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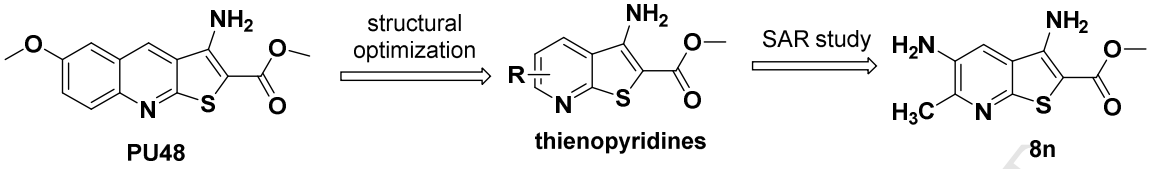
Graphical abstract



PU48

IC_{50} on UT-$B_{(rat)}$: 0.22 μM
QPlogS: -3.001

thienopyridines

8n

IC_{50} on UT-$B_{(rat)}$: 0.54 μM
QPlogS: -1.753
(10 fold incresed in water solubility)

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Discovery and optimization of thienopyridine derivatives as novel urea transporter inhibitors

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Running title: Thienopyridine urea transporter inhibitors

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Abstract:

Urea transporters (UTs) play an important role in the urine concentrating mechanism and are recognized as novel targets for developing small molecule inhibitors with salt-sparing diuretic activity. Thienoquinoline derivatives, a class of novel UT-B inhibitors identified by our group, play a significant diuresis in animal model. However, the poor solubility and low bioavailability limited its further development. To overcome these shortcomings, the structure modification of thienoquinoline was carried out in this study, which led to the discovery of novel thienopyridine derivatives as specific urea transporter inhibitors. Further optimization obtained the promising preclinical candidate **8n** with not only excellent inhibition effect on urea transporters and diuretic activity on rat model, but also suitable water solubility and Log P value.

Key words:

Urea transporter inhibitor; Thienoquinoline; Thienopyridine; Diuretics; Structure-activity relationship

1. Introduction

Diuretics are mainly used to treat diseases such as edema, hypertension and heart failure and are often used for long-term therapy[1]. At present, the commonly used diuretics in clinical include loop diuretics (e.g. furosemide), thiazides (e.g. hydrochlorothiazide), aldosterone antagonists (e.g. spironolactone) and osmotic dehydrating agents (e.g. mannitol)[2]. All of these drugs induce a diuresis by mechanism in which water excretion following salt excretion, which may result in several adverse effects, involving hypokalemia, hyponatremia, hyperuricemia, hyperlipidemia and a decrease in glucose tolerance, while long-term using[3-7]. Moreover, electrolyte imbalance may increase risks of arrhythmia and sudden death[8,9]. Therefore, it would be desirable to find novel diuretics that do not cause electrolyte disturbances.

Except for electrolytes, urea is also a major solute in hyperosmolar renal medulla and plays a critical role in urinary concentrating mechanism and in maintenance of water balance[10]. The intrarenal urea recycling is facilitated by transmembrane urea transporters (UTs) expressed in renal tubule epithelial cells (UT-A isoforms) and renal vasa recta microvessels (UT-B isoform)[11-13]. Study on knock-out mice lacking of UT-B[14] or various UT-A isoforms[15-17] showed that UT functional deletion led to urea-selective urinary concentrating defects associated with urinary hypoosmolality. Meanwhile, the glomerular filtration rate and clearance rate of the principal solutes (Na^+, K^+, Cl^-), except for urine, were not changed[18,19]. These evidences suggested that UT inhibitors would be developed as novel diuretics with a mechanism different from currently used salt-excreting diuretics, therefore would not disturb the balance of electrolyte metabolism *in vivo* as they did and would be more suitable for long-term therapy [20].

Several classes of UT inhibitors, including phenyl-sulfoxyoxozoles(**a**, Fig. 1)[21], benzenesulfon-anilides (**b**, Fig. 1)[21], phthalazinamines (**c**, Fig. 1)[21,22], aminobenzimidazoles (**d**, Fig. 1)[21], thiazolothienopyrimidines (**e**, Fig. 1)[23-26], hydroxyquinolines (**f**, Fig. 1)[25], aminothiazolones (**g**, Fig. 1)[25], thioureas (**h**, Fig. 1)[27,28], fluorenones (**i**, Fig. 1)[29] and so on, were identified by Verkman's group over the past decade via high-throughput screening of small-molecule libraries or preliminary structural modification of the hits. Although some of the molecules mentioned above exhibited potent inhibitory effect to UTs with IC_{50} values at micromolar

or submicromolar level *in vitro*, they were shown to have very low or no diuretic effect in experimental animals, which obstructed their further development.

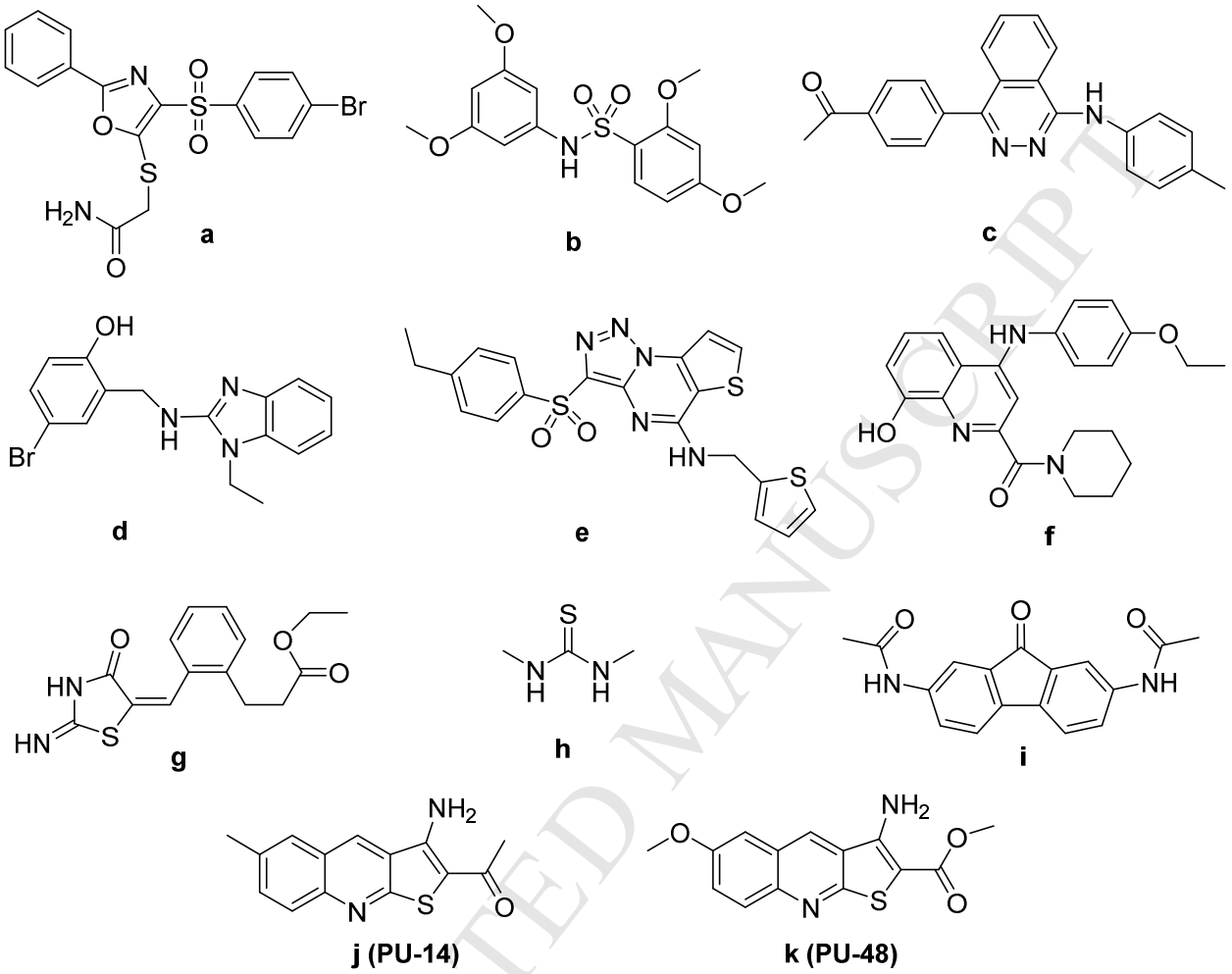


Fig. 1. Chemical structures of several known UT inhibitors.

In our previous work, we identified a thienoquinoline UT inhibitor named PU-14 (**j**, Fig. 1) [30] by screening a commercially available collection of drug-like compounds with erythrocyte lysis model. **PU-14**, containing a new thienoquinoline scaffold different from those reported by Verkman's group, exhibited an excellent inhibitory effect on both rat and human UT-B *in vitro* with IC_{50} value of 3.51 and 1.72 μmol/L, respectively. Based on these results, we conducted an extensive screen of thienoquinolin analogs and got a more superior small-molecular UT inhibitor **PU-48** (**k**, Fig. 1) [31], which was over 10 times more potent than **PU-14**. **PU-48** significantly increased urine output and decreased urine osmolality in an *in vivo* rat model without affecting electrolyte metabolism and notable toxicity. Moreover, the poor solubility and low bioavailability limited further development of

PU-48. To overcome these shortcomings, the systematical structure modification of **PU-48** was firstly carried out in the present study, which led to the discovery of novel thienopyridine derivatives as UT-B inhibitor. Further optimization obtained the promising preclinical candidate **8n** with not only excellent inhibition effect on UT-B and diuretic activity on rat model, but also suitable water solubility and Log P value (Fig. 2).

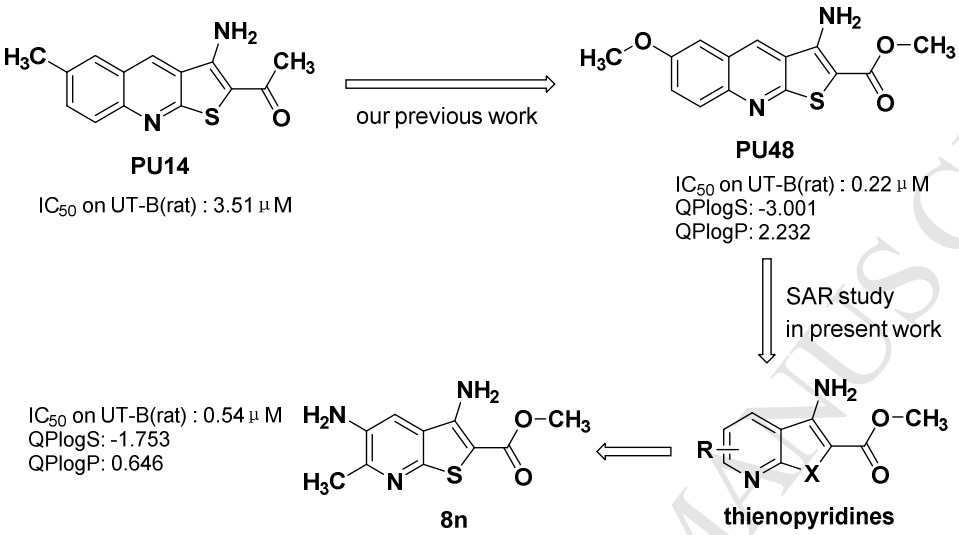


Fig.2. Overview of present study.

2. Results and discussions

Optimization of PU48

In our previous study, the modification of amino group in **PU14** had already been investigated. All the changes including removing amino group, replacing it by hydroxyl group, alkylating or acetylating it led to a dramatically decrease or loss of the urea transporter UT-B inhibition activity. So in present study, the further optimization of **PU-48** was mainly focused on two aspects, ester group at 2-position of the thiophene ring (R_1) and substituents at benzene ring (R_2) (Fig. 3).

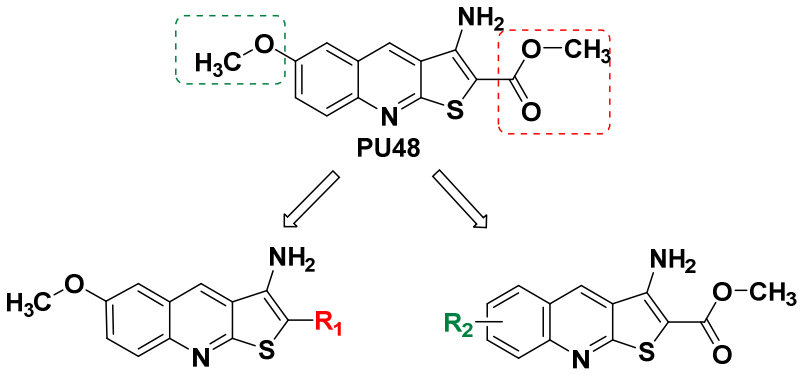
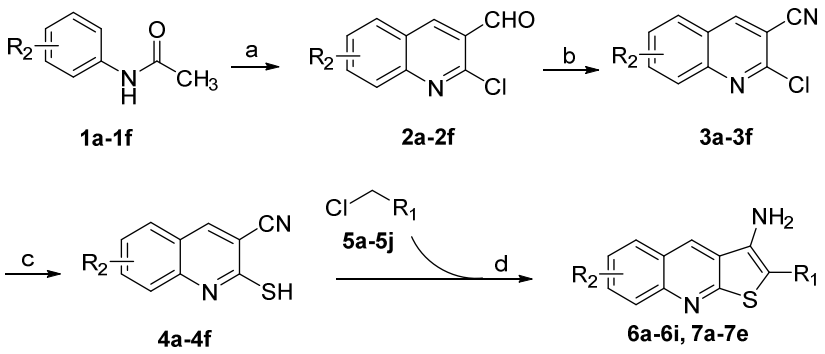


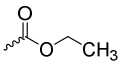
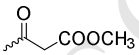
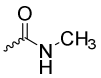
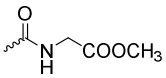
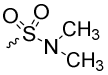
Fig. 3. The structural modification strategy of lead **PU-48**.

For the modification of R_1, ethyl ester group (**6a**) was firstly selected to replace methyl ester of **PU-48** to investigate the effect of chain length on the UT-B inhibition activity. Secondly, as ester group usually easily hydrolyze *in vivo*, it was modified with its bioisosteres such as amide groups (**6b-6e**), sulfonamides (**6f**) and acetyl groups (**6g, 6h**) to improve the stability *in vivo* and *in vitro*. Finally, since carboxyl acid was thought to be the metabolite of ester *in vivo*, the possible metabolites **6i** was designed to exam whether this metabolite could inhibit UT-B or not.

For the optimization of R_2, methyloxy group of **PU-48** was removed (**7a**), replaced by electro-donating (**7b**) or electro-withdrawing (**7c, 7d**) groups to test the influence of different substituents on the UT-B inhibition activity. Furthermore, multi-substituted derivative **(7e)** was also designed to examine the steric effect on activity.

As outlined in Scheme 1, the preparation of targeted compounds **6** and **7** referred to the synthesis of **PU-48** in our previous work [32]. A variety of substituted acetanilide (**1a-1f**) was treated with DMF and phosphorus oxychloride at 75 ℃ to obtain **2a-2f**, which was condensed with hydroxylamine to afford oxime and subsquently treated with thionyl chloride to yield the corresponding 3-cyano-quinolone derivatives **3a-3f**. Without further purification, sulfhydrylation of **3a-3f** with thiourea afforded the 2-mercapto-3-cyanoquinoline derivatives **4a-4f**. Then, 2-mercapto-3-cyano-6-methoxyquinoline (**4f**) was reacted with corresponding materials containing chloroacetyl or chloromethanesulfonyl (**5a-5i**) under basic conditions in methanol to generate the target compounds **6a-6i**. Similarly, **4a-4e** was reacted with methyl chloroacetate (**5j**) under the same conditions to afford **7a-7e**.



Compd.	R_1	R_2	Compd.	R_1	R_2
6a		6-OCH_3	**6h**		6-OCH_3
6b		6-OCH_3	**6i**		6-OCH_3
6c		6-OCH_3	**7a**		H
6d		6-OCH_3	**7b**		6-CH_3
6e		6-OCH_3	**7c**		6-Br
6f		6-OCH_3	**7d**		6-F
6g		6-OCH_3	**7e**		6-CH_3,8-CH_3

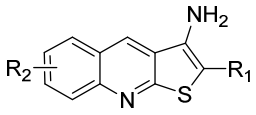
Scheme 1. Synthesis of compounds **6a-6j** and **7a-7e**. Reagents and conditions: (a) DMF, $POCl_3$, 75 ℃; (b) $NH_2OH{\cdot}HCl$, H_2O, THF, r.t.; $SOCl_2$, $CHCl_3$, reflux; (c) NH_2CSNH_2, EtOH, reflux; (d) Et_3N or MeONa, MeOH, reflux.

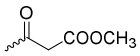
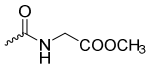
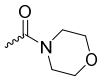
The UT-B inhibition activity of target compounds **6a-6i** and **7a-7e** was evaluated with an erythrocyte lysis assay established with rat erythrocyte as previously reported [31]. The lead compound **PU-48** was used as the positive control. The results are shown in Table 1.

Unfortunately, almost all the modification of 2-ester group in **PU48** resulted in the loss of

activity, even a small change in methyl ester to ethyl ester (**6a**). Replacement of R_1 with acetyl group (**6g**) caused a 5-fold decrease in efficiency compared to **PU-48**. These results implicated that methoxycarbonyl at this position was necessary for the inhibition effect on UT-B.

Table 1. Inhibition potency of compounds **6a-6i** and **7a-7e** to rat UT-B.



Compd.	R_1	R_2	IC_{50} (μM)	Compd.	R_1	R_2	IC_{50} (μM)
PU48	$COOCH_3$	6-OCH_3	0.22	**6h**		6-OCH_3	>10
6a	$COOC_2H_5$	6-OCH_3	>10	**6i**	COOH	6-OCH_3	>10
6b	$CONH_2$	6-OCH_3	>10	**7a**	$COOCH_3$	H	0.22
6c	$CONHCH_3$	6-OCH_3	>10	**7b**	$COOCH_3$	6-CH_3	0.23
6d		6-OCH_3	>10	**7c**	$COOCH_3$	6-Br	0.27
6e		6-OCH_3	>10	**7d**	$COOCH_3$	6-F	0.16
6f	$SO_2N(CH_3)_2$	6-OCH_3	>10	**7e**	$COOCH_3$	6-CH_3,8-CH_3	0.61
6g	$COCH_3$	6-OCH_3	1.63				

Results of optimization on R_2 indicated that no-substituted (**7a**) and methyl-substituted (**7b**) at the 6-position maintained the activity, bromine-substituted (**7c**) caused a slight decrease in activity, and fluorine-substituted (**7d**) resulted in a slight improvement in activity, comparing with **PU-48.** These results suggested that the electronic properties of substituents attached to the phenyl ring exerted little influence on the inhibition activity. However, the 6-,8-disubstitued compound **7e** was lower the activity by almost 3-fold compared with the 6-monosubstitued **7b**, manifesting that the large steric hindrance at 8-position of quinoline ring is unfavorable for the activity.

Discovery of thienopyridines UT inhibitors.

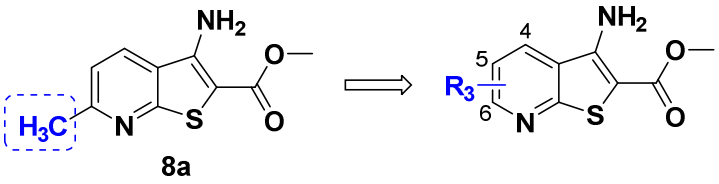
Based on the above SAR results that altering substituents on the benzene ring led to little

changes in potency, we surmised that removing benzene ring could maintain the UT-B inhibition activity. To prove this conjecture, thienopyridine analogue **8a** was designed by replacing the benzene ring of **PU48** with methyl group. It is really delightful that **8a** showed a favorable IC_{50} value of 1.30 μM, remaining at micromolar level (Table 2). This encouraging finding illustrated our hypothesis and inspired us to turn our focus to thienopyridines. For one point of view, it was a new and simplified structural skeleton used as UT inhibitors. On the other hand, the strong intermolecular π-π action due to the tricyclic aromatic conjugation system led to poor solubility of **PU48**. Removing one aromatic benzene ring could reduce such π-π action thus improve solubility to some extent.

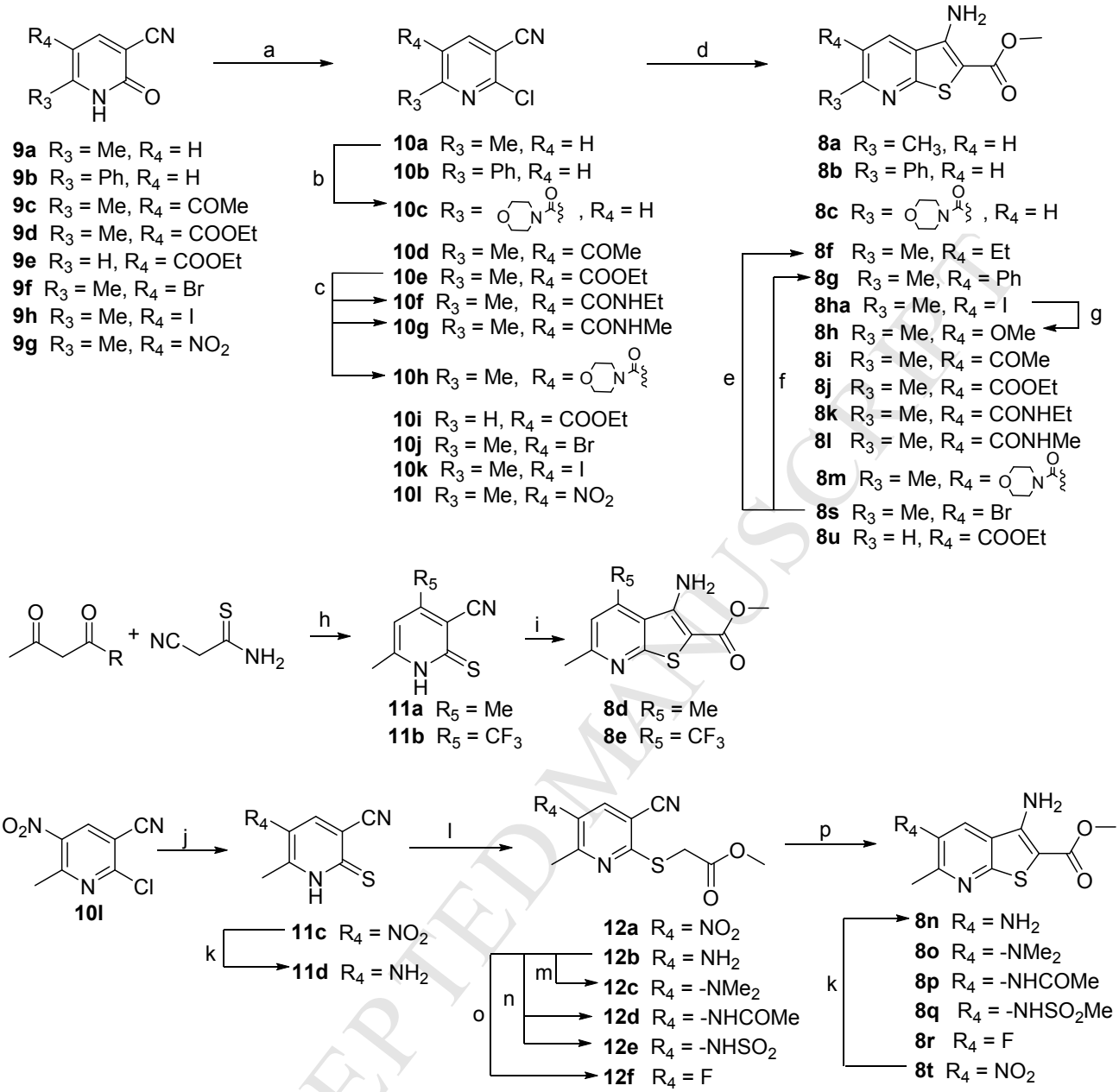
SAR study of thienopyridines UT inhibitors.

Based on the results of SAR study on **PU48**, the structural modification of compound **8a** was mainly focused on the substituent at pyridine ring. The methyl group was modified with various groups at different position of pyridine ring while the thiophene ring remained intact. The detailed synthetic routes for thienopyridine analogs are delineated in Scheme 2. Various of substituted 2-oxopyridine-3-carbonitrile **9**, which was prepared by literature procedures [33], was treated with phosphorus oxychloride in hot 1,4-dioxane to afford the corresponding **10**[34]. The synthesis of **8a-8c**, **8i-8m, 8s** and **8u** were performed by treating substituted 2-chloronicotinonitriles (**10)** with methyl thioglycolate under basic conditions [35] while **8d** and **8e** were prepared from substituted 2-thioxopyridine-3-carbonitrile (**11)**[32] reacted with methyl chloroacetate. However, for some compounds such as **8o-8r**, the substituents at pyridine ring derived from amino group. In such cases, functional group transformation should be implemented before thiophene ring closing in order to avoid additional side reactions. Therefore in regard to preparation of **8o-8r**, **11d,** obtained by reduction of **11c**, was converted to immediate **12b** first via reacting with methyl chloroacetate under mild conditions. **12b** then underwent conversion of functional groups to afford **12c-12f** followed by ring-closing reaction using sodium methoxide as base. For other compounds such as **8f-8h** and **8n**, the functional group transformation was conducted after the ring closing of thiophene. Under the catalysis of palladium-tetrakis, **8s** coupled with triethylsilane or phenylboronic acid to afford **8f** and **8g**, respectively. **8h** was acquired by treating **8ha** with sodium methoxide with copper powder as catalyst in DMF and methanol at 90℃. Finally, reduction of the nitro group of **8t** using Fe/NH_4Cl to

obtain the corresponding amino analogue **8n**.



Compd.	R_3	Compd.	R_3
8a	6-CH_3	**8l**	5-$CONHCH_3$, 6-CH_3
8b	6-Ph	**8m**	5-(morpholine-N-formyl), 6-CH_3
8c	6-(morpholine-N-formyl)	**8n**	5-NH_2, 6-CH_3
8d	4-CH_3, 6-CH_3	**8o**	5-$N(CH_3)_2$, 6-CH_3
8e	4-CF_3, 6-CH_3	**8p**	5-$NHCOCH_3$, 6-CH_3
8f	5-CH_2CH_3, 6-CH_3	**8q**	5-$NHSO_2CH_3$, 6-CH_3
8g	5-Ph, 6-CH_3	**8r**	5-F, 6-CH_3
8h	5-OCH_3,6-CH_3	**8s**	5-Br, 6-CH_3
8i	5-$COCH_3$, 6-CH_3	**8t**	5-NO_2, 6-CH_3
8j	5-$CO_2C_2H_5$, 6-CH_3	**8u**	5-$COOC_2H_5$
8k	5-$CONHC_2H_5$, 6-CH_3		

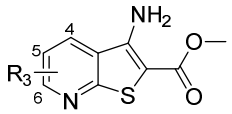


Scheme 2. Synthetic routes for thienopyridines **8a-8u**. Reagents and conditions: (a) $POCl_3$, 1,4-dioxane, 80 ℃, 3 h; (b) CrO_3, H_2SO_4, r.t., 24 h; $SOCl_2$, reflux, 8 h; morpholine, Et_3N, CH_2Cl_2, r.t., 1 h; (c) LiOH, THF, r.t., 30 min; $SOCl_2$, CH_2Cl_2, DMF, reflux, 8 h; amine, Et_3N, 0 ℃ ~ r.t., 30 min; (d) $HSCH_2COOCH_3$, Et_3N, MeOH, reflux, 6 h; (e) triethylsilane, $(PPh_3)_4Pd$, BINAP, K_3PO_4, toluene-H_2O, Ar protect, 100 ℃, 16 h; (f) phenylboronic acid, $(PPh_3)_4Pd$, K_2CO_3, 1,4-dioxane-H_2O, 100 ℃, 8 h; (g) Cu, MeONa, DMF, MeOH, 90 ℃, 24 h; (h) DABCO, EtOH, reflux, 6 h; 1N HCl, r.t., 2 h; (i) $ClCH_2COOMe$, MeONa, MeOH, reflux, 2 h; (j) thiourea, EtOH, reflux, 2 h; (k) Fe, NH_4Cl, MeOH-H_2O, 60 ℃, 2 h; (l) $ClCH_2COOMe$, Et_3N, MeOH, reflux, 6 h; (m) HCHO, $Na(CN)BH_3$,

CH_3COOH, MeCN, 0 ℃ ~ r.t., 12 h; (n) MeCOCl or $MeSO_2Cl$, Et_3N, CH_2Cl_2, 0 ℃~ r.t., 30 min; (o) $NaNO_2$, HBF_4, H_2O, 0 ~ 5 ℃, 3 h; toluene, 90 ℃, 1 h; (p) MeONa, MeOH, r.t., 1 h.

The inhibition effects of all these thienopyridines to rat UT-B were presented in Table 2. Our optimization of **8a** was started with investigation of monosubstitution at the 6-position of pyridine ring. Replacement of methyl group at 6-position in **8a** with phenyl (**8b**) resulted in a two-fold-decrease in activity while **8c** with morpholine-4-carbonyl at this position dramatically reduced the inhibitory potency (IC_{50} > 10 μM). This finding indicated that alkyl with low steric hindrance substituted at the 6-position was more beneficial. With this result in hand, we next incorporate substituents at 4- or 5-position of pyridine ring in **8a** while keeping methyl at 6-position intact. As illustrated in Table 2, introduction of electro-donating alkyl (**8d**) or electro-withdrawing trifluoromethyl (**8e**) at the 4-position caused 2-fold and 5-fold losses of potency respectively. However, **8f** (IC_{50} = 0.71 μM)**,** obtained by adding alkyl at the 5-position of pyridine ring, turned out to be more potent than **8a**. In light of this result, we focused our attention to the moiety at 5-position of **8a**.Various types of substituents including halide, electron-withdrawing, electron-donating and sterically crowded ones were tested. As shown in Table 2, substitution at 5-position with electron-donating groups such as alkyl (**8f, 8g**), methoxyl (**8h**), amino (**8n**), dimethylamino (**8o**) and acetamino (**8p**) resulted in only slightly reduced or even improved (IC_{50} < 1 μM for **8f** and **8n**) inhibitory activity to UT-B compared to **8a**. However, most of the derivatives with electro-withdrawing groups, such as sulfonamido (**8q**), carbamoyl (**8k-8m**), halide (**8r**, **8s**) and nitro group (**8t**), at 5-position of pyridine ring were much less potent (IC_{50} > 10 μM for **8k-8m, 8r** and **8t**) than **8a**. Interestingly, incorporation of acetyl (**8i**) or ethoxycabonyl (**8j**), which are also electro-withdrawing groups, led to 2-3 fold increase in potency. These findings implied that inhibition activities to UT-B of thienopyridines were influenced remarkably by electric effects of group at 5-position of pyridine ring. Finally, the necessity of methyl at 6-position of pyridine ring was detected. Deletion of the methyl group at 6-position of the pyridine ring, exemplified by the 5-monosubstituted analogue (**8u**), resulted in a substantial loss (>10 fold) of potency, suggesting that substitution of methyl at 6-position of pyridine ring is required for activity.

Table 2. Inhibitory potency of compounds with thienopyridine core to rat UT-B.



Compd.	R_3			IC_{50} (μM)
	4-	5-	6-	
8a	H	H	CH_3	1.30
8b	H	H	Ph	3.36
8c	H	H		>10
8d	CH_3	H	CH_3	2.54
8e	CF_3	H	CH_3	6.12
8f	H	C_2H_5	CH_3	0.71
8g	H	Ph	CH_3	3.29
8h	H	OCH_3	CH_3	5.46
8i	H	$COCH_3$	CH_3	0.91
8j	H	$COOC_2H_5$	CH_3	0.47
8k	H	$CONHC_2H_5$	CH_3	>10
8l	H	$CONHCH_3$	CH_3	>10
8m	H		CH_3	>10
8n	H	NH_2	CH_3	0.54
8o	H	$N(CH_3)_2$	CH_3	3.75
8p	H	$NHCOCH_3$	CH_3	3.47
8q	H	$NHSO_2CH_3$	CH_3	6.42
8r	H	F	CH_3	>10
8s	H	Br	CH_3	8.47
8t	H	NO_2	CH_3	>10
8u	H	$COOC_2H_5$	H	>10
PU-48				0.22

Solubility and Log P prediction of represented compounds.

Water solubility and Log P value, served as important parameters influencing PK profile of a molecule, were predicted using the Qikprop module from Schrödinger for the compounds with IC_{50} value under 1 μM**.** As illustrated in Table 3, the predictive log S value of most thienopyridines (**8f, 8i**, **8n**) is lower than that of thienoquinolines (**7a-7e**), indicating that modification of thienoquinolines to thienopyridines do improve the water solubility. Among them, **8n** was particularly noteworthy since it was predictable to be more than 10 times soluble in water compared to **PU48**. In the meanwhile, **8n** had a predictive Log P value of 0.646 and favorable molecular weight (237 Da) which coincided with the drug-like properties described by Lipinski and Veber.

Table 3. Predictive water-solubility and Log P of selected compounds.

Comd.	QPlogS[a]	QPlogP[a]
PU48	-3.001	2.232
7a	-2.772	2.125
7b	-3.334	2.442
7c	-3.627	2.695
7d	-3.137	2.361
7e	-3.838	2.816
8f	-2.838	2.107
8i	-2.252	1.067
8j	-3.114	1.631
8n	-1.753	0.646

[a] predicted by Qikprop module from Schrödinger.

Diuretic effect on rat model of thienoquinolines.

To evaluate *in vivo* diuretic activity of **8n**, rats were kept in metabolic cages with being fed ad libitum. Urine output was checked before experiments to make sure for urine output to be similar in each rat. **8n** at 100 mg/kg or 40% 2-hydroxypropyl-bcyclodextrin as vehicle control was

subcutaneously administrated. Urine samples were collected for 10 h after drug administration. The experimental results showed **8n** significantly increased urine output (Fig. 4), which suggest that **8n** has significant diuretic effect on rats.

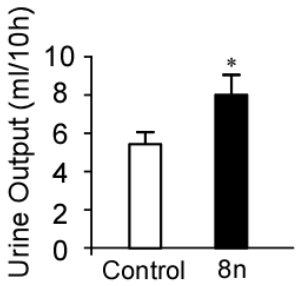


Fig 4. Diuretic effect of **8n** on rat. Urine of SD rats was collected in metabolic cages for 10 h after subcutaneous injection of 100 mg/kg of **8n** or 40% 2-hydroxypropyl-bcyclodextrin as vehicle control. Mean + SD (n=5) **P* < 0.05 compared with control.

3. Conclusion

A novel type of UT inhibitors with thienopyridine scaffold was identified in present study based on the structural modification of **PU-48**. Further structure-activity analysis resulted in a promising preclinical candidate **8n**, which possesses not only excellent inhibition effect on UT-B and diuretic activity on rat model but also favorable water solubility and suitable Log P value. These results provide a new lead compound and support further development of thienopyridine analogs as potential salt-sparing diuretics by targeting kidney urea transporters.

4. Experimental section

4.1 General

All reagents and solvents were purchased from commercial sources and were used without further purification. Melting points were determined on X4 microscope and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCEIII 400 MHz and 100 MHz spectrometer respectively. High resolution mass spectrum (HRMS) was recorded on a Thermo Scientific Orbitrap Elite MS.

4.2 General procedure for the synthesis of compounds **2a-2f**

$POCl_3$ (9.0 mL, 96 mmol) was added dropwise to DMF (2.8 mL, 36 mmol) precooled at 0 ℃. Followed by adding acetanilide (10 mmol), the mixture was heated to 75 ℃ and stirred at that temperature for 8 h. After been cooled to room temperature, the mixture was poured to 100 mL of ice-water. The precipitate was obtained by suction filtration, washed with cold water and dried to afford the product. ^{1}H NMR and ^{13}C NMR data of selected products are shown as follows.

4.2.1 2-Chloro-6-methylquinoline-3-carbaldehyde (**2b**)

Yield 83%. Mp: 125-126 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.57 (s, 1H), 8.69 (s, 1H), 7.98 (d, *J* = 8.5 Hz, 1H), 7.73 (dd, *J* = 10.9, 2.2 Hz, 2H), 2.59 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.70, 149.63, 148.60, 139.92, 138.76, 136.31, 128.75, 128.59, 126.95, 126.67, 21.91.

4.2.2 2-Chloro-6-methoxyquinoline-3-carbaldehyde (**2f**)

Yield 75%. Mp: 143-145 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.48 (s, 1H), 8.57 (s, 1H), 7.89 (d, *J* = 9.2Hz, 1H), 7.45 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.12 (d, *J* = 2.8 Hz, 1H), 3.89 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.75, 159.15, 148.01, 146.18, 138.98, 130.27, 128.12, 126.91, 126.77, 106.79, 56.12.

4.3 General procedure for the synthesis of compounds **3a-3f**

To a solution of hydroxylamine hydrochloride (520 mg, 7.5 mmol) in 1 mL water, compound **2a-2f** (1.65 g, 7.5 mmol) in 15 mL THF was added. The reaction mixture was stirred at room temperature for 1 h. THF was removed under vacuum. The residue was stirred in 10 mL water for 10 min. Solid precipitated was filtered and dried. The dried solid was dissolved in 15 mL of chloroform and $SOCl_2$ (1.4 mL, 15 mmol) was add slowly to the solution. After refluxing for 1 h, the mixture was concentrated and the residue was recrystallized with ethanol to give pure product. ^{1}H NMR and ^{13}C NMR data of selected products are shown as follows.

4.3.1 2-Chloro-6-methylquinoline-3-carbonitrile (**3b**)

Yield 98%. Mp: 155-156 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H), 7.94 (d, *J* = 8.7 Hz, 1H), 7.73 (dd, *J* = 8.7, 1.8 Hz, 1H), 7.63 (s, 1H), 2.57 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 147.67,

147.20, 144.40, 139.52, 136.52, 128.83, 127.13, 125.51, 115.55, 108.03, 21.92.

4.3.2 2-Chloro-6-methoxyquinoline-3-carbonitrile (**3f**)

Yield 98%. Mp: 158-160 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (s, 1H), 7.94 (d, *J* = 8.5 Hz, 1H), 7.53 (d, *J* = 7.7 Hz, 1H), 7.10 (s, 1H), 3.96(s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.58, 146.04, 144.78, 143.46, 130.57, 127.02, 126.80, 115.60, 108.32, 105.51, 56.23.

4.4 General procedure for the synthesis of compounds **4a-4f**

A mixture of **3a-3f** (1.59 g, 7.3 mmol) and thiourea (1.11 g, 14.6 mmol) in 20 mL of ethanol was refluxed for 1 h. After cooled to room temperature, water (50 mL) was added and the mixture was stirred for another 30 min. The product was yielded by filtration. ^{1}H NMR and ^{13}C NMR data of selected products are shown as follows.

4.4.1 2-Mercapto-6-methylquinoline-3-carbonitrile (**4b**)

Yield 98%. Mp: 229-231 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 14.21 (s, 1H), 8.70 (s, 1H), 7.73-7.63 (m, 2H), 7.57 (d, *J* = 8.3 Hz, 1H), 2.42 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.26, 144.92, 139.39, 137.24, 135.59, 129.04, 121.88, 117.43, 117.22, 116.28, 21.40.

4.4.2 2-Mercapto-6-methoxyquinoline-3-carbonitrile (**4f**)

Yield 98%. Mp: 209-211 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 14.22 (s, 1H), 8.67 (s, 1H), 7.63 (d, *J* = 9.1 Hz, 1H), 7.50 (dd, *J* = 9.1, 2.8 Hz, 1H), 7.39 (d, *J* = 2.8 Hz, 1H), 3.87 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.84, 157.02, 144.48, 136.42, 126.10, 122.87, 118.93, 117.43, 116.61, 109.65, 56.63.

4.5 General procedure for the synthesis of compounds **6a-6i** and **7a-7e**

To a solution of **4a-4f** (1.58 g, 7.3 mmol) in 15 mL of methanol, MeONa (7.3 mmol) and corresponding material with chloroacetyl (11.0 mmol) were added. The reaction mixture was refluxed for 3 h. After cooled to room temperature, water (15 mL) was added and the mixture was stirred for 5 min. The precipitate was filtered and recrystallized by ethanol to afford pure product.

4.5.1 Ethyl 3-amino-6-methoxythieno[2,3-*b*]quinoline-2-carboxylate (**6a**)

Yield 96%. Mp: 257 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.03 (s, 1H), 7.96 (d, *J* = 9.3 Hz, 1H), 7.52 (dd, *J* = 9.3, 2.3 Hz, 1H), 7.43 (s, 2H), 7.34 (s, 1H), 4.31 (q, *J* = 7.0 Hz, 2H), 3.95 (s, 3H), 1.33 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.41, 157.14, 157.02, 145.44, 144.98, 129.71, 129.66, 126.75, 126.13, 124.23, 106.24, 97.55, 56.04. HRMS *m/z*: calcd for $C_{15}H_{15}N_2O_3S$ $[M+H]^+$: 303.0798; found: 303.0791.

4.5.2 3-Amino-6-methoxythieno[2,3-*b*]quinoline-2-carboxamide (**6b**)

Yield 96%. Mp: > 300 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (s, 1H), 7.97 (d, *J* = 9.2 Hz, 1H), 7.50 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.36 (d, *J* = 2.8 Hz, 1H), 7.34 (s, 2H), 7.21 (s, 2H), 3.94 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.41, 157.14, 157.02, 145.44, 144.98, 129.71, 129.66, 126.75, 126.13, 124.23, 106.24, 97.55, 56.04. HRMS*m/z*: calcd for $C_{13}H_{12}N_3O_2S$ $[M+H]^+$: 274.0645; found: 274.0641.

4.5.3 3-Amino-6-methoxy-*N*-methylthieno[2,3-*b*]quinoline-2-carboxamide (**6c**)

Yield 95%. Mp: 225-228 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (s, 1H), 7.97 (d, *J* = 9.2 Hz, 1H), 7.72 (d, *J* = 4.4 Hz, 1H), 7.50 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.36 (d, *J* = 2.8 Hz, 1H), 7.35 (s, 2H), 3.95 (s, 3H), 2.77 (d, *J* = 4.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.86, 157.03, 157.01, 144.87, 144.80, 129.72, 129.69, 126.83, 126.17, 124.17, 106.21, 97.77, 56.04, 26.57. HRMS *m/z*: calcd for $C_{14}H_{14}N_3O_2S$ $[M+H]^+$: 288.0801; found: 288.0799.

4.5.4 Methyl (3-amino-6-methoxythieno[2,3-*b*]quinoline-2-carbonyl)glycinate (**6d**)

Yield 60%. Mp: 209-210 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.95 (s, 1H), 8.16 (t, *J* = 5.6 Hz, 1H), 7.98 (d, *J* = 9.6 Hz, 2H), 7.51 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.38 (s, 2H), 7.37 (s, 1H), 3.97 (s, 2H), 3.95 (s, 3H), 3.67(s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.00, 165.76, 157.11, 156.98, 145.84, 145.07, 129.87, 129.71, 126.54, 126.19, 124.41, 106.27, 96.60, 56.06, 52.19, 41.56. HRMS *m/z*: calcd for $C_{16}H_{16}N_3O_4S$ $[M+H]^+$: 346.0856; found: 346.0848.

4.5.5 (3-Amino-6-methoxythieno[2,3-*b*]quinolin-2-yl)(morpholino)methanone (**6e**)

Yield 95%. Mp: 244-245 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.97 (s, 1H), 7.95 (d, J = 9.2 Hz, 1H), 7.48 (dd, J = 9.2, 2.4 Hz, 1H), 7.33 (d, J = 2.4 Hz, 1H), 6.65 (s, 2H), 3.94 (s, 3H), 3.66 (s, 4H), 3.64 (s, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.61, 157.58, 157.00, 144.42, 141.57, 129.69, 129.50, 126.76, 126.16, 124.07, 106.15, 99.45, 66.69, 56.03, 45.86. HRMS *m/z*: calcd for $C_{17}H_{18}N_3O_3S$ $[M+H]^+$: 344.1063; found: 344.1066.

4.5.6 3-Amino-6-methoxy-*N*,*N*-dimethylthieno[2,3-*b*]quinolin-2-sulfonamide (**6f**)

Yield 78%. Mp: > 300 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.09 (s, 1H), 8.00 (d, J = 9.2 Hz, 1H), 7.56 (d, J = 9.2 Hz, 1H), 7.36 (s, 1H), 6.89 (s, 2H), 3.96 (s, 3H), 2.80 (s, 6H). ^{13}C NMR (100 MHz, DMSO-$_{d6}$) δ 157.26, 156.78, 145.04, 143.91, 131.02, 129.72, 126.23, 125.97, 125.07, 106.17, 95.27, 56.09, 38.28. HRMS m/z: calcd for $C_{14}H_{16}N_3O_3S_2$ $[M+H]^+$: 338.0628; found: 338.0619.

4.5.7 1-(3-amino-6-methoxythieno[2,3-*b*]quinoline-2-yl)ethan-1-one (**6g**)

Yield 93%. Mp: 294-295 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.12 (s, 1H), 8.19 (s, 2H), 7.96 (d, J = 9.2 Hz, 1H), 7.52 (dd, J = 9.2, 1.8 Hz, 1H), 7.32 (d, J = 1.8 Hz, 1H), 3.94 (s, 3H), 2.38 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 192.05, 157.49, 157.13, 147.89, 145.84, 131.59, 129.73, 126.10, 125.92, 125.12, 106.28, 104.08, 56.09, 29.69. HRMS *m/z*:calcd for $C_{14}H_{13}N_2O_2S$ $[M+H]^+$: 273.0692; found: 273.0688.

4.5.8 Methyl(3-amino-6-methoxythieno[2,3-*b*]quinoline-2-yl)-3-oxopropanoate (**6h**)

Yield 75%. Mp: > 300 ℃.^{1}H NMR (400 MHz, DMSO-d_6) δ 9.12 (s, 1H), 8.38 (s, 2H), 7.96 (d, J = 9.2 Hz, 1H), 7.54 (d, J = 9.2 Hz, 1H), 3.95 (s, 3H), 3.82 (s, 2H), 3.36 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ186.50, 168.20, 157.57, 157.19, 149.54, 146.05, 131.83, 129.70, 126.09, 125.50, 125.38, 106.33, 103.15, 56.09, 52.44, 48.11. HRMS *m/z*: calcd for $C_{16}H_{15}N_2O_4S$ $[M+H]^+$: 331.0747; found: 331.0742.

4.5.9 Methyl 3-aminothieno[2,3-*b*]quinoline-2-carboxylate (**7a**)

Yield 71%. Mp: 238-241 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.20 (s, 1H), 8.23-7.95 (m, 2H), 7.86 (t, J = 7.3 Hz, 1H), 7.63 (t, J = 7.3 Hz, 1H), 7.51 (s, 2H), 3.82 (s, 3H). ^{13}C NMR (100 MHz,

DMSO-d_6) δ 165.66, 160.74, 149.27, 148.49, 132.87, 132.12, 130.17, 128.65, 126.80, 126.25, 125.36, 93.52, 52.41. HRMS *m/z*: calcd for $C_{13}H_{11}N_2O_2S$ $[M+H]^+$: 259.0536; found: 259.0531.

4.5.10 Methyl 3-amino-6-methylthieno[2,3-*b*]quinoline-2-carboxylate (**7b**)

Yield 89%. Mp: 278-291 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.08 (s, 1H), 7.94 (d, *J* = 8.7 Hz, 1H), 7.81 (s, 1H), 7.70 (dd, *J* = 8.7, 1.8 Hz, 1H), 7.49 (s, 2H), 3.82 (s, 3H), 2.54 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.71, 158.15, 157.56, 148.34, 145.84, 131.17, 130.13, 126.45, 125.30, 110.44, 106.77, 93.88, 56.51, 52.36. HRMS *m/z*: calcd for $C_{14}H_{13}N_2O_2S$ $[M+H]^+$: 273.0692; found: 273.0688.

4.5.11 Methyl 3-amino-6-bromothieno[2,3-*b*]quinoline-2-carboxylate (**7c**)

Yield 39%. Mp: > 300 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.14 (s, 1H), 8.32 (s, 1H), 8.06-7.84 (m, 2H), 7.55 (s, 2H), 3.86 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.59, 160.26, 147.24, 146.74, 134.05, 131.10, 130.79, 129.87, 125.90, 125.57, 118.53, 93.23, 51.55. HRMS *m/z*: calcd for $C_{13}H_{10}BrN_2O_2S$ $[M+H]^+$: 336.9641; found: 336.9637.

4.5.12 Methyl 3-amino-6-fluorothieno[2,3-*b*]quinoline-2-carboxylate (**7d**)

Yield 23%. Mp: > 300 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.17 (s, 1H), 8.14 (dd, *J* = 9.2, 5.4 Hz, 1H), 8.04-7.69 (m, 2H), 7.53 (s, 2H), 3.86 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.65, 160.23, 159.25, 157.79, 147.09, 145.56, 131.38, 130.58, 125.83, 124.87, 121.69, 111.56, 51.52. HRMS *m/z*: calcd for $C_{13}H_{10}FN_2O_2S$ $[M+H]^+$: 277.0441; found: 277.0436.

4.5.13 Methyl 3-amino-6,8-dimethythieno[2,3-*b*]quinoline-2-carboxylate (**7e**)

Yield 78%. Mp: 259-260 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (s, 1H), 7.47 (d, *J* = 14.9 Hz, 2H), 6.05 (s, 2H), 3.92 (s, 3H), 2.80 (s, 3H), 2.50 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.28, 166.30, 159.44, 147.71, 146.36, 136.32, 135.60, 133.67, 128.79, 125.44, 125.12, 124.91, 52.07, 21.89, 18.51. HRMS *m/z*: calcd for $C_{15}H_{15}N_2O_2S$ $[M+H]^+$: 287.0849; found: 287.0843.

4.6 The procedure for the synthesis of 3-amino-6-methoxythieno[2,3-*b*]quinoline-2-carboxylic acid

(**6i**)

A mixture of **PU-48** (288 mg, 1 mmol) and NaOH (160 mg, 4 mmol) in water (5 mL) and ethanol (20 mL) was heated under reflux for 3 h. 25 mL of cold water was added slowly and the mixture was stirred in an ice bath for 5 min. Add hydrochloric acid to adjust the pH to 4-5, filter with suction to provide **6i** as an orange solid (200 mg, 73%). Mp: 224-225 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.01 (s, 1H), 7.97 (d, *J* = 9.2 Hz, 1H), 7.52 (dd, *J* = 9.2, 2.4 Hz, 1H), 7.35 (d, *J* = 2.4 Hz, 1H), 3.95 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.68, 157.76, 157.02, 147.17, 145.20, 130.38, 129.68, 126.25, 125.97, 124.65, 95.38, 56.04. HRMS *m/z*: calcd for $C_{13}H_{11}N_2O_3S$ $[M+H]^+$: 275.0485; found: 275.0483.

4.7 General procedure for the synthesis of **10a, 10b, 10d, 10e and 10i-10l.**

To a solution of **9a-9h** (10 mmol) in 10 mL of 1,4-dioxane, $POCl_3$ (2.3 mL, 25 mmol) was added dropwise. The reaction mixture was stirred at 80 ℃ for 1 h. After cooled to room temperature, the mixture was poured to 50 mL of ice water. 20 N NaOH aqueous solution was added to adjust pH = 7. Solid was precipitated, filtered and purified by column chromatography (eluent: petroleum ether/ethyl acetate = 10:1) to provide the title compound. ^{1}H NMR and ^{13}C NMR data of selected products are shown as follows.

4.7.1 2-Chloro-6-methylnicotinonitrile (**10a**)

Yield 77%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (d, *J* = 7.9 Hz, 1H), 7.24 (d, *J* = 7.9 Hz, 1H), 2.63 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.99, 151.93, 142.37, 121.96, 114.96, 107.61, 24.77.

4.7.2 5-Acetyl-2-chloro-6-methylnicotinonitrile (**10d**)

Yield 93%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 1H), 2.83(s, 3H), 2.64 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.87, 163.84, 153.29, 142.65, 131.29, 114.15, 107.96, 29.37, 25.13.

4.7.3 Ethyl 6-chloro-5-cyano-2-methylnicotinate (**10e**)

Yield 95%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (s, 1H), 4.43 (q, *J* = 6.6 Hz, 2H), 2.91 (s, 3H),

1.43 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.30, 163.72, 153.77, 144.65, 124.64, 114.15, 108.12, 62.34, 25.21, 14.19.

4.7.4 Ethyl 6-chloro-5-cyanononitrile (**10i**)

Yield 81%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.17 (d, *J* = 2.3 Hz, 1H), 8.59 (d, *J* = 2.3 Hz, 1H), 4.48 (d, *J* = 7.1 Hz, 2H), 1.45 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.58, 156.30, 153.60, 143.33, 125.48, 113.87, 110.94, 62.59, 14.19.

4.7.5 2-Chloro-6-methyl-5-nitronicotinonitrile (**10l**)

Yield 87%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (s, 1H), 2.97 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.03, 154.72, 143.66, 138.81, 112.85, 109.29, 24.49.

4.8 Synthesis of 2-Chloro-6-(morpholine-4-carbonyl)nicotinonitrile (**10c**)**.**

To a solution of **10a** (765 mg, 5 mmol) in 10 mL of concentrated H_2SO_4, which was pre-cooled to 0 ℃, CrO_3 (750 mg, 7.5 mmol) was added batchwise. The mixture was stirred at 0 ℃ for 1 h and then warmed to room temperature for another 12 h. The reaction mixture was added dropwise to 20 mL of ice water while stirring. After filtration, the filter cake dissolved in 10 mL of $POCl_3$ and stirred at 90 ℃ for 40 min. The resulted mixture was cooled and poured to ice water. The precipitate was filtered, dried and then dissolved in DCM (15 mL). $SOCl_2$ (357 mg, 3 mmol) was added to the solultion followed by 5 drops of DMF. The mixture was heated at 40 ℃ for 8 h. After cooled to 0 ℃, a solution of morpholine (348 mg, 4 mmol) and Et_3N (404 mg, 4 mmol) in dichloromethane (10 mL) was added. The resulted mixture was stirred for further 30 min and then poured to 20 mL of water. The mixture was extracted with dichloromethane (20 mL ×3), dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography (eluent: petroleum ether/ethyl acetate = 5:1) to provide the title product. Yield 88%. Mp: 118-119 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, *J* = 7.9 Hz, 1H), 7.81-7.73 (m, 1H), 3.80 (s, 4H), 3.71 (s, 2H), 3.63 (d, *J* = 2.5 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.99, 156.36, 151.32, 143.70, 122.90, 114.16, 111.56, 66.76, 66.64, 47.71, 43.00. HRMS *m/z*: calcd for $C_{11}H_{11}ClN_3O_2$ $[M+H]^+$: 252.0534; found: 252.0530.

4.9 General procedure for the synthesis of **10f-10h.**

15 mL of 3 N LiOH aqueous solution was added drop wise to the solution of **10e** (2.5 mmol) in THF (35 mL). The mixture was stirred at room temperature for 10 min and then concentrated in vacuum to remove THF. 1 N hydrochloric acid was added to adjust pH = 3. The solid precipitated was filtered, dried and then dissolved in DCM (15 mL). $SOCl_2$ (357 mg, 3 mmol) was added to the solultion followed by 5 drops of DMF. The mixture was heated at 40 ℃ for 8 h. After cooled to 0 ℃, a solution of amine (4 mmol) and Et_3N (404 mg, 4 mmol) in dichloromethane (10 mL) was added. The resulted mixture was stirred for further 30 min and then poured to 20 mL of water. The mixture was extracted with dichloromethane (20 mL ×3), dried over anhydrous Na_2SO_4 and concentrated to provide the title products without further purification.

4.10 General procedure for the synthesis of **8a-8c, 8i-8m, 8s and 8u.**

A mixture of **10a-10k** (1 mmol), methyl thioglycolate (159 mg, 1.5 mmol) and Et_3N (152 mg, 1.5 mmol) in 10 mL of methanol was stirred at 80 ℃ for 6~8 h. Methanol was removed under vacuum. The residue was purified by column chromatography to provide the title compound.

4.10.1 Methyl 3-amino-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8a**)

Yield 75%. Mp: 137 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.40 (d, *J* = 8.3 Hz, 1H), 7.41-7.11 (m, 3H), 3.79 (s, 3H), 2.58 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.30, 160.63, 159.88, 152.94, 148.49, 136.10, 131.96, 123.67, 119.93, 113.89, 92.72, 51.75, 24.72. HRMS *m/z*: calcd for $C_{10}H_{10}N_2O_2S$ $[M+H]^+$: 223.0536; found: 223.0530.

4.10.2 Methyl 3-amino-6-phenylthieno[2,3-*b*]pyridine-2-carboxylate (**8b**)

Yield 70%. Mp: 220-222 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (d, *J* = 7.0 Hz, 2H), 7.99 (d, *J* = 8.5 Hz, 1H), 7.77 (d, *J* = 8.5 Hz, 1H), 7.50 (dq, *J* = 14.2, 7.0 Hz, 3H), 5.95 (s, 2H), 3.93 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.21, 160.45, 157.73, 148.28, 138.19, 132.90, 130.32, 129.38, 127.55, 124.88, 116.83, 51.86. HRMS *m/z*: calcd for $C_{15}H_{13}N_2O_2S$ $[M+H]^+$: 285.0692; found: 285.0688.

4.10.3 Methyl 3-amino-6-(morpholine-4-carbonyl)thieno[2,3-b]pyridine-2-carboxylate (**8c**).

Yield 70%. Mp: 188-189 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (d, *J* = 8.3 Hz, 1H), 7.65 (d, *J* = 8.3 Hz, 1H), 6.06 (s, 2H), 3.92 (s, 3H), 3.85 (d, *J* = 1.8 Hz, 4H), 3.71 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.74, 165.50, 159.27, 153.61, 145.96, 130.53, 125.86, 119.18, 99.62, 66.97, 66.76, 51.85, 47.86, 42.93. HRMS *m/z*: calcd for $C_{14}H_{15}N_3O_4S$ $[M+H]^+$: 322.0856; found: 322.0854.

4.10.4 Methyl 5-acetyl-3-amino-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8i**).

Yield 79%. Mp: 192 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.06 (d, *J* = 4.1 Hz,1H), 7.39 (s, 2H), 3.80 (s, 3H), 2.71 (d, *J* = 2.0 Hz, 3H), 2.64 (d, *J* = 1.1 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 199.89, 165.00, 161.41, 159.57, 148.42, 133.96, 128.66, 123.69, 93.43, 51.85, 29.60, 25.61. HRMS *m/z*: calcd for $C_{12}H_{12}N_2O_3S$ $[M+H]^+$: 265.0641; found: 265.0639.

4.10.5 5-Ethyl 2-methyl 3-amino-6-methylthieno[2,3-*b*]pyridine-2,5-dicarboxylate (**8j**).

Yield 73%. Mp: 193-194 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (s, 1H), 6.05 (s, 2H), 4.43 (q, *J* = 7.1 Hz, 2H), 3.91 (s, 3H), 2.95 (s, 3H), 1.44 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.18, 165.60, 162.93, 160.95, 146.42, 132.11, 123.11, 121.45, 98.17, 61.56, 51.75, 25.63, 14.33. HRMS *m/z*: calcd for $C_{13}H_{14}N_2O_4S$ $[M+H]^+$: 295.0747; found: 295.0743.

4.10.6 Methyl 3-amino-5-(ethylcarbamoyl)-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8k**).

Yield 89%. Mp: 274-276 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.53 (s, 2H), 7.32 (s, 2H), 3.80 (s, 3H), 3.29 (dd, *J* = 13.0, 7.0 Hz, 2H), 2.61 (s, 3H), 1.16 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.91, 165.17, 159.72, 157.65, 148.34, 130.74, 129.48, 123.40, 93.40, 51.85, 34.46, 23.31, 15.07. HRMS *m/z*: calcd for $C_{13}H_{15}N_3O_3S$ $[M+H]^+$: 294.0907; found: 294.0901.

4.10.7 Methyl 3-amino-5-(methylcarbamoyl)-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8l**).

Yield 87%. Mp: 287 ℃ (decomposed). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.54 (s, 1H), 8.46 (d, *J* = 4.4 Hz, 1H), 7.30 (s, 2H), 3.79 (s, 3H), 2.81 (d, *J* = 4.6 Hz, 3H), 2.61 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.62, 165.16, 159.79, 157.76, 148.31, 130.85, 129.26, 123.40, 93.47, 51.86, 26.54, 23.39. HRMS *m/z*: calcd for $C_{12}H_{13}N_3O_3S$ $[M+H]^+$: 280.0750; found: 280.0749.

4.10.8 Methyl 3-amino-6-methyl-5-(morpholine-4-carbonyl)thieno[2,3-*b*]pyridine-2-carboxylate (**8m**).

Yield 87%. Mp: 215 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (s, 1H), 6.11 (s, 2H), 3.90-3.74 (m, 7H), 3.67-3.53 (m, 2H), 3.27 (d, *J* = 4.1 Hz, 2H), 2.63 (s, 3H),.^{13}C NMR (100 MHz, $CDCl_3$) δ 168.31, 165.63, 160.88, 157.77, 146.21, 127.48, 126.72, 122.90, 98.52, 66.76, 51.73, 47.45, 42.30, 22.72. HRMS *m/z*: calcd for $C_{15}H_{17}N_3O_4S$ $[M+H]^+$: 336.1013; found: 336.1017.

4.10.9 Methyl 3-amino-5-bromo-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8s**).

Yield 83%. Mp: 187 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.80 (d, *J* =6.6 Hz, 1H), 7.25 (s, 2H), 3.79 (s, 3H), 2.66 (d, *J* = 3.9 Hz, 3H).^{13}C NMR (100 MHz, DMSO-d_6) δ 164.97, 158.15, 147.16, 135.07, 125.83, 116.99, 94.37, 51.90, 25.55. HRMS *m/z*: calcd for $C_{10}H_9BrN_2O_2S$ $[M+H]^+$: 300.9641; found: 300.9639.

4.10.10 5-Ethyl 2-methyl 3-aminothieno[2,3-*b*]pyridine-2,5-dicarboxylate (**8u**).

Yield 72%. Mp: 174-176 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.25 (d, *J* = 1.8 Hz, 1H), 8.64 (d, *J* = 1.8 Hz,1H), 6.15 (s, 2H), 4.46 (q, *J* = 7.1 Hz, 2H), 3.92 (s, 3H), 1.45 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.44, 165.21, 164.47, 150.93, 146.35, 130.82, 124.84, 121.76, 99.16, 66.76, 61.73, 51.83, 14.30. HRMS *m/z*: calcd for $C_{12}H_{12}N_2O_4S$ $[M+H]^+$: 281.0591; found: 281.0594.

4.11 General procedure for the synthesis of **8d** and**8e.**

To a solution of acetyl- or trifuluroacetyl acetone (1 mmol) and DABCO (220 mg, 1 mmol) in 30 mL of ethanol, 2-cyanoethanethioamide (120 mg, 1.2 mmol) was added. The mixture was stirred under reflux for 6 h. The solvent was removed in vacuum. Add 1 N HCl (50 mL) to the residue and stirred the resulted suspension for 2 h at room temperature. The solid was filtered and washed with water to provide **11a** or **11b** without further purification. Dissolve **11a** or **11b** in 15 mL of methanol. Methyl chloroacetate (162 mg, 1.5 mmol) and MeONa (81 mg, 1.5 mmol) was added to the solution and was stirred for 3 h at room temperature. Water was added and the precipitate was filtered and recrystallized with ethyl acetate/petroleum ether to afford the pure product.

4.11.1 Methyl 3-amino-4,6-dimethylthieno[2,3-*b*]pyridine-2-carboxylate (**8d**).

Yield 82%. Mp: 184-185 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.86 (s, 1H), 6.17 (s, 2H), 3.89 (s, 3H), 2.74 (s, 3H), 2.60 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.13, 161.41, 159.75, 149.12, 143.63, 122.33, 121.77, 119.49, 51.51, 24.40, 20.14. HRMS *m/z*: calcd for $C_{11}H_{13}N_2O_2S$ $[M+H]^+$: 237.0692; found: 237.0688.

4.11.2 Methyl 3-amino-6-methyl-4-(trifluoromethyl)thieno[2,3-*b*]pyridine-2-carboxylate (**8e**).

Yield 79%. Mp: 190-192 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (s, 1H), 6.39 (s, 2H), 3.93 (s, 3H), 2.76 (s, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ 165.75, 162.79, 159.91, 145.81, 130.90, 128.84, 124.29, 116.30, 116.24, 51.84, 24.72. HRMS *m/z*: calcd for $C_{11}H_{10}F_3N_2O_2S$ $[M+H]^+$: 291.0410; found: 291.0407.

4.12 The procedure for the synthesis of methyl 3-amino-5-ethyl-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8f**).

A mixture of **8s** (301 mg, 1 mmol), $B(C_2H_5)_3$ (66 mg, 0.67 mmol), $(PPh_3)_4Pd$ (58 mg, 0.05 mmol), BINAP (31 mg, 0.05 mmol), K_3PO_4 (425 mg, 2 mmol), toluene (10 mL) and water (1 mL) was stirred at 100 ℃ under argon for 16 h followed by cooling to room temperature. Water (10 mL) was added and the mixture was extracted with ethyl acetate (10 mL × 3). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography (eluent: petroleum ether/ethyl acetate = 6:1) to provide **8f** as a yellow solid (156 mg, 62%). Mp: 191 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (s, 1H), 7.23 (s, 2H), 3.78 (s, 3H), 2.70 (d, *J* = 7.0 Hz, 2H), 2.56 (s, 3H), 1.25 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.37, 159.54, 157.01, 148.50, 133.64, 130.12, 124.47, 92.93, 51.68, 25.24, 22.84, 13.95. HRMS *m/z*: calcd for $C_{12}H_{14}N_2O_2S$ $[M+H]^+$: 251.0849; found: 251.0843.

4.13 The procedure for the synthesis of methyl 3-amino-6-methyl-5-phenylthieno[2,3-*b*]pyridine-2-carboxylate (**8g**).

A mixture of **8s** (301 mg, 1 mmol), $PhB(OH)_2$ (244 mg, 2 mmol), $(PPh_3)_4Pd$ (58 mg, 0.05 mmol),

K_2CO_3 (277 mg, 2 mmol), 1,4-dioxane (5 mL) and water (1 mL) was stirred at 100 ℃ for 8 h followed by cooling to room temperature. Water (10 mL) was added and the mixture was extracted with ethyl acetate (10 mL × 3). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography (eluent: petroleum ether/ethyl acetate = 6:1) to provide **8g** as a yellow solid (272 mg, 91%). Mp: 201 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ7.74 (s, 1H), 7.53-7.39 (m, 3H), 7.36 (dd, *J* = 5.2, 3.0 Hz, 2H), 3.91 (s, 3H), 2.61 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.90, 159.35, 158.37, 146.46, 139.44, 133.41, 129.89, 129.21, 128.57, 127.79, 124.48, 97.97, 51.67, 24.08. HRMS *m/z*: calcd for $C_{16}H_{14}N_2O_2S$ $[M+H]^+$: 299.0849; found: 299.0848.

4.14 The procedure for the synthesis of methyl 3-amino-5-methoxy-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8h**).

A mixture of compound **8ha** (348 mg, 1 mmol, prepared from **10k** by the method similar to 4.10), MeONa (162 mg, 3 mmol), copper powder (107 mg, 2 mmol), methanol (15 mL) and DMF (15 mL) was stirred at 90 ℃ for 24 h followed by cooling to room temperature. Water (10 mL) was added and the mixture was extracted with ethyl acetate (10 mL × 3). The mixture was filtered over diatomite and concentrated. Water (30 mL) was then added to the residue and the mixture was extracted with ethyl acetate (20 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography (eluent: petroleum ether/ethyl acetate = 3:1) to provide **8h** as a yellow solid (212 mg, 84%). Mp: 179 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (s, 1H), 7.20 (s, 2H), 3.87 (s, 3H), 3.78 (s, 3H), 2.46 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.31, 152.06, 151.90, 150.56, 148.37, 124.83, 111.46, 93.91, 56.22, 51.69, 20.33. HRMS *m/z*: calcd for $C_{11}H_{12}N_2O_3S$ $[M+H]^+$: 253.0641; found: 253.0639.

4.15 The procedure for the synthesis of 6-methyl-5-nitro-2-thioxo-1,2-dihydropyridine-3-carbonitrile (**11c**).

A mixture of **10l** (3.916 g, 20 mmol), thiourea (3.04 g, 40 mmol) and ethanol (50 mL) was refluxed for 3 h. Water (50 mL) was added. The mixture was cooled to room temperature and filtered. The filter cake was dried to provide **11c** as a bright yellow solid (3.705 g, 75%), which was not

purified and directly used in the next reaction.

4.16 The procedure for the synthesis of 5-amino-6-methyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile (**11d**).

A mixture of compound11c (1.95 g, 10 mmol), ferric powder (1.84 g, 33 mmol), NH_4Cl (2.67 mg, 50 mmol), methanol (20 mL) and water (10 mL) was drastically stirred at 60 ℃ for 3 h. After cooled to room temperature, the mixture was filtered over diatomite. Add 30 mL of water to the filtrate and extract with ethyl acetate (30 mL × 3). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated to provide **11d** as a yellow solid (1.58 g, 96%), which was not purified and directly used in the next reaction.

4.17 The procedure for the synthesis of **12a** and **12b**.

A mixture of **11c or 11d** (5 mmol), methyl chloroacetate (810 mg, 7.7 mmol), Et_3N (760 mg, 7.6 mmol) and methanol (20 mL) was stirred at 50 ℃ for 2 h. Methanol was removed under vacuum and the residue was purified by column chromatography (eluent: petroleum ether/ethyl acetate = 5:1) to provide **12a** or **12b.**

Methyl 2-((5-amino-3-cyano-6-methylpyridin-2-yl)thio)acetate (**12b)**. Yield 84%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.04 (s, 1H), 3.95 (s, 2H), 3.77 (s, 5H), 2.41 (s, 3H).

4.18 The procedure for the synthesis of methyl 2-((3-cyano-5-(dimethylamino)-6-methylpyridin-2-yl)thio)acetate (**12c**).

To a solution of compound **12b** (356 mg, 1.5 mmol) in 15 mL of acetonitrile was added aqueous solution of formaldehyde (15 mL, *w* = 37%), $NaCNBH_3$ (284 mg, 4.5 mmol) and 10 drops of acetic acid. The mixture was stirred at room temperature for 12 h. Water (20 mL) was added and the suspension was extracted with dichloromethane (20 mL × 3). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated to afford **12c** without further purification.

4.19 The procedure for the synthesis of methyl

2-((5-acetamido-3-cyano-6-methylpyridin-2-yl)thio)acetate (**12d**) and methyl 2-((3-cyano-6-methyl-5-(methylsulfonamido)pyridin-2-yl)thio)acetate (**12e**).

To a solution of compound **12b** (356 mg, 1.5 mmol) and Et_3N (228 mg, 2.25 mmol) in dichloromethane (15 mL) precooled to 0 ℃, acetylchloride or methylsufonylchloride (2.25 mmol) was added dropwise. The mixture was then stirred at room temperature for 30 min. Water (15 mL) was added and the suspension was extracted with dichloromethane (15 mL × 3). The combined organic phase was dried over anhydrous Na_2SO_4 and concentratedto afford **12d** or **12e** without further purification.

4.20 The procedure for the synthesis of methyl 2-((3-cyano-5-fluoro-6-methylpyridin-2-yl)thio)acetate (**12f**).

A mixture of compound **12b** (475 mg, 2 mmol), aqueous solution of HBF_4 (10 mL, *w* = 40%) and water (15 mL) was cooled in an ice water bath to 0 ℃. $NaNO_2$ (345 mg, 5 mmol) in 2 mL of water was added dropwise. The resulted mixture was stirred at 0 ℃ for 3 h. Diethyl ether was added and yellow solid precipitated. Filter the mixture and dissolve the filter cake with toluene (10 mL). The solution then was heated and refluxed for 1h followed by cooling to room temperature. Water (15 mL) was added and the suspension was extracted with ethyl acetate (15 mL × 3). The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated to afford **12f** without further purification.

4.21 The preparation of **8o-8r and 8t**.

A mixture of **12a or 12c-12f** (prepared by procedure 4.17-4.20), MeONa (122 mg, 2.25 mmol) and methanol (15 mL) was stirred at room temperature for 1 h followed by concentrating. The residue was purified by column chromatography provide **8o-8r and 8t**.

4.21.1 Methyl 3-amino-5-(dimethylamino)-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8o**).

Yield 41%. Mp: 204 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.20 (s, 1H), 7.23 (s, 2H), 3.78 (s, 3H), 2.70 (s, 6H), 2.56 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.38, 157.00, 153.08, 148.49, 146.35, 124.63, 120.82, 93.41, 51.66, 44.52, 22.12. HRMS *m/z*: calcd for $C_{12}H_{15}N_3O_2S$ $[M+H]^+$: 266.0958;

found: 266.0955.

4.21.2 Methyl 5-acetamido-3-amino-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8p**).

Yield 42%. Mp: 221 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.67 (s, 1H), 8.50 (s, 1H), 7.27 (s, 2H), 3.79 (s, 3H), 2.51 (s, 3H), 2.13 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.27, 165.27, 156.21, 156.09, 148.29, 129.74, 128.39, 124.39, 93.59, 51.78, 23.59, 21.91. HRMS *m/z*: calcd for $C_{12}H_{13}N_3O_3S$ $[M+H]^+$: 280.0750; found: 280.0749.

4.21.3 Methyl 3-amino-6-methyl-5-(methylsulfonamido)thieno[2,3-*b*]pyridine-2-carboxylate (**8q**).

Yield 34%. Mp: > 252 ℃ (decomposed). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.50 (s, 1H), 8.42 (s, 1H), 7.34 (s, 2H), 3.79 (s, 3H), 3.12 (s, 3H), 2.62 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.17, 158.32, 157.14, 148.22, 129.56, 129.11, 124.80, 93.78, 51.83, 41.26, 22.22. HRMS *m/z*: calcd for $C_{11}H_{13}F_3N_2O_4S_2$ $[M+H]^+$: 316.0420; found: 316.0423.

4.21.4 Methyl 3-amino-5-fluoro-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8r**).

Yield 32%. Mp: 201 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (d, *J* = 9.2 Hz, 1H), 5.84 (s, 2H), 3.91 (s, 3H), 2.66 (d, *J* = 3.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.64, 156.93, 155.29, 154.42, 150.07, 149.87, 145.66, 124.28, 114.41, 114.19, 100.02, 51.75, 18.75. HRMS *m/z*: calcd for $C_{10}H_9FN_2O_2S$ $[M+H]^+$: 241.0442; found: 241.0435.

4.21.5 Methyl 3-amino-6-methyl-5-nitrothieno[2,3-*b*]pyridine-2-carboxylate (**8t**).

Yield 88%. Mp: 182 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.34 (s, 1H), 7.48 (s, 2H), 3.80 (s, 3H), 2.83 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.69, 162.53, 154.57, 147.96, 142.87, 129.46, 124.38, 94.92, 52.05, 24.90. HRMS *m/z*: calcd for $C_{10}H_9N_3O_4S$ $[M+H]^+$: 268.0387; found: 268.0386.

4.22 3,5-diamino-6-methylthieno[2,3-*b*]pyridine-2-carboxylate (**8n**).

Prepared as 4.16. Yield 96%. Mp: 217 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.49 (s, 1H), 7.05 (s, 2H), 5.20 (s, 2H), 3.76 (s, 3H), 2.41 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.61, 148.91, 148.41, 147.41, 140.40, 125.09, 113.00, 93.44, 51.57, 21.66. HRMS *m/z*: calcd for $C_{10}H_{11}N_3O_2S$

$[M+H]^+$: 238.0645; found: 238.0639.

4.23 UT-B inhibition assay

UT-B inhibition activity was determined with an erythrocyte lysis assay as previously described [31]. Testing compounds were dissolved in DMSO. Blood sample was collected from 8-week-old male SD rats. Erythrocytes were diluted to a hematocrit value of 2% in hyperosmolar PBS containing 1.25 M urea and 5 mM glucose. Erythrocyte suspensions were preserved at ambient temperature for 2 h by periodic pipette mixture. 100 μL of the erythrocyte suspension was added to a 96-well microplate, followed by 1 μL of testing compound. After 6 min of incubation, 20 μL of the compound-treated erythrocyte suspension was quickly transferred to a 96-well black wall microplate which contained 180 μL of isotonic PBS and the resulted mixture was mixed sufficiently. Erythrocyte lysis was quantified from a single time point measure of absorbance at 710 nm with a plate reader (BioTek, Winooski, VT) within 5 min. Each assay plate included negative no-lysis controls (1.25 M urea + isotonic PBS with 1% DMSO) and positive full-lysis controls (1.25 M urea + distilled H_2O with 1% DMSO) that were mixed with DMSO vehicle-treated erythrocyte suspension. The percentage of erythrocyte lysis in each test well was calculated using control values from the same plate as: $\%lysis = 100\%(A_{neg}-A_{test})/(A_{neg}-A_{pos})$, where A_{test} , A_{neg} and A_{pos} referred to the absorbance values from a test well, a negative no-lysis control well and a positive full-lysis control well, respectively.

4.24 Diuretic effect on rat model of **8n**.

Adult male SD rats (body weight 200 ~ 250 g) were adapted in metabolic cages (Harvard Apparatus, Holliston, MA) for 4 days with a standard synthetic rodent diet. Water and food were provided ad libitum during the whole study. Bladder was emptied by gentle abdominal massage. Then, urine was collected with metabolic cages. **8n** in 40% 2-hydroxypropyl-bcyclodextrin was administered by subcutaneous injection. 40% 2-hydroxypropyl-bcyclodextrin was used as a vehicle control.

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Highlights

•Discovery of a novel structural type of urea transporter inhibitor with thienopyridine scaffold through SAR study of **PU48**.
• Most of the thienopyridines showed significant inhibition activity on UT-B *in vitro*.
•Compound **8n** exhibited potent activities both *in vitro* and *in vivo*.
•Compound **8n** showed predictable higher water solubility than **PU48**.