

Synthesis of methyl 3-amino-4-(aryl)thiophene-2-carboxylates **34a**²⁶ and **34b** were performed according to reported procedures.²⁶

Methyl 3-amino-4-(4'-chlorophenyl)thiophene-2-carboxylate 34a.²⁶ Beige solid; mp 104–105 $^{\circ}\text{C}$ (lit.,²⁶ 106 $^{\circ}\text{C}$); δ_{H} (300 MHz, CDCl $_3$) 3.86 (3H, s, OMe), 5.57 (2H, br s, NH $_2$), 7.23 (1H, s, C5-H), 7.37 (2H, d, J = 8.7 Hz, 3',5'Ar-H), 7.43 (2H, d, J = 8.7 Hz, 2',6'Ar-H); δ_{C} (75 MHz, CDCl $_3$) 51.39 (Me), 101.69 (C2), 128.88 (C5), 129.39 (C2', C6'), 129.44 (C3', C5'), 132.08 (C4), 132.82 (C1'), 134.00 (C4'), 151.27 (C3), 165.03 (C=O); m/z (ESI+) 268 (16%, (M + H) $^{+}$), 236 (100, M – MeO); t_{R} = 13.20 min.

Methyl 3-amino-4-(3',4'-dichlorophenyl)thiophene-2-carboxylate 34b. White solid; mp 135–137 $^{\circ}\text{C}$; δ_{H} (300 MHz, DMSO- d_6) 3.76 (3H, s, OMe), 6.38 (2H, br s, NH $_2$), 7.45 (1H, dd, J = 8.0, 2.0 Hz, 6'Ar-H), 7.69 (1H, d, J = 2.0 Hz, 2'Ar-H), 7.71 (1H, d, J = 8.0 Hz, 5'Ar-H), 7.78 (1H, s, C5-H); δ_{C} (75 MHz, DMSO- d_6) 51.65 (Me), 99.91 (C2), 128.75 (C6'), 130.31 (C2'), 130.73 (C4'), 130.77 (C5), 131.39 (C4), 131.60 (C5'), 131.99 (C3'), 135.08 (C1'), 152.58 (C3), 164.64 (C=O); m/z (ESI+) 302 (12%, (M + H) $^{+}$), 270 (100, M – MeO); t_{R} = 12.79 min.

Synthesis of 3-amino-4-(aryl)thiophene-2-carboxylic acids **35a** and **35b** were performed as described for preparation of **14a** and **14b**.

3-Amino-4-(4'-chlorophenyl)thiophene-2-carboxylic acid 35a (reported as reaction intermediate and not isolated).^{20,55} (5.38 g, 85%) White solid; mp 160–162 $^{\circ}\text{C}$; δ_{H} (300 MHz, DMSO- d_6) 6.45 (2H, br s, NH $_2$), 7.49–7.56 (4H, m, 4'-ClC $_6\text{H}_4$), 7.64 (1H, s, C5-H), 11.86 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 101.28 (C2), 129.32 (C2', C6'), 130.00 (C5), 130.15 (C3', C5'), 132.08 (C4), 132.68 (C1'), 133.62 (C4'), 152.19 (C3), 166.00 (C=O); m/z (ESI+) 254 (18%, (M + H) $^{+}$), 236 (100, M – OH); t_{R} = 10.69 min.

3-Amino-4-(3',4'-dichlorophenyl)thiophene-2-carboxylic acid 35b (5.52 g, 77%). Beige solid; mp 127–129 $^{\circ}\text{C}$; δ_{H} (300 MHz, DMSO- d_6) 7.46 (1H, dd, J = 8.0, 2.0 Hz, 6'Ar-H), 7.70 (1H, d, J = 8.0 Hz, 5'Ar-H), 7.70 (1H, d, J = 2.0 Hz, 2'Ar-H), 7.73 (1H, s, C5-H), 7.74 (2H, br s, NH $_2$), 12.49 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 101.46 (C2), 128.67 (C6'), 130.21 (C2'), 130.59 (C4'), 130.79 (C5), 130.81 (C5'), 131.36 (C4), 131.96 (C3'), 135.35 (C1'), 152.16 (C3), 165.94 (C=O); m/z (ESI+) 287 (12%, M $^{+}$), 270 (100, M – OH); t_{R} = 11.53 min.

Synthesis of 7-(aryl)-1H-thieno[3,2-*d*][1,3]oxazine-2,4-diones **36a** and **36b** were performed as described for preparation of **15a** and **15b**.

7-(4'-Chlorophenyl)-1H-thieno[3,2-*d*][1,3]oxazine-2,4-dione 36a.^{20,55} (1.23 g, 73%) White solid; mp 214–216 $^{\circ}\text{C}$ (lit.,²⁰ 245 $^{\circ}\text{C}$, lit.,⁵⁵ > 260 $^{\circ}\text{C}$); δ_{H} (300 MHz, DMSO- d_6) 7.49 (2H, d, J = 8.8

Hz, 3',5'Ar-H), 7.54 (2H, d, J = 8.8 Hz, 2',6'Ar-H), 8.25 (1H, s, C6-H), 11.86 (1H, br s, NH); δ_{C} (75 MHz, DMSO- d_6) 107.66 (C4a), 129.20 (C2', C6'), 129.35 (C7), 130.97 (C3', C5'), 131.03 (C1'), 133.66 (C4'), 136.87 (C6), 147.24 (C7a), 149.41 (C2), 156.06 (C4); m/z (ESI+) 279 (24%, M $^{+}$), 251 (100, M – CO); t_{R} = 10.18 min.

7-(3',4'-Dichlorophenyl)-1H-thieno[3,2-*d*][1,3]oxazine-2,4-dione 36b (1.36 g, 72%). Beige solid; mp 249–251 $^{\circ}\text{C}$; δ_{H} (300 MHz, DMSO- d_6) 7.43 (1H, dd, J = 8.0, 2.0 Hz, 6'Ar-H), 7.70 (1H, d, J = 2.0 Hz, 2'Ar-H), 7.73 (1H, d, J = 8.0 Hz, 5'Ar-H), 8.32 (1H, s, C6-H), 11.96 (1H, br s, NH); δ_{C} (75 MHz, DMSO- d_6) 107.73 (C4a), 129.58 (C6'), 129.64 (C7), 131.25 (C2'), 131.27 (C5'), 131.71 (C4'), 131.82 (C3'), 132.50 (C1'), 137.70 (C6), 146.70 (C7a), 149.24 (C2), 155.77 (C4); m/z (ESI+) 313 (13%, M $^{+}$), 627 (13, 2M + H), 285 (100, M – CO); t_{R} = 11.14 min.

Synthesis of 4-(aryl)-3-[3-(substituted)ureido]thiophene-2-carboxylic acids **37–42** were performed as described for preparation of **16–21**.

4-(4'-Chlorophenyl)-3-(3-hexylureido)thiophene-2-carboxylic acid 37.²¹ (195 mg, 80%) White crystals; mp 176–178 $^{\circ}\text{C}$; δ_{H} (300 MHz, DMSO- d_6) 0.86 (3H, t, J = 6.4 Hz, Me), 1.12–1.30 (8H, m, $\text{Me}(\text{CH}_2)_4\text{CH}_2\text{NH}$), 2.84 (2H, m, $\text{CH}_2\text{CH}_2\text{NH}$), 6.88 (1H, t, J = 5.9 Hz, NHCH_2), 7.37 (2H, d, J = 7.8 Hz, 3',5'Ar-H), 7.42 (2H, d, J = 7.8 Hz, 2',6'Ar-H), 7.79 (1H, s, C5-H), 8.34 (1H, br s, NHCO), 13.15 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 14.42 (C6''), 22.51 (C5''), 26.31 (C3''), 30.08 (C2''), 31.49 (C4''), 39.47 (C1''), 118.68 (C2), 128.59 (C2', C6'), 128.70 (C3', C5'), 129.01 (C5), 131.83 (C4'), 135.76 (C1'), 138.35 (C4), 142.42 (C3), 154.72 (NHCO), 164.24 (COOH); m/z (ESI+) 381 (25%, (M + H) $^{+}$), 761 (17, 2M + H), 295 (53, M – C $_6\text{H}_{13}$), 236 (100, M – C $_6\text{H}_{13}$, NH, CO $_2$); t_{R} = 12.71 min.

3-(3-Benzylureido)-4-(4'-chlorophenyl)thiophene-2-carboxylic acid 38.²¹ (225 mg, 91%) White crystals; mp 196–198 $^{\circ}\text{C}$ (lit.,²¹ 216 $^{\circ}\text{C}$); δ_{H} (300 MHz, DMSO- d_6) 4.10 (2H, d, J = 5.9 Hz, CH $_2$), 7.06–7.32 (5H, m, Ph), 7.38–7.46 (5H, m, 4'-ClC $_6\text{H}_4$, NHCH_2), 7.82 (1H, s, C5-H), 8.49 (1H, br s, NHCO), 13.23 (1H, br s, COOH); δ_{C} (75 MHz, DMSO- d_6) 43.06 (CH $_2$), 119.38 (C2), 127.05 (C4''), 127.32 (C2'', C6''), 128.54 (C3'', C5''), 128.75 (C2', C6'), 128.88 (C3', C5'), 129.09 (C5), 131.92 (C4'), 135.68 (C1'), 138.63 (C4), 140.70 (C1''), 142.13 (C3), 155.03 (NHCO), 164.17 (COOH); m/z (ESI+) 387 (40%, (M + H) $^{+}$), 773 (36, 2M + H), 295 (51, M – C $_7\text{H}_7$), 236 (100, M – C $_7\text{H}_7$, NH, CO $_2$); t_{R} = 10.41 min.

3-(3-Benzyl-3-ethylureido)-4-(4'-chlorophenyl)thiophene-2-carboxylic acid 39.²¹ (212 mg, 80%) White crystals; mp 157–159 $^{\circ}\text{C}$; δ_{H} (500 MHz, DMSO- d_6) 1.09 (3H, t, J = 6.3 Hz, Me), 3.24 (2H, q, J = 6.6 Hz, MeCH_2N), 4.37 (2H, s, PhCH_2N), 7.02–7.32 (5H, m, Ph), 7.43 (2H, d, J = 8.8 Hz, 3',5'Ar-H), 7.46 (2H, d, J = 8.8 Hz, 2',6'Ar-H), 7.83 (1H, s, C5-H), 8.52 (1H, br s, NHCO), 13.23 (1H, br s, COOH); δ_{C} (126 MHz, DMSO- d_6) 13.04 (Me), 40.77 (MeCH_2N), 48.40 (PhCH_2N), 119.87 (C2), 126.85 (C4''), 127.18 (C2'', C6''), 128.14 (C3'', C5''), 128.27 (C2', C6'), 128.28 (C5), 128.66 (C3', C5'), 131.56 (C4'), 134.97 (C1'), 138.44 (C4), 138.48 (C1''), 142.51 (C3), 154.37 (NHCO), 163.87 (COOH); m/z (ESI+) 415 (100%, (M + H) $^{+}$), 829 (21, 2M + H), 236 (37, M – C $_7\text{H}_7$, EtN, CO $_2$); t_{R} = 12.74 min.

4-(3',4'-Dichlorophenyl)-3-(3-hexylureido)thiophene-2-carboxylic acid 40 (220 mg, 83%). White crystals; mp 189–191 $^{\circ}\text{C}$; δ_{H} (300 MHz, DMSO- d_6) 0.85 (3H, t, J = 7 Hz, Me), 1.02–1.39 (8H, m,

Me(CH₂)₄CH₂NH), 2.85 (2H, m, CH₂CH₂NH), 7.01 (1H, t, *J* = 5.5 Hz, NHCH₂), 7.38 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.57 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 7.62 (1H, d, *J* = 2.0 Hz, 2'-Ar-H), 7.89 (1H, s, C5-H), 8.42 (1H, br s, NHCO), 13.19 (1H, br s, COOH); δ_C (75 MHz, DMSO-d₆) 14.42 (C6''), 22.48 (C5''), 26.29 (C3''), 30.14 (C2''), 31.47 (C4''), 39.53 (C1''), 118.34 (C2), 127.16 (C6'), 128.59 (C2'), 129.67 (C5), 129.82 (C4'), 130.72 (C5'), 131.31 (C3'), 136.81 (C4), 137.56 (C1'), 142.41 (C3), 154.63 (NHCO), 164.21 (COOH); *m/z* (ESI+) 415 (76%, (M + H)⁺), 829 (37, 2M + H), 329 (100, M - C₆H₁₃), 270 (96, M - C₆H₁₃, NH, CO₂); *t*_R = 12.65 min.

3-(3-Benzylureido)-4-(3',4'-dichlorophenyl)thiophene-2-carboxylic acid 41 (221 mg, 82%). Off white crystals; mp 182–184 °C; δ_H (300 MHz, DMSO-d₆) 4.12 (2H, d, *J* = 5.9 Hz, CH₂), 7.03–7.35 (5H, m, Ph), 7.40 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.51 (1H, t, *J* = 5.9 Hz, NHCH₂), 7.59 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 7.67 (1H, d, *J* = 2.0 Hz, 2'-Ar-H), 7.91 (1H, s, C5-H), 8.54 (1H, br s, NHCO), 12.69 (1H, br s, COOH); δ_C (75 MHz, DMSO-d₆) 43.09 (CH₂), 119.18 (C2), 127.04 (C4''), 127.19 (C2'', C6''), 127.36 (C6'), 128.58 (C3'', C5''), 128.80 (C2'), 129.80 (C5), 129.83 (C4'), 130.90 (C5'), 131.42 (C3'), 137.15 (C4), 137.52 (C1'), 140.57 (C1''), 142.09 (C3), 154.98 (NHCO), 164.12 (COOH); *m/z* (ESI+) 421 (58%, (M + H)⁺), 841 (24, 2M + H), 329 (83, M - C₇H₇), 270 (100, M - C₇H₇, NH, CO₂); *t*_R = 9.60 min.

3-(3-Benzyl-3-ethylureido)-4-(3',4'-dichlorophenyl)thiophene-2-carboxylic acid 42 (236 mg, 82%). Off white crystals; mp 171–173 °C; δ_H (300 MHz, DMSO-d₆) 1.09 (3H, t, *J* = 6.8 Hz, Me), 3.25 (2H, q, *J* = 6.8 Hz, MeCH₂N), 4.40 (2H, s, PhCH₂N), 6.91–7.37 (5H, m, Ph), 7.43 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.63 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 7.70 (1H, d, *J* = 2.0 Hz, 2'-Ar-H), 7.94 (1H, s, C5-H), 8.55 (1H, br s, NHCO), 13.36 (1H, br s, COOH); δ_C (75 MHz, DMSO-d₆) 13.48 (Me), 41.32 (MeCH₂N), 49.00 (PhCH₂N), 120.31 (C2), 127.35 (C4''), 127.51 (C2'', C6''), 127.70 (C6'), 128.70 (C3'', C5''), 129.01 (C2'), 129.54 (C5), 129.97 (C4'), 130.98 (C5'), 131.50 (C3'), 137.17 (C4), 137.44 (C1'), 138.86 (C1''), 142.85 (C3), 154.80 (NHCO), 164.28 (COOH); *m/z* (ESI+) 449 (100%, (M + H)⁺), 897 (9, 2M + H), 270 (10, M - C₇H₇, EtN, CO₂); *t*_R = 13.02 min.

Ethyl 3-amino-5-(3',4'-dichlorophenyl)furan-2-carboxylate 46. To a stirred ice-cooled solution of triphenylphosphine (5.12 g, 19.5 mmol) in anhydrous THF (70 mL), diethyl azodicarboxylate (3.40 g, 19.5 mmol) was added dropwise. After 10 min, ethyl glycolate (2.03 g, 19.5 mmol) was added dropwise, then **44**²⁸ (3.21 g, 15.0 mmol) was added portionwise. The reaction mixture was allowed to warm to rt, and stirred for 15 h. Sodium hydride (55–65% in mineral oil, 1.80 g, 42.0 mmol) was added, and the reaction was further stirred for 6 h. The reaction mixture was treated with water (10 mL), and the solvent was removed by vacuum distillation. The obtained residue was dissolved in EtOAc (70 mL), washed with water (50 mL), dried (MgSO₄), and concentrated. The crude material was purified by flash chromatography (SiO₂, *n*-hexane–EtOAc = 3 : 1). (3.90 g, 87%); white solid; mp 153–155 °C; δ_H (300 MHz, CDCl₃) 1.42 (3H, t, *J* = 7.1 Hz, Me), 4.40 (2H, q, *J* = 7.1 Hz, CH₂O), 4.64 (2H, br s, NH₂), 6.39 (1H, s, C4-H), 7.47 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 7.55 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.81 (1H, d, *J* = 2.0 Hz, 2'-Ar-H); δ_C (75 MHz, CDCl₃) 14.65 (Me), 60.13 (CH₂), 100.98 (C4), 124.03 (C6'), 126.16 (C3), 126.62 (C2'), 129.38 (C1'), 130.76 (C5'),

132.98 (C4'), 133.16 (C3'), 144.74 (C2), 153.45 (C5), 160.37 (C=O); *m/z* (ESI+) 300 (83%, (M + H)⁺), 599 (6, 2M + H), 254 (100, M - EtO); *t*_R = 13.72 min.

Ethyl 5-(3',4'-dichlorophenyl)-3-(phenoxycarbonylamino)-furan-2-carboxylate 47. To a stirred ice-cooled solution of **46** (1.00 g, 3.33 mmol), and pyridine (264 mg, 3.33 mmol) in anhydrous DCM (20 mL), phenyl chloroformate (525 mg, 3.33 mmol) was added dropwise. The reaction mixture was stirred at rt for 12 h. The solvent was evaporated under vacuum, and the residue was dissolved in EtOAc (50 mL), washed with 1 M HCl (2 × 25 mL), brine (25 mL), dried (MgSO₄), and the solvent was removed by vacuum distillation. The obtained material was triturated with *n*-hexane (20 mL), collected by filtration and dried. (1.20 g, 86%); pale yellow solid; mp 120–121 °C; δ_H (300 MHz, CDCl₃) 1.46 (3H, t, *J* = 7.1 Hz, Me), 4.46 (2H, q, *J* = 7.1 Hz, CH₂O), 7.18–7.45 (5H, m, Ph), 7.48 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 7.51 (1H, s, C4-H), 7.57 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.85 (1H, d, *J* = 2.0 Hz, 2'-Ar-H), 8.76 (1H, br s, NHCO); δ_C (75 MHz, CDCl₃) 14.51 (Me), 61.19 (CH₂), 102.14 (C4), 121.43 (C2'', C6''), 124.09 (C6'), 126.04 (C4''), 126.68 (C2'), 128.48 (C3), 129.01 (C1'), 129.50 (C3'', C5''), 130.93 (C5'), 133.36 (C4'), 133.42 (C3'), 136.53 (C2), 150.40 (C1'), 151.38 (NHCO), 153.68 (C5), 160.12 (C=O); *m/z* (ESI+) 420 (100%, (M + H)⁺), 374 (7, M - EtO); *t*_R = 17.56 min.

General procedures for synthesis of ethyl 3-[3-(substituted)-ureido]-5-(3',4'-dichlorophenyl)furan-2-carboxylate 48 and 49

To a stirred solution of **47** (300 mg, 0.71 mmol) in anhydrous DMSO (10 mL) under a nitrogen atmosphere, the appropriate amine (0.75 mmol) was added dropwise. The reaction mixture was stirred at rt for 2 h, then EtOAc (50 mL) was added. The organic layer was washed with 2 M HCl (2 × 30 mL), 1 M NaOH (2 × 30 mL), brine (30 mL), dried (MgSO₄), and the solvent was removed by vacuum distillation. The obtained residues were purified by flash chromatography (SiO₂, *n*-hexane–EtOAc = 1 : 1).

Ethyl 3-(3-benzylureido)-5-(3',4'-dichlorophenyl)furan-2-carboxylate 48 (283 mg, 92%). White solid; mp 211–213 °C; δ_H (300 MHz, DMSO-d₆) 1.33 (3H, t, *J* = 7.1 Hz, Me), 4.33 (2H, d, *J* = 4.7 Hz, PhCH₂NH), 4.35 (2H, q, *J* = 7.1 Hz, CH₂O), 7.23–7.38 (5H, m, Ph), 7.71 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 7.76 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.87 (1H, s, C4-H), 8.02 (1H, d, *J* = 2.0 Hz, 2'-Ar-H), 8.06 (1H, t, *J* = 5.8 Hz, NHCH₂), 8.66 (1H, br s, NHCO); δ_C (75 MHz, DMSO-d₆) 14.88 (Me), 43.43 (PhCH₂NH), 60.70 (CH₂O), 104.25 (C4), 125.12 (C6'), 126.71 (C2'), 127.36 (C4''), 127.75 (C2'', C6''), 128.58 (C3), 128.83 (C3'', C5''), 129.77 (C1'), 131.81 (C5'), 132.17 (C4'), 132.51 (C3'), 138.53 (C2), 140.12 (C1''), 152.57 (C5), 154.45 (NHCO), 159.57 (C=O); *m/z* (ESI+) 433 (100%, (M + H)⁺), 865 (27, 2M + H), 341 (8, M - C₇H₇), 254 (59, M - C₇H₇, NCO, EtO); *t*_R = 15.99 min.

Ethyl 3-(3-benzyl-3-ethylureido)-5-(3',4'-dichlorophenyl)-furan-2-carboxylate 49 (278 mg, 85%). Reddish liquid; δ_H (500 MHz, CDCl₃) 1.25 (3H, t, *J* = 7.3 Hz, MeCH₂N), 1.36 (3H, t, *J* = 7.3 Hz, MeCH₂O), 3.42 (2H, q, *J* = 7.3 Hz, MeCH₂N), 4.36 (2H, q, *J* = 7.3 Hz, CH₂O), 4.62 (2H, s, PhCH₂N), 7.25–7.36 (5H, m, Ph), 7.48 (1H, d, *J* = 8.0 Hz, 5'-Ar-H), 7.60 (1H, dd, *J* = 8.0, 2.0 Hz, 6'-Ar-H), 7.71 (1H, s, C4-H), 7.89 (1H, d, *J* = 2.0 Hz, 2'-Ar-H), 8.84 (1H, br s, NHCO); δ_C (126 MHz, CDCl₃) 13.16 (MeCH₂N), 14.51 (MeCH₂O),

42.03 (MeCH₂N), 50.02 (PhCH₂N), 60.80 (CH₂O), 102.98 (C4), 124.12 (C6'), 126.73 (C2'), 127.49 (C4''), 127.58 (C2'', C6''), 127.76 (C3), 128.76 (C3'', C5''), 129.44 (C1'), 130.87 (C5'), 133.11 (C4'), 133.28 (C3'), 137.42 (C2), 137.70 (C1''), 152.94 (C5), 154.11 (NHCO), 159.64 (C=O); *m/z* (ESI+) 461 (100%, (M + H)⁺), 921 (8, 2M + H); *t_R* = 17.00 min.

3-Benzyl-6-(3',4'-dichlorophenyl)furo[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione 50. To a stirred solution of **48** (130 mg, 0.3 mmol) in MeOH (10 mL), NaOH (20 mg, 0.5 mmol) in water (10 mL) was added. The reaction mixture was stirred at 70 °C for 3 h. The mixture was concentrated *in vacuo*. The residue was diluted with water (10 mL), and washed with EtOAc (20 mL). The aqueous layer was cooled in ice bath and acidified with KHSO₄ (saturated aqueous solution) to pH 3–4. The precipitated solid was collected by filtration, washed with cold water (20 mL), and *n*-hexane (20 mL).

(93 mg, 80%); white solid; mp 281–283 °C dec; δ_{H} (500 MHz, DMSO-*d*₆) 5.04 (2H, s, CH₂), 7.23–7.32 (6H, m, C7–H, Ph), 7.76 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.88 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 8.20 (1H, d, *J* = 2.0 Hz, 2'Ar-H), 11.87 (1H, br s, NH); δ_{C} (126 MHz, DMSO-*d*₆) 43.13 (CH₂), 98.87 (C7), 125.03 (C6'), 126.89 (C2'), 127.04 (C4''), 127.37 (C2'', C6''), 128.27 (C3'', C5''), 128.81 (C7a), 129.83 (C1'), 131.39 (C5'), 132.12 (C4'), 132.45 (C3'), 137.35 (C1''), 137.79 (C4a), 150.88 (C4), 153.00 (C2), 156.43 (C6); *m/z* (ESI+) 387 (68%, (M + H)⁺), 773 (29, 2M + H), 186 (100); *t_R* = 13.16 min.

General procedures for synthesis of 3-[3-(substituted)ureido]-5-(3',4'-dichlorophenyl)furan-2-carboxylic acid **51** and **52**

To a stirred ice-cooled solution of the appropriate ester **48** or **49** (1.00 mmol), and THT (5 mL) in anhydrous DCM (5 mL), AlCl₃ (1.33 g, 10.0 mmol) was added portionwise. The reaction mixture was stirred at rt for 72 h (TLC monitoring, TLC samples were diluted with MeOH). The reaction mixture was concentrated under vacuum, then cold water (10 mL) was added followed by 1 M HCl to pH 4–5. The mixture was extracted with EtOAc (3 × 25 mL). The combined organic layers were dried (MgSO₄), and the solvent was removed by vacuum distillation. The crude material was purified using preparative RP-HPLC.

3-(3-Benzylureido)-5-(3',4'-dichlorophenyl)furan-2-carboxylic acid 51 (100 mg, 25%). White crystals; mp 195–197 °C dec; δ_{H} (500 MHz, DMSO-*d*₆) 4.31 (2H, d, *J* = 5.7 Hz, CH₂), 7.23–7.37 (5H, m, Ph), 7.71 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.77 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 7.84 (1H, s, C4–H), 8.03 (1H, d, *J* = 2.0 Hz, 2'Ar-H), 8.06 (1H, t, *J* = 5.7 Hz, NHCH₂), 8.65 (1H, br s, NHCO), 13.23 (1H, br s, COOH); δ_{C} (125 MHz, DMSO-*d*₆) 42.92 (CH₂), 103.73 (C4), 124.55 (C6'), 126.11 (C2'), 126.83 (C4''), 127.24 (C2'', C6''), 127.51 (C3), 128.32 (C3'', C5''), 129.51 (C1'), 131.30 (C5'), 131.44 (C4'), 131.99 (C3'), 137.28 (C2), 139.72 (C1''), 151.55 (C5), 154.05 (NHCO), 160.57 (COOH); *m/z* (ESI+) 405 (90%, (M + H)⁺), 809 (14, 2M + H), 313 (93, M – C₇H₇), 254 (100, M – C₇H₇, NH, CO₂); *t_R* = 13.60 min.

3-(3-Benzyl-3-ethylureido)-5-(3',4'-dichlorophenyl)furan-2-carboxylic acid 52 (120 mg, 28%). Pale yellow crystals; mp 160–162 °C dec; δ_{H} (500 MHz, DMSO-*d*₆) 1.15 (3H, t, *J* = 7.2

Hz, Me), 3.38 (2H, q, *J* = 6.9 Hz, MeCH₂N), 4.56 (2H, s, PhCH₂N), 7.25–7.37 (5H, m, Ph), 7.72 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.79 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 7.82 (1H, s, C4–H), 8.06 (1H, d, *J* = 2.0 Hz, 2'Ar-H), 8.79 (1H, br s, NHCO), 13.46 (1H, br s, COOH); δ_{C} (125 MHz, DMSO-*d*₆) 13.11 (Me), 41.82 (MeCH₂N), 49.39 (PhCH₂N), 103.45 (C4), 124.62 (C6'), 126.23 (C2'), 126.92 (C4''), 127.16 (C2'', C6''), 127.94 (C3), 128.50 (C3'', C5''), 129.39 (C1'), 131.31 (C5'), 131.60 (C4'), 132.02 (C3'), 137.95 (C2), 137.99 (C1''), 151.99 (C5), 153.06 (NHCO), 161.20 (COOH); *m/z* (ESI+) 433 (66%, (M + H)⁺), 865 (8, 2M + H), 389 (100, [M + H] – CO₂); *t_R* = 13.64 min.

Ethyl 2-amino-5-(3',4'-dichlorophenyl)furan-3-carboxylate 54. To a stirred ice-cooled solution of **43**²⁷ (4.83 g, 18.0 mmol) in anhydrous DMF (13 mL) under a nitrogen atmosphere, ethyl cyanoacetate (2.05 g, 18.0 mmol), and diethylamine (3.95 g, 54.0 mmol) were added slowly. The reaction mixture was allowed to warm to rt, and stirred for 2 h. The mixture was diluted with DCM (100 mL), washed with 2 M HCl (2 × 50 mL), dried (MgSO₄), and concentrated under vacuum till half of the volume. Trifluoroacetic acid (50 mL) was added in one portion to the solution. The reaction was stirred at rt for 40 h. The solvent was removed by vacuum distillation. The residue was dissolved in DCM (50 mL), cautiously washed with NaHCO₃ (saturated aqueous solution, 50 mL), the organic layer was dried (MgSO₄), and concentrated. The crude material was purified by flash chromatography (SiO₂, *n*-hexane–EtOAc = 6 : 1). (2.15 g, 40%); white solid; mp 110–111 °C; δ_{H} (300 MHz, CDCl₃) 1.36 (3H, t, *J* = 7.1 Hz, Me), 4.29 (2H, q, *J* = 7.1 Hz, CH₂O), 5.62 (2H, br s, NH₂), 6.78 (1H, s, C4–H), 7.29 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 7.38 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.57 (1H, d, *J* = 2.0 Hz, 2'Ar-H); δ_{C} (75 MHz, CDCl₃) 14.54 (Me), 59.84 (CH₂), 91.80 (C3), 106.46 (C4), 121.60 (C6'), 124.09 (C2'), 129.87 (C4'), 130.10 (C1'), 130.61 (C5'), 132.90 (C3'), 141.30 (C5), 161.55 (C2), 164.83 (C=O); *m/z* (ESI+) 300 (100%, (M + H)⁺), 599 (79, 2M + H), 254 (91, M – EtO); *t_R* = 15.98 min.

Ethyl 2-[bis(phenoxycarbonyl)amino]-5-(3',4'-dichlorophenyl)furan-3-carboxylate 55. To a stirred ice-cooled solution of **54** (1.00 g, 3.33 mmol), and pyridine (528 mg, 6.66 mmol) in anhydrous DCM (30 mL), phenyl chloroformate (1.05 g, 6.66 mmol) was added dropwise, and the reaction mixture was stirred at rt for 12 h. The solvent was evaporated under vacuum. The residue was dissolved in EtOAc (60 mL), washed with 1 M HCl (2 × 30 mL), brine (30 mL), dried (MgSO₄), and the solvent was removed by vacuum distillation. The crude material was triturated with *n*-hexane (30 mL), collected by filtration and dried. (1.48 g, 82%); white solid; mp 166–168 °C; δ_{H} (300 MHz, CDCl₃) 1.29 (3H, t, *J* = 7.1 Hz, Me), 4.32 (2H, q, *J* = 7.1 Hz, CH₂O), 7.02 (1H, s, C4–H), 7.08–7.34 (10H, m, 2 Ph), 7.42 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.46 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 7.72 (1H, d, *J* = 2.0 Hz, 2'Ar-H); δ_{C} (75 MHz, CDCl₃) 14.31 (Me), 61.37 (CH₂), 107.34 (C4), 114.59 (C3), 121.10 (C2'', C6'', C2''', C6'''), 123.24 (C6'), 125.91 (C2'), 126.59 (C4'', C4'''), 128.97 (C4'), 129.57 (C3'', C5'', C3''', C5'''), 130.98 (C5'), 132.67 (C1'), 133.40 (C3'), 145.63 (C5), 149.04 (N(C=O)₂), 149.64 (C2), 150.24 (C1'', C1'''), 161.41 (C=O); *m/z* (ESI+) 540 (100%, (M + H)⁺), 494 (13, M – EtO); *t_R* = 16.97 min.

General procedures for synthesis of ethyl 2-[3-(substituted)-ureido]-5-(3',4'-dichlorophenyl)furan-3-carboxylate **57** and **58**

To a stirred solution of **55** (300 mg, 0.55 mmol) in anhydrous DMSO (10 mL) under a nitrogen atmosphere, the appropriate amine (2.20 mmol) was added dropwise. The reaction mixture was stirred at rt for 2 h, then EtOAc (50 mL) was added. The organic layer was washed with 2 M HCl (2 × 30 mL), 1 M NaOH (2 × 30 mL), brine (30 mL), dried (MgSO₄), and the solvent was removed by vacuum distillation. The product was purified from the *sym*-urea side product using flash chromatography (SiO₂, EtOAc–THF = 4 : 1).

Ethyl 2-(3-benzylureido)-5-(3',4'-dichlorophenyl)furan-3-carboxylate **57 (203 mg, 85%).** White solid; mp 223–225 °C dec; δ_{H} (500 MHz, DMSO-*d*₆) 1.28 (3H, t, *J* = 7.0 Hz, Me), 4.25 (2H, q, *J* = 7.0 Hz, CH₂O), 4.33 (2H, d, *J* = 5.8 Hz, PhCH₂NH), 7.26–7.38 (6H, m, C4–H, Ph), 7.63 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 7.66 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.89 (2H, m, 2'Ar-H, NHCH₂), 9.27 (1H, br s, NHCO); δ_{C} (125 MHz, DMSO-*d*₆) 14.28 (Me), 43.00 (PhCH₂NH), 59.95 (CH₂O), 99.11 (C3), 107.18 (C4), 122.86 (C6'), 124.32 (C2'), 126.93 (C4''), 127.27 (C2'', C6''), 128.37 (C3'', C5''), 129.29 (C4'), 129.96 (C1'), 131.04 (C5'), 131.78 (C3'), 139.39 (C1''), 143.37 (C5), 152.04 (NHCO), 153.33 (C2), 162.92 (C=O); *m/z* (ESI+) 433 (100%, (M + H)⁺), 865 (60, 2M + H), 341 (15, M – C₇H₇), 254 (30, M – C₇H₇, NCO, EtO); *t*_R = 15.31 min.

Ethyl 2-(3-benzyl-3-ethylureido)-5-(3',4'-dichlorophenyl)furan-3-carboxylate **58 (208 mg, 82%).** Reddish liquid; δ_{H} (500 MHz, CDCl₃) 1.26 (3H, t, *J* = 7.0 Hz, MeCH₂N), 1.33 (3H, t, *J* = 7.0 Hz, MeCH₂O), 3.41 (2H, q, *J* = 7.0 Hz, MeCH₂N), 4.27 (2H, q, *J* = 7.0 Hz, CH₂O), 4.64 (2H, s, PhCH₂N), 6.82 (1H, s, C4–H), 7.28–7.37 (5H, m, Ph), 7.40 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.49 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 7.72 (1H, d, *J* = 2.0 Hz, 2'Ar-H), 9.34 (1H, br s, NHCO); δ_{C} (126 MHz, CDCl₃) 13.15 (MeCH₂N), 14.39 (MeCH₂O), 42.24 (MeCH₂N), 50.11 (PhCH₂N), 60.56 (CH₂O), 97.53 (C3), 104.74 (C4), 122.60 (C6'), 124.92 (C2'), 127.29 (C4''), 127.56 (C2'', C6''), 128.65 (C3'', C5''), 129.71 (C4'), 130.68 (C5'), 130.89 (C1'), 133.01 (C3'), 136.96 (C1''), 144.79 (C5), 151.77 (NHCO), 155.08 (C2), 165.16 (C=O); *m/z* (ESI+) 461 (100%, (M + H)⁺), 921 (25, 2M + H); *t*_R = 16.94 min.

Synthesis of 2-[3-(substituted)ureido]-5-(3',4'-dichlorophenyl)furan-3-carboxylic acid **59** and **60** were performed as described for preparation of **51** and **52**.

2-(3-Benzylureido)-5-(3',4'-dichlorophenyl)furan-3-carboxylic acid **59 (73 mg, 18%).** White crystals; mp 200–202 °C dec; δ_{H} (500 MHz, DMSO-*d*₆) 4.32 (2H, d, *J* = 6.0 Hz, CH₂), 7.25–7.38 (6H, m, C4–H, Ph), 7.61 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 7.66 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.86 (1H, d, *J* = 2.0 Hz, 2'Ar-H), 7.95 (1H, t, *J* = 5.7 Hz, NHCH₂), 9.29 (1H, br s, NHCO), 12.68 (1H, br s, COOH); δ_{C} (125 MHz, DMSO-*d*₆) 42.95 (CH₂), 99.52 (C3), 107.73 (C4), 122.74 (C6'), 124.10 (C2'), 126.91 (C4''), 127.25 (C2'', C6''), 128.36 (C3'', C5''), 128.97 (C4'), 130.15 (C1'), 131.10 (C5'), 131.72 (C3'), 139.42 (C1''), 142.85 (C5), 151.98 (NHCO), 153.27 (C2), 164.63 (COOH); *m/z* (ESI+) 405 (100%, (M + H)⁺), 809 (54, 2M + H), 313 (71, M – C₇H₇), 295 (24, M – C₇H₇, H₂O), 254 (16, M – C₇H₇, NH, CO₂); *t*_R = 12.54 min.

2-(3-Benzyl-3-ethylureido)-5-(3',4'-dichlorophenyl)furan-3-carboxylic acid **60 (95 mg, 22%).** White crystals; mp 175–177 °C

dec; δ_{H} (500 MHz, DMSO-*d*₆) 1.11 (3H, t, *J* = 7.2 Hz, Me), 3.34 (2H, q, *J* = 7.2 Hz, MeCH₂N), 4.56 (2H, s, PhCH₂N), 7.26–7.38 (6H, m, C4–H, Ph), 7.65 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 7.67 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.91 (1H, d, *J* = 2.0 Hz, 2'Ar-H), 9.25 (1H, br s, NHCO), 12.77 (1H, br s, COOH); δ_{C} (125 MHz, DMSO-*d*₆) 13.10 (Me), 41.35 (MeCH₂N), 49.11 (PhCH₂N), 104.55 (C3), 107.98 (C4), 123.00 (C6'), 124.45 (C2'), 127.09 (C4''), 127.29 (C2'', C6''), 128.44 (C3'', C5''), 129.47 (C4'), 130.00 (C1'), 131.16 (C5'), 131.81 (C3'), 138.04 (C1''), 144.24 (C5), 152.81 (NHCO), 152.87 (C2), 164.54 (COOH); *m/z* (ESI+) 433 (100%, (M + H)⁺), 865 (16, 2M + H), 254 (5, M – C₇H₇, EtN, CO₂); *t*_R = 13.71 min.

Methyl 3,4-dichlorobenzenecarbodithioate **62.** To a stirred mixture of sulfur (3.52 g, 110 mmol), and NEt₃ (15.2 g, 150 mmol) in DMF (25 mL), 3,4-dichlorobenzyl chloride **61** (9.77 g, 50.0 mmol) was added dropwise. The reaction mixture was stirred at 60 °C for 6 h then cooled in an ice bath. Iodomethane (7.81 g, 55.0 mmol) was added slowly maintaining the temperature below 10 °C. The reaction was further stirred for 1 h then filtered. The filtrate was poured into stirred ice-cooled water (100 mL). The precipitated bright red crystals were collected by filtration, washed with water, and dried. (10.4 g, 88%); red solid; mp 60–62 °C; δ_{H} (500 MHz, CDCl₃) 2.78 (3H, s, SMe), 7.46 (1H, d, *J* = 8.0 Hz, C5–H), 7.83 (1H, dd, *J* = 8.0, 2.0 Hz, C6–H), 8.10 (1H, d, *J* = 2.0 Hz, C2–H); δ_{C} (126 MHz, CDCl₃) 20.77 (Me), 125.68 (C6), 128.58 (C2), 130.22 (C5), 132.83 (C4), 136.62 (C3), 144.03 (C1), 225.21 (C=S); *m/z* (ESI+) 236 (22%, M⁺), 187 (100, M – CH₂, Cl); *t*_R = 16.53 min.

Methyl 4-amino-2-(3',4'-dichlorophenyl)-1,3-thiazole-5-carboxylate **64.** To a stirred ice-cooled solution of sodium (0.81 g, 35.0 mmol), and cyanamide (1.27 g, 30.0 mmol) in anhydrous MeOH (50 mL) under a nitrogen atmosphere, compound **62** (7.11 g, 30 mmol) was added portionwise, and the reaction mixture was stirred at 75 °C for 3 h. The solvent was removed under vacuum, and the residue was triturated with ether, and filtered. The intermediate **63** was dissolved in MeOH (50 mL) and methyl bromoacetate (6.88 g, 45.0 mmol) was added dropwise. The reaction mixture was stirred at rt for 2 h then NEt₃ (12.5 mL, 90.0 mmol) was added, and the reaction was further stirred for 12 h. The solvent was removed by vacuum distillation, and the crude material was purified by flash chromatography (SiO₂, *n*-hexane–EtOAc = 3 : 1). (3.18 g, 35%); yellow solid; mp 156–158 °C; δ_{H} (500 MHz, CDCl₃) 3.83 (3H, s, OMe), 5.88 (2H, br s, NH₂), 7.48 (1H, d, *J* = 8.0 Hz, 5'Ar-H), 7.69 (1H, dd, *J* = 8.0, 2.0 Hz, 6'Ar-H), 8.00 (1H, d, *J* = 2.0 Hz, 2'Ar-H); δ_{C} (126 MHz, CDCl₃) 51.70 (Me), 94.46 (C5), 125.56 (C6'), 128.22 (C2'), 130.98 (C5'), 132.68 (C4'), 133.50 (C3'), 135.32 (C1'), 163.12 (C4), 164.33 (C=O), 167.46 (C2); *m/z* (ESI+) 303 (17%, (M + H)⁺), 344 (100, M + H, MeCN), 271 (6, M – MeO); *t*_R = 14.39 min.

4-Amino-2-(3',4'-dichlorophenyl)-1,3-thiazole-5-carboxylic acid **65.** To a stirred solution of **64** (3.03 g, 10.0 mmol) in MeOH (30 mL), KOH (2.24 g, 40.0 mmol) in water (30 mL) was added. The reaction mixture was stirred at reflux for 2 h then the MeOH was evaporated by vacuum distillation. The residue was diluted with water (10 mL), and washed with EtOAc (20 mL). The aqueous layer was cooled in an ice bath and acidified by KHSO₄ (saturated aqueous solution) to pH 3–4. The precipitate was collected by filtration, washed with cold water (20 mL), *n*-hexane (20 mL), and

dried over CaCl_2 in amber glass vacuum desiccator. (1.91 g, 66%); yellow solid; mp 131–133 °C dec; δ_{H} (500 MHz, DMSO-d_6) 6.98 (2H, br s, NH_2), 7.77 (1H, d, $J = 8.0$ Hz, 5'-Ar-H), 7.87 (1H, dd, $J = 8.0, 2.0$ Hz, 6'-Ar-H), 8.09 (1H, d, $J = 2.0$ Hz, 2'-Ar-H), 12.92 (1H, br s, COOH); δ_{C} (126 MHz, DMSO-d_6) 93.37 (C5), 126.27 (C6'), 127.48 (C2'), 131.53 (C5'), 132.09 (C4'), 132.76 (C3'), 133.65 (C1'), 163.25 (C4), 164.65 (C=O), 165.42 (C2); m/z (ESI+) 289 (20%, $(\text{M} + \text{H})^+$), 330 (100, $\text{M} + \text{H}$, MeCN), 271 (11, $\text{M} - \text{OH}$); $t_{\text{R}} = 12.12$ min.

2-(3',4'-Dichlorophenyl)-4H-[1,3]thiazolo[4,5-d][1,3]oxazine-5,7-dione 66. The compound was prepared as described for preparation of **15a** and **15b**. (1.32 g, 70%); yellow solid; mp 219–221 °C dec; δ_{H} (500 MHz, DMSO-d_6) 7.77 (1H, d, $J = 8.0$ Hz, 5'-Ar-H), 8.15 (1H, dd, $J = 8.0, 2.0$ Hz, 6'-Ar-H), 8.37 (1H, d, $J = 2.0$ Hz, 2'-Ar-H), 11.11 (1H, br s, NH); δ_{C} (126 MHz, DMSO-d_6) 95.81 (C7a), 128.16 (C6'), 129.87 (C2'), 130.72 (C5'), 131.21 (C4'), 131.99 (C3'), 133.07 (C1'), 153.29 (C5), 153.56 (C3a), 164.45 (C7), 165.28 (C2); m/z (ESI+) 314 (100%, M^+), 355 (94, $\text{M} + \text{MeCN}$), 629 (18, $2\text{M} + \text{H}$); $t_{\text{R}} = 11.75$ min.

Synthesis of 2-(3',4'-dichlorophenyl)-4-[3-(substituted)ureido]-1,3-thiazole-5-carboxylic acids **67** and **68** were performed as described for preparation of **16–21**.

4-(3-Benzylureido)-2-(3',4'-dichlorophenyl)-1,3-thiazole-5-carboxylic acid 67 (65 mg, 24%). Yellow crystals; mp 213–215 °C; δ_{H} (500 MHz, DMSO-d_6) 4.48 (2H, d, $J = 5.4$ Hz, CH_2), 7.27–7.40 (5H, m, Ph), 7.75 (1H, d, $J = 8.0$ Hz, 5'-Ar-H), 7.92 (1H, dd, $J = 8.0, 2.0$ Hz, 6'-Ar-H), 8.18 (1H, d, $J = 2.0$ Hz, 2'-Ar-H), 8.53 (1H, t, $J = 5.7$ Hz, NHCH_2), 9.04 (1H, br s, NHCO), 13.80 (1H, br s, COOH); δ_{C} (125 MHz, DMSO-d_6) 43.38 (CH_2), 102.27 (C5), 126.64 (C6'), 126.99 (C4''), 127.13 (C2'', C6''), 127.93 (C2'), 128.48 (C3'', C5''), 131.45 (C5'), 131.79 (C4'), 132.30 (C3'), 134.40 (C1'), 139.30 (C1''), 152.25 (NHCO), 154.44 (C4), 164.02 (COOH), 165.77 (C2); m/z (ESI+) 422 (100%, $(\text{M} + \text{H})^+$), 843 (11, $2\text{M} + \text{H}$), 330 (23, $\text{M} - \text{C}_7\text{H}_7$), 271 (24, $\text{M} - \text{C}_7\text{H}_7$, NH, CO_2); $t_{\text{R}} = 14.98$ min.

4-(3-Benzyl-3-ethylureido)-2-(3',4'-dichlorophenyl)-1,3-thiazole-5-carboxylic acid 68 (63 mg, 22%). Yellow crystals; mp 191–193 °C; δ_{H} (500 MHz, DMSO-d_6) 1.14 (3H, t, $J = 6.9$ Hz, Me), 3.37 (2H, q, $J = 6.9$ Hz, MeCH_2N), 4.58 (2H, s, PhCH_2N), 7.26–7.38 (5H, m, Ph), 7.81 (1H, d, $J = 8.0$ Hz, 5'-Ar-H), 7.95 (1H, dd, $J = 8.0, 2.0$ Hz, 6'-Ar-H), 8.18 (1H, d, $J = 2.0$ Hz, 2'-Ar-H), 9.64 (1H, br s, NHCO), 13.57 (1H, br s, COOH); δ_{C} (125 MHz, DMSO-d_6) 13.16 (Me), 41.44 (MeCH_2N), 49.12 (PhCH_2N), 102.30 (C5), 126.41 (C6'), 127.05 (C4''), 127.27 (C2'', C6''), 127.61 (C2'), 128.43 (C3'', C5''), 131.59 (C5'), 132.15 (C4'), 132.74 (C3'), 133.76 (C1'), 138.24 (C1''), 152.83 (NHCO), 154.86 (C4), 163.98 (COOH), 164.25 (C2); m/z (ESI+) 450 (100%, $(\text{M} + \text{H})^+$), 899 (4, $2\text{M} + \text{H}$), 271 (4, $\text{M} - \text{C}_7\text{H}_7$, EtN, CO_2); $t_{\text{R}} = 13.14$ min.

Biology

Transcription assay. The assay was performed as described previously³⁶ with slight modifications. *E. coli* RNA polymerase holoenzyme was purchased from Epicentre Biotechnologies (Madison, WI). Final concentrations in a total volume of 30 μL were one unit of RNA polymerase (0.5 μg) which was used along with 60 nCi of $[5,6\text{-}^3\text{H}]\text{-UTP}$, 400 μM of ATP, CTP and GTP as well as 100 μM of UTP, 20 units of RNase inhibitor (RiboLock,

Fermentas), 10 mM DTT, 40 mM tris-HCl (pH 7.5), 150 mM KCl, 10 mM MgCl_2 and 0.1% CHAPS. As a DNA template 3500 ng of religated pcDNA3.1/V5-His-TOPO were used per reaction. Prior to starting the experiment, the compounds were dissolved in DMSO (final concentration during experiment: 2%). Dilution series of the compounds were prepared using a liquid handling system (Janus, PerkinElmer, Waltham, MA). The components described above (including the compounds) were preincubated in absence of NTPs and DNA for 10 min at 25 °C. Transcription reaction was started by the addition of a mixture containing DNA template and NTPs and incubated for 10 min at 37 °C. The reaction was stopped by the addition of 10% TCA, followed by a transfer of this mixture to a 96-well Multiscreen GFB plate (Millipore, Billerica, MA) and incubation for 45 min at 4 °C. The plate underwent several centrifugation and washing steps with 10% TCA and 95% EtOH to remove residual unincorporated ^3H -UTP. After that the plate was dried for 30 min at 50 °C and 30 μL of scintillation fluid (Optiphase Supermix, PerkinElmer) was added to each well. After 10 min the wells were assayed for presence of ^3H -RNA by counting using Wallac MicroBeta TriLux system (Perkin Elmer). To obtain inhibition values for each sample, their counts were related to DMSO control.

Determination of IC_{50} values. Three different concentrations of the compound were chosen (two samples for each concentration) in the linear range of the log dose response curve (20–80% inhibition) including concentrations above and below the IC_{50} value. Values of percent inhibition were plotted *versus* the inhibitor molar concentrations on a semi-log plot. IC_{50} values were calculated as the molar concentration causing 50% inhibition of RNAP activity. At least three independent determinations were performed for each compound (standard deviation < 20%).

Minimal inhibitory concentration determinations. MIC values were determined in 96-well plates (Sarstedt, Nümbrecht, Germany) against *Staphylococcus aureus subsp. aureus* (Newman strain), *Bacillus subtilis subsp. subtilis*, *Pseudomonas aeruginosa* PAO1, *E. coli* K12, *E. coli* TolC, and the Rif-resistant *E. coli* TolC mutants: *E. coli* TolC β Q513L and *E. coli* TolC β H526Y. As bacteria start OD_{600} 0.03 was used in a total volume of 200 μL in lysogeny broth (LB) medium containing the compounds dissolved in DMSO (maximal DMSO concentration in the experiment: 1%). Final compound concentrations (in duplicates) were prepared by serial dilution ranging from 0.02–100 $\mu\text{g mL}^{-1}$ depending on their antibacterial activity and solubility in growth medium. The ODs were measured using a POLARstar Omega (BMG labtech, Offenburg, Germany) after inoculation and after incubation for 18 h at 37 °C with 50 rpm (200 rpm for *P. aeruginosa* PAO1). Given MIC values are means of two independent determinations (three if $\text{MIC} < 10 \mu\text{g mL}^{-1}$) and defined as the lowest concentration of compound that reduced OD_{600} by $\geq 95\%$.

Selection of Rif-resistant *E. coli* TolC spontaneous mutants. An *E. coli* TolC culture with an OD_{600} 0.70 in LB was subcultured to fresh medium containing 3-fold the MIC of Rif every 24 h with a dilution factor of 1 : 5. The cultures were incubated at 37 °C for 24 h with shaking. After 4 cycles the bacteria were transferred on LB agar plates containing 3-fold the MIC of Rif. The plates were incubated at 37 °C for 24 h. Single colonies were picked and transferred to liquid culture in the presence of 3-fold

the MIC of Rif. Rif-resistant mutants were characterized by sequencing of RNAP rpoB.

MIC determinations in presence of polymyxin B nonapeptide (PMBN) or phenyl-arginine- β -naphthylamide (PA β N). The same procedures followed as mentioned above with minor modifications: before inoculation, bacteria were cultured in LB medium containing PMBN (1 $\mu\text{g mL}^{-1}$) or PA β N (20 $\mu\text{g mL}^{-1}$) (10 $\mu\text{g mL}^{-1}$ in case of *E. coli* TolC) for 2 h and subsequently diluted with the same medium to OD₆₀₀ 0.06. Inocula of 100 μL were added to the wells containing 100 μL of the specific concentrations of the compounds in PMBN/PA β N containing medium. MIC₅₀ values were determined for *E. coli* K12, and *E. coli* D22.

Determination of resistance rate. Procedures were performed according to a described method¹² with modifications. Defined numbers of *E. coli* TolC cells (10^4 – 10^{12}) were incubated in LB in presence of the $2 \times$ MIC of Rif, Myx B or compound **30** in parallel (16 h, 37 °C, 50 rpm, 0.5% DMSO). On each of the three following days, a fraction of each sample was supplemented with fresh compound containing LB followed by recultivation (conditions as before). The final cultures were plated on LB agar to determine the bacterial start concentration which was needed to yield at least one colony on the plates. This threshold was determined to be the resistance rate.

Cytotoxicity. HEK 293 cells, a Human Embryonic Kidney 293 cell line, (2×10^5 cells per well) were seeded in 24-well, flat-bottomed plates. Culturing of cells, incubations and OD measurements were performed as described previously⁵⁷ with slight modifications. 24 h after seeding the cells, the incubation was started by the addition of compounds in a final DMSO concentration of 1%. The living cell mass was determined after 24, 48 and 72 h followed by the calculation of LD₅₀ values.

Computational chemistry

All computational work was performed using Molecular Operating Environment (MOE) version 2010.10, Chemical Computing Group Inc., 1010 Sherbrooke St. West, Suite 910, Montreal, Quebec, H3A 2R7, Canada.

Similarity analysis. A database containing the compounds **11**, **21**, **30**, and **42** was created and 2D fingerprint GpiDAPH3 (graph pi-donor-acceptor-polar-hydrophobe-3 point pharmacophore) was calculated for all entries. Compound **11** was selected as reference structure and sent to MOE window. In the database viewer window, similarity search was performed by setting the fingerprint system to GpiDAPH3, and using the similarity metric Tanimoto coefficient (T_C) to measure similarity between molecules. T_C values range from 0 (no similarity) to 1 (complete similarity).

Flexible alignment. Four ligands (**10**, **20**, **29**, and **41** representing classes I–IV respectively) were sketched using molecular builder of MOE, and each structure was subjected to energy minimization up to a gradient 0.05 kcal mol^{−1} Å using the MMFF94x force field. The compounds were aligned using the flexible alignment mode of MOE with stochastic conformational search option was turned on, and configuration limit was set to 200 and iteration limit was set to 1000. Alignment had the best similarity score was retained and refined by MOE.

Preparation of protein structure for docking. X-ray crystal structure of the *T. Thermophilus* RNA polymerase holoenzyme in complex with dMyx B (Protein Data Bank (PDB) code 3EQL)⁷ was used to perform the molecular docking study. In the sequence editor panel of MOE, chains C and D (corresponding to β and β' subunits respectively) were selected, and all other chains were deleted. Hydrogen atoms were added to the receptor atoms, and the potential of protein was fixed.

Ligand-receptor docking. The binding site was set to dummy atoms which were identified by the site finder mode, and the amino acid residues were chosen where dMyx B binds in the switch region. Docking placement was triangle matcher with rotate bonds option was turned on, the 1st rescoring was ASE with force field refinement, and the 2nd rescoring was alpha HB.

Calculation of angle. Each structure was loaded from a previously prepared database of the target compounds into the MOE window, then it was subjected to energy minimization up to gradient 0.05 kcal mol^{−1} Å using the MMFF94x force field. Angle between the aryl group and the ureido side chain was determined by activating the measure button and choosing angles option, then selecting carbon atom no. 1 of the aryl group, the corresponding carbon atom on the heterocyclic ring, and nitrogen atom no. 1 of the ureido group respectively.

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