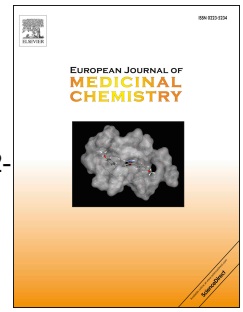
# Journal Pre-proof




Design, synthesis and biological evaluation of diamino substituted cyclobut-3-ene-1,2-dione derivatives for the treatment of drug-resistant tuberculosis

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# Design, synthesis and biological evaluation of diamino substituted cyclobut-3-ene-1,2-dione derivatives for the treatment of drug-resistant tuberculosis

Peng Li[1,2], Bin Wang[3], Gang Li[1,2], Lei Fu[3], Dongfeng Zhang[1,2], Ziyun Lin[1,2], Haihong Huang[1,2,*], Yu Lu[3,*]

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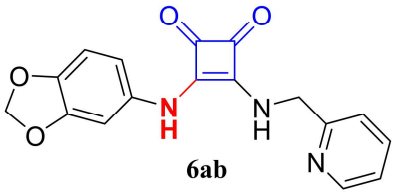
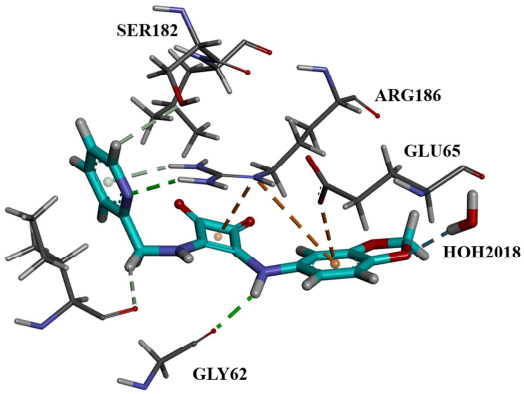



**6ab**

DS-TB: MIC < 0.5 μg/mL
DR-TB: MIC < 0.5 μg/mL
Lipophilicity: ClogP 0.89
Good druggability profiles
Potential *Mtb* ATP synthase inhibitor




The predicted binding mode of **6ab** with *Mtb* ATP synthase

# Design, synthesis and biological evaluation of diamino substituted cyclobut-3-ene-1,2-dione derivatives for the treatment of drug-resistant tuberculosis

Peng Li[1,2], Bin Wang[3], Gang Li[1,2], Lei Fu[3], Dongfeng Zhang[1,2], Ziyun Lin[1,2], Haihong Huang[1,2,*], Yu Lu[3,*]

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**ABSTRACT:**

*Mycobacterium tuberculosis* (*Mtb*) ATP synthase is an important target for treating drug-resistant infections and sterilizing the bacteria, spurring intensive efforts to develop new TB therapeutics based on this target. In this work, four novel series including furan-2(5*H*)-ketone (**3**, **4**), maleimide (**5**) and squaramide (**6**) derivatives were designed, respectively, through the strategy of scaffold morphing and hydrogen-bond introduction, using the selective *Mtb* ATP synthase inhibitor compound **2** as the lead compound. The result demonstrated that diamino substituted cyclobut-3-ene-1,2-dione compounds **6ab** and **6ah** displayed good to excellent *in vitro* anti-TB activities (MIC 0.452 ~ 0.963 μg/mL) with low cytotoxicity ($IC_{50}$ > 64 μg/mL). In addition, not only did compound **6ab** show effective activity against clinically isolated resistant strains, it also revealed good druggability profiles

including improved metabolic stability, no hERG channel inhibition potential, and acceptable oral bioavailability. The preliminary result of docking study and *in vitro* anti-bedaquiline-resistant strain test compared to compound **2** suggested that *Mtb* ATP synthase is most likely the target of compound **6ab**. The structure-activity relationship laid a good foundation for the identification of novel squaramides as a potential treatment of drug-resistant tuberculosis.

## KEYWORDS

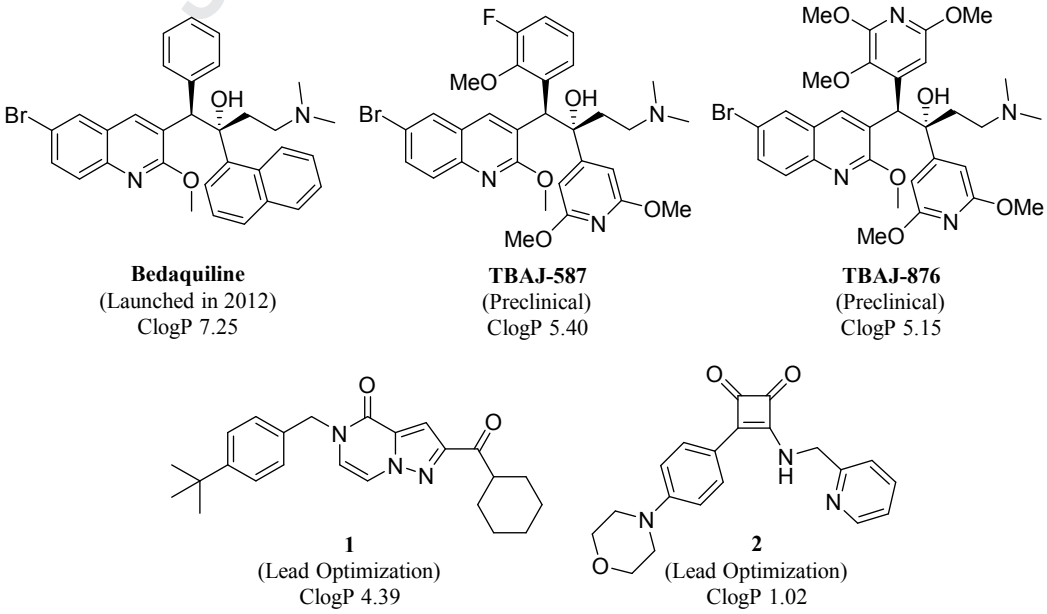
Squaramide; Drug-resistant tuberculosis; Structure-activity relationship; ATP synthase inhibitor.

### 1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). Worldwide, TB is one of the top 10 causes of death and the leading cause of death from a single infectious agent (above HIV/AIDS) [1]. In recent years, the emergence and spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB have presented great challenges to disease treatment and prevention [2, 3]. Due to the high mortality and socioeconomic burden, drug-resistant TB has become a serious social health and safety risk factor [4, 5]. In light of this gloomy scenario, new anti-TB drugs to tackle drug-resistant infections are urgent needed [6].

The energy metabolism pathway, essential for bacterial growth and survival, has emerged as a novel target pathway in TB drug discovery recently [7]. As part of the energy metabolism pathway, mycobacterial ATP synthase plays an important role for the survival of bacteria under both replicating and non-replicating states [8]. *Mtb* ATP synthase is a membrane-bound enzyme composed of multiple structural fragments which utilize the electrochemical gradient across the membrane to produce ATP [9, 10]. This target has been validated for the development of promising new anti-TB drugs [11], especially in complete sterilization [8]. One example of targeting bacterial energetics for the treatment of drug-resistant TB [12] is bedaquiline (Fig. 1), the first

selective inhibitor of the *Mtb* ATP synthase. Bedaquiline was approved as the first new anti-TB drug after rifampicin's launch in the 1960s [13]. The unique mechanism of action renders bedaquiline effective against drug-resistant TB [14, 15]. In addition, arylquinoline compounds TBAJ-587 and TBAJ-876, as bedaquiline analogs, were in preclinical studies with improved activity and reduced lipophilicity, exemplified by a lower ClogP compared to bedaquiline [16, 17]. The purpose of increasing polarity was to reduce cardiotoxicity related to bedaquiline's high lipophilicity [18]. Despite all the progress, new chemical entities are still needed to address the safety and cross-resistance issues. High-throughput screening has provided an efficient way to discover *Mtb* ATP synthase inhibitors with novel scaffolds distinct from arylquinolines [19]. Recently, compound **1** and **2** (Fig. 1), obtained from a high-throughput screening as non-quinolines *Mtb* ATP synthase inhibitors, were under lead optimization [20, 21]. Specifically, compound **2** (monoamino-cyclobut-3-ene-1,2-dione) with very low lipophilicity displayed efficiency in a mouse TB infection model and showed cross-resistance to some extent against a bedaquiline-resistant mutant, which indicated that this compound might occupy a different binding site of the ATP synthase compared to that of bedaquiline. Therefore, it is worth conducting a lead optimization to identify novel *Mtb* ATP synthase inhibitors based on compound **2** with lower lipophilicity and cardiotoxicity.




**Bedaquiline**
(Launched in 2012)
ClogP 7.25

**TBAJ-587**
(Preclinical)
ClogP 5.40

**TBAJ-876**
(Preclinical)
ClogP 5.15

**1**
(Lead Optimization)
ClogP 4.39

**2**
(Lead Optimization)
ClogP 1.02

**Fig. 1**. Structures of representative *Mtb* ATP synthase inhibitors.

It was found that the two carbonyls in the cyclobut-3-ene-1,2-dione scaffold exhibited no interaction with *Mtb* ATP synthase according to the docking study of compound **2** [21]. This provided the possibility for scaffold morphing to afford the furan-2(5*H*)-ones (**3** and **4**) (Fig. 2). In addition, maleimide and squaramide fragments were widely used in a variety of drug molecules as promising pharmacophores [22, 23]. Both fragments generally showed good metabolic stability [24-26]. To reduce the high *in vitro* metabolic clearance rate of compound **2**, we designed maleimide **5** and squaramide **6** derived from cyclobut-3-ene-1,2-dione by introducing NH group to increase the additional hydrogen-bond interaction between the compound and ATP synthase (Fig. 2).

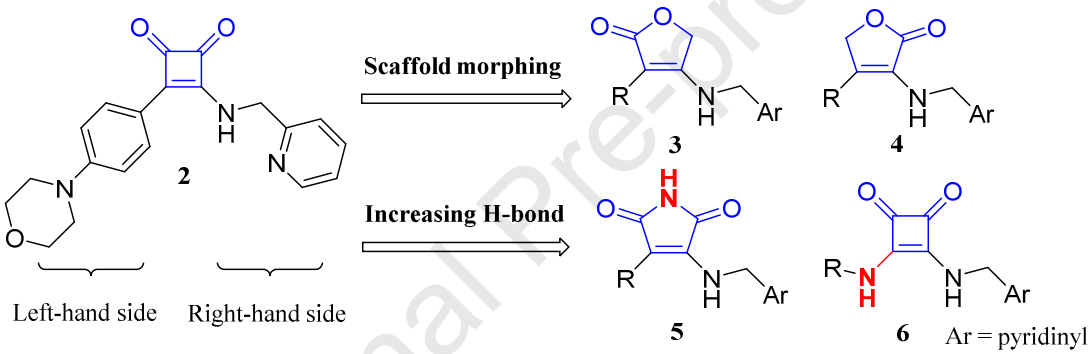


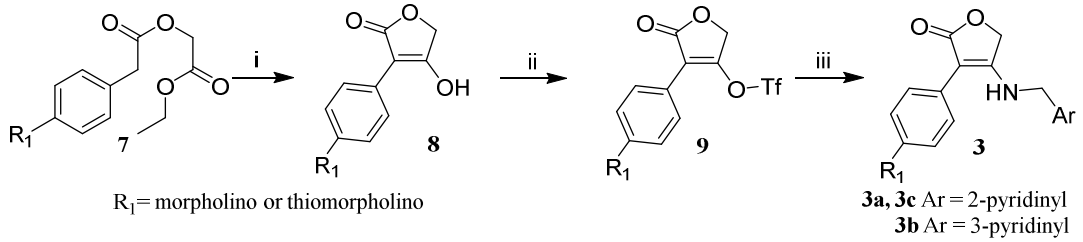

**Fig. 2**. Structure modification of compound **2** through scaffold morphing and increasing H-bond strategy.

Herein, we identified squaramide compound **6ab** as a promising anti-TB lead compound with good anti-TB activity, improved pharmacokinetic and safety profiles through a preliminary druggability evaluation.

## 2. Results and discussion

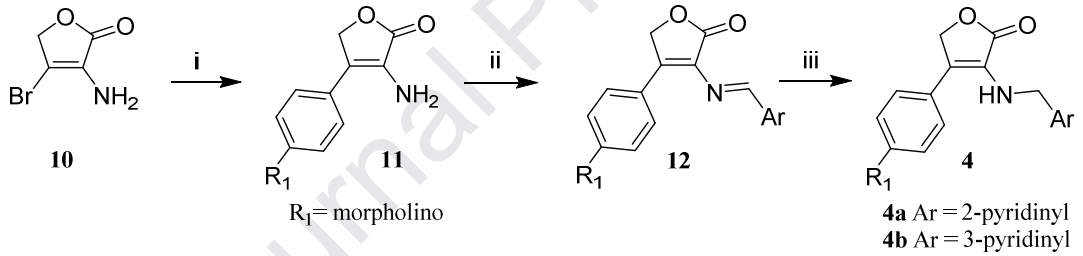
### *2.1. Chemistry*

The target compound **3** was synthesized following the procedure as outlined in **Scheme 1**. 2-Ethoxy-2-oxoethyl 2-phenylacetate derivative **7** was treated with *t*-BuOK to afford furanone **8** [27]. The sulfonylation of **8** in the presence of trifluoromethanesulfonic anhydride ($Tf_2O$) and DIPEA led to the intermediate **9** [28]. The replacement of **9** with the corresponding amines via conjugated addition–elimination in isopropanol resulted in the target compound **3**.



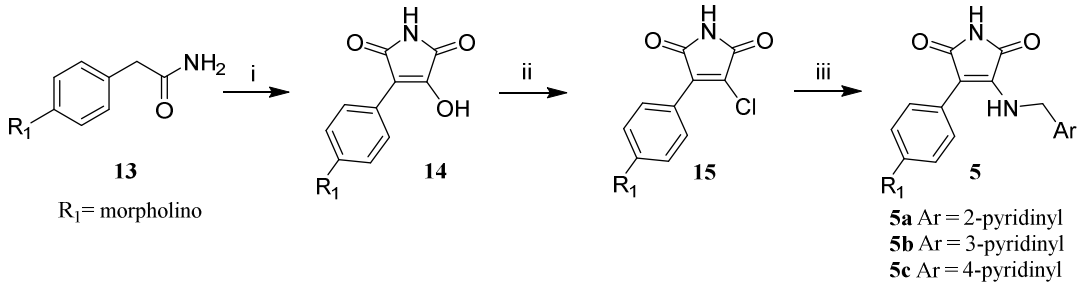

**Scheme 1**. Synthesis of the target compound **3**. Reagents and conditions: i) *t*-BuOK, THF, argon, rt, 12 h, 52-62%; ii) $Tf_2O$, DIPEA, $CH_2Cl_2$, argon, -78 □, 2 h, 54-63%; iii) the corresponding amine, isopropanol, argon, 85 □, 3 h, 30-40%.

The synthetic route of compound **4** is shown in **Scheme 2**. The Suzuki coupling reaction of 3-amino-4-bromofuran-2(5*H*)-one **10** and (4-morpholinophenyl)boronic acid in the presence of $Pd(PPh_3)_4$ and $Na_2CO_3$ led to the intermediate **11**. The condensation of **11** and the corresponding aldehyde was catalyzed by AcOH under microwave (MW) condition to afford the intermediate **12**. Reduction reaction of **12** with $NaBH_3CN$ in 1,2-dichloroethane under argon resulted in the target compound **4**.



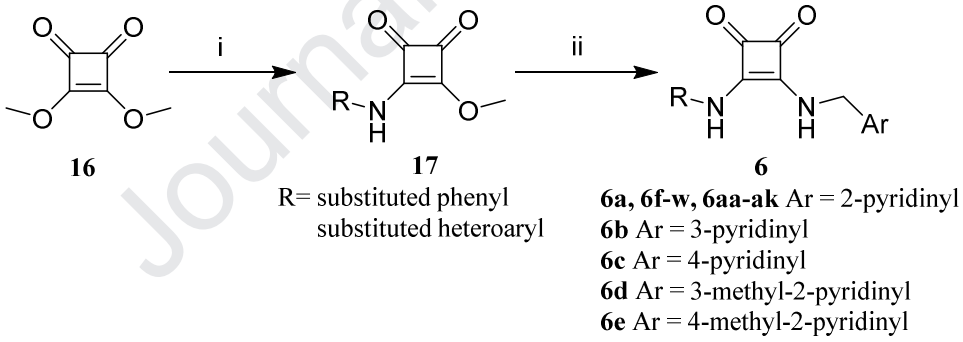

**Scheme 2**. Synthesis of the target compound **4**. Reagents and conditions: i) (4-morpholinophenyl)boronic acid, $Pd(PPh_3)_4$, $Na_2CO_3$, 1,4-dioxane, $H_2O$, argon, 70 □, 12 h, 61%; ii) the corresponding aldehyde, AcOH, MeOH, argon, MW, 100 □, 1 h, 57-89%; iii) $NaBH_3CN$, 1,2-dichloroethane, argon, reflux, 6 h, 37-66%.

The synthetic route for the target compound **5** is illustrated in **Scheme 3**. The heterocyclization of substituted 2-phenylacetamide **13** with diethyl oxalate in the presence of *t*-BuOK afforded intermediate **14**, which was then converted to vinyl chloride **15** by oxalyl chloride in $CH_2Cl_2$ [29]. The replacement of **15** with the corresponding amines via addition–elimination under MW condition led to the formation of target compound **5**.




**Scheme 3**. Synthesis of the target compound **5**. Reagents and conditions: i) diethyl oxalate, *t*-BuOK, THF, argon, 0 □ ~ rt, overnight, 93%; ii) oxalyl chloride, DMF, $CH_2Cl_2$, argon, 0 □, rt, 4 h, 75%; iii) the corresponding amine, DMF, MW, 150 □, 0.5 h, 27-37%.

The preparation of the target compound **6** is described in **Scheme 4**. Treatment of 3,4-dimethoxy-3-cyclobutene-1,2-dione **16** with the substituted aniline in ethanol afforded the intermediate amido-ester **17**. The treatment of **17** with the desired alkylamine in ethanol afforded the target compound **6** via addition–elimination [30]. The structures of all new target compounds are characterized by $^{1}$H NMR, $^{13}$C NMR and HRMS.



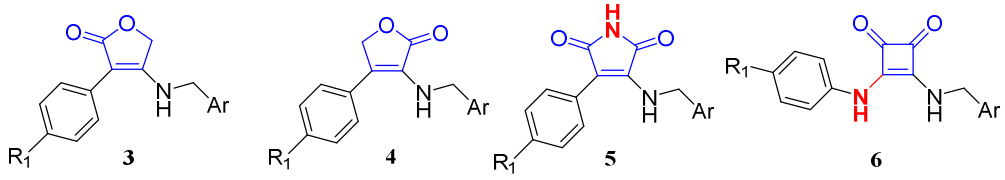

**Scheme 4**. Synthesis of the target compound **6**. Reagents and conditions: i) the requisite substituted aniline, ethanol, rt, overnight; ii) the corresponding amine, ethanol, rt, overnight, 40-94% (two steps).

*2.2. Identification of squaramide as a promising lead scaffold*

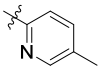
The first effort is to explore the privileged scaffold through the focused structure-activity relationship (SAR) studies around compound **2** by keeping the pyridine group on the right-hand side and morpholine or thiomorpholine substituents on the left. As the change of scaffold may affect the substrate-enzyme interaction, the position of the nitrogen on pyridine group was adjusted. As shown in **Table 1**, each

series displayed various *in vitro* anti-TB activities. Furan-2(5*H*)-one series (**3** and **4**) and maleimide series (**5**) dramatically lost activity with MIC > 32 μg/mL. We were pleased to find that squaramide series (**6**), as exemplified by compounds **6a** and **6b**, displayed an MIC of 3.745 μg/mL and 1.544 μg/mL, respectively. The position of the N atom in the pyridine is also crucial for the activity. For example, compounds with the 2-pyridinyl substituent could keep the activity (e.g. **6b**: MIC = 1.544 μg/mL) whereas compound **6c** lost the anti-TB activity (MIC > 32 μg/mL). In addition, the substituent on the pyridine ring could affect the activity shown by **6d** containing the 3-methyl-2-pyridinyl group with moderate activity while **6e** containing 4-methyl-2-pyridinyl group displayed no activity. Subsequently, compounds with an electron-donating or electron-withdrawing group on the phenyl ring on the left-hand side were evaluated against TB. Compounds with electron-withdrawing group such as chloro and trifluoromethyl (**6k**, **6l**) displayed a decreased activity compared to the compounds with electron-donating groups (**6f**, **6g**, **6h**), such as the substituted amino and methoxy groups. Among compounds **6h**, **6i** and **6j** with a methoxy group at a different position on the phenyl ring, only compound **6h** with the methoxy group at the 4-position displayed a moderate anti-TB activity with an MIC of 3.417 μg/mL. The aforementioned SAR result indicated that the rigid four-member ring (squaramide scaffold) and pyridinyl methyl amino group on the right-hand side played a crucial role on the anti-TB activity.

**Table 1** *In vitro* anti-TB activities of the target compounds.




| Compds. | $R_1$ | Ar | MIC[1] (μg/mL) | Compds. | $R_1$ | Ar | MIC[1] (μg/mL) |
|---|---|---|---|---|---|---|---|
| **3a** |  |  | >32 | **3b** |  |  | >32 |
| **3c** |  |  | >32 | **4a** |  |  | >32 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **4b** |  | | >32 | **5a** |  |  | >32 |
| **5b** |  | | >32 | **5c** |  |  | >32 |
| **6a** |  |  | 3.745 | **6b** |  |  | 1.544 |
| **6c** |  |  | >32 | **6d** |  |  | 3.646 |
| **6e** |  |  | >32 | **6f** |  |  | 3.758 |
| **6g** |  |  | 1.996 | **6h** | 4-$CH_3O$ |  | 3.417 |
| **6i** | 3-$CH_3O$ |  | 6.024 | **6j** | 2-$CH_3O$ |  | >32 |
| **6k** | Cl |  | 7.698 | **6l** | $CF_3$ |  | 14.896 |
| **2** | | | 0.122 | **INH** | | | 0.038 |

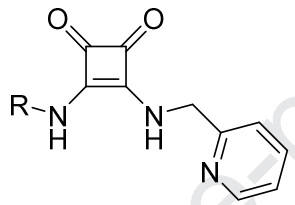
[1] Minimum inhibitory concentration against *M. tuberculosis* H37Rv.

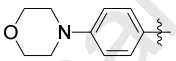
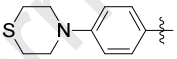
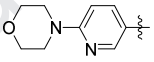
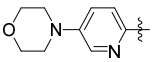
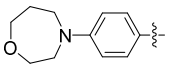
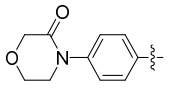
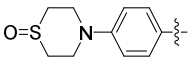
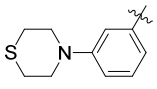
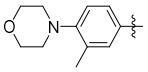
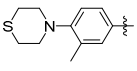
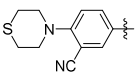
To further understand the SAR of squaramide series (**6**), structural modification on the left-hand side was explored by introducing substituted aromatic rings or heteroaryl rings. As displayed in **Table 2**, compounds (**6m**, **6n**) with pyridinyl instead of phenyl lost activity (MIC > 32 μg/mL). Expanding morpholine to 1,4-oxazepane (**6o**, MIC 6.649 μg/mL) or inserting carbonyl on the morpholine ring (**6p**, MIC 7.751 μg/mL) led to a weaker activity compared to **6a**. Sulfoxide **6q** had a much lower activity compared to its parent **6b**. Compound **6r** with a meta-substituent had no activity (MIC > 32 μg/mL) while the para-substituent was beneficial (**6b**). It was noted that compounds with two substituents at the ortho-position exhibited an improved activity compared to the compounds with mono-substituent, exemplified by **6s** (MIC 2.452 μg/mL) vs **6a** (MIC 3.745 μg/mL), and **6t** (MIC 0.496 μg/mL**)** vs **6b** (MIC 1.544 μg/mL), respectively. Moreover, compared to compound **6t**, compounds **6u**, **6v** and **6w** with substituents such as cyano, trifluoromethyl and methoxy, respectively, displayed a reduced activity. Compound **6w** with the methoxy ortho-substitution totally lost activity.

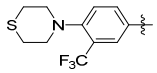
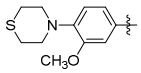
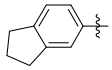
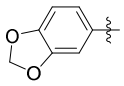
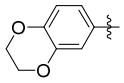
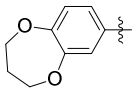
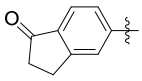
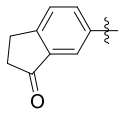
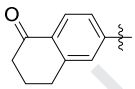
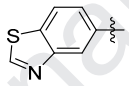
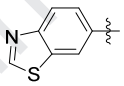
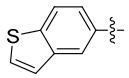
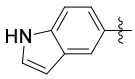
With this critical SAR between the substituent size, position and anti-TB activity

in hand, a series novel squaramide with benzo aliphatic ring (**6aa**, **6ae**, **6af**, **6ag**) or benzo heterocyclic ring (**6ab**, **6ac**, **6ad**, **6ah**, **6ai, 6aj** and **6ak**) on the left-hand side were further investigated. We were pleased to find that all compounds exhibited good anti-TB activity with MICs ranging from 0.452 ~ 3.456 μg/mL, except compounds containing benzothiophene (**6aj**) or benzopyrrole (**6ak**). Particularly, these active compounds showed no cytotoxicity against Vero cell ($IC_{50}$ > 64 μg/mL) and low lipophilicity with ClogP ranging from 0.84 ~ 1.81, indicating these compounds may display favorable pharmacokinetic properties.

**Table 2** The MIC, $IC_{50}$ and ClogP values of squaramide compounds



| Compds. | R | ClogP[1] | MIC[2] (μg/mL) | $IC_{50}$(Vero) (μg/mL) |
|---|---|---|---|---|
| **6a** |  | 0.25 | 3.745 | >64 |
| **6b** |  | 1.08 | 1.544 | >64 |
| **6m** |  | 0.27 | >32 | ND[3] |
| **6n** |  | 0.27 | >32 | ND |
| **6o** |  | 0.30 | 6.649 | ND |
| **6p** |  | 0.39 | 7.751 | ND |
| **6q** |  | -0.63 | 15.828 | ND |
| **6r** |  | 1.08 | >32 | ND |
| **6s** |  | 0.75 | 2.452 | >64 |
| **6t** |  | 1.58 | 0.496 | >64 |
| **6u** |  | 1.33 | 1.232 | >64 |

| | | | | |
|---|---|---|---|---|
| **6v** |  | 2.58 | 9.402 | ND |
| **6w** |  | 1.21 | >32 | ND |
| **6aa** |  | 1.81 | 0.979 | >64 |
| **6ab** |  | 0.89 | 0.452 | >64 |
| **6ac** |  | 0.84 | 1.885 | >64 |
| **6ad** |  | 1.00 | 2.222 | >64 |
| **6ae** |  | 0.92 | 3.456 | >64 |
| **6af** |  | 0.92 | 1.832 | >64 |
| **6ag** |  | 1.33 | 0.739 | >64 |
| **6ah** |  | 1.52 | 0.963 | >64 |
| **6ai** |  | 1.52 | 1.909 | >64 |
| **6aj** |  | 1.98 | >32 | ND |
| **6ak** |  | 0.78 | >32 | ND |
| **2** | | 1.02 | 0.122 | >64 |
| **INH** | | | 0.038 | |

[1] Calculated with ChemDraw Professional 16.0. [2] Minimum inhibitory concentration against *M. tuberculosis* H37Rv. [3] ND, not determined.

*2.3. Evaluation of compound **6ab** as a potential lead for treatment of TB*

Squaramide compounds with good *in vitro* activities (MIC < 1 μg/mL) were selected for further tests against two MDR (XDR)-TB clinical isolated strains (**Table 3**). All compounds demonstrated potent activities against drug-resistant TB strains with MIC < 1 μg/mL. The results indicated that this scaffold has the potential to treat

drug-resistant tuberculosis.

**Table 3** Activity of compounds against the selected clinical isolates of *M. tuberculosis*.

| Compds. | MIC (μg/mL) | | |
|---|---|---|---|
| | H37Rv[1] | 13946[2] | 14862[3] |
| **2** | 0.120 | 0.107 | 0.114 |
| **6t** | 0.748 | 0.257 | 0.207 |
| **6aa** | 1.753 | 0.903 | 0.940 |
| **6ab** | 0.483 | 0.470 | 0.499 |
| **6ag** | 0.962 | 0.488 | 0.895 |
| **6ah** | 3.634 | 3.531 | 3.374 |
| **INH** | 0.019 | 2.264 | >10 |
| **RFP** | 0.015 | >10 | 9.317 |

[1] Retested. [2] Resistance to isoniazid (INH), streptomycin (SM), rifampicin (RFP), ethambutol (EMB), rifabutin (RBT), paza-aminosalicylate (PAS) and ofloxacin (OLFX). [3] Resistance to INH, SM, RFP, EMB, PAS, prothionamide and capreomycin (CPM).

Due to the poor pharmacokinetic profile of the lead compound **2** [21], some representative squaramide compounds were chosen for the investigation of metabolic stability. As shown in **Table 4**, compound **6a** exhibited significantly improved stability both in mouse and human hepatocytes with much longer half-life ($t_{1/2}$ > 93.2 min) and lower intrinsic clearance (Clint < 7.4 μL/min/million cell), compared to compound **2**. As such, the design strategy of bringing more polarity appeared to improve the metabolic stability. The poor metabolic stability of compounds **6b** and **6t** may be attributed to the oxidation of sulfur. Compound **6aa** was unstable in both mouse and human hepatocytes. Compounds **6ag** and **6ab,** showed good to excellent stability in human hepatocytes, compared to compound **2**. The above results encouraged us to further explore the safety profiles of this squaramide series.

**Table 4** Mouse and human hepatocytes stability of selected compounds.

| Compds. | $t_{1/2}$ (min) | | Clint (μL/min/million cell) | |
|---|---|---|---|---|
| | Mouse | Human | Mouse | Human |
| **2** | 17.1 | 31.7 | 40.6 | 21.9 |
| **6a** | >93.2 | >93.2 | <7.4 | <7.4 |
| **6b** | 12.6 | 12.1 | 55.1 | 57.2 |
| **6t** | 5.4 | 9.0 | 127.8 | 77.0 |
| **6aa** | 11.1 | 10.3 | 62.4 | 67.1 |
| **6ab** | 17.0 | >93.2 | 40.8 | <7.4 |
| **6ag** | 10.3 | 41.9 | 67.2 | 16.6 |

Subsequently, selected compounds with good anti-TB activity and metabolic stability were screened for hERG inhibitory activity (**Table 5**). The low inhibition of hERG ($IC_{50}$ > 10 μM) indicated that squaramides **6** may display a low risk of blocking the cardiac potassium channel which may cause the QT prolongation.

**Table 5** The hERG inhibition of squaramide compounds

| Compds. | hERG $IC_{50}$ (μM) | Compds. | hERG $IC_{50}$ (μM) |
|---|---|---|---|
| **2** | >30 | **6aa** | >30 |
| **6a** | >30 | **6ab** | >30 |
| **6b** | >30 | **6ah** | >10 |
| **6t** | >10 | **Dofetilide**[1] | 0.013 |

[1] Positive control.

To further evaluate the druggability of compound **6ab**, the pharmacokinetic studies in BALB/c mice was conducted, following a single oral or an intravenous administration (**Table 6**). Compound **6ab** displayed a good PK profile as reflected by a high plasma exposure ($AUC_{0-\infty}$ = 2198 ng h/mL) and a high maximal plasma concentration ($C_{max}$ = 1333 ng/mL) after an oral administration at 50 mg/kg. In addition, compound **6ab** had a moderate clearance with CL of 51.4 mL/min/kg after an intravenously administration at a dose of 5 mg/kg. Thus far, all the above results supported compound **6ab** to be further evaluated as a promising potential candidate for the DR-TB treatment.

**Table 6** Mouse pharmacokinetic parameters of compound **6ab**

| | dose (mg/kg) | $C_{max}$ (ng/mL) | $t_{max}$ (h) | $t_{1/2}$[1] (h) | $AUC_{0-t}$ (ng·h/mL) | $AUC_{0-\infty}$[2] (ng·h/mL) | $MRT_{0-\infty}$[3] (h) | Clearance (mL/min/kg) | F[4] (%) |
|---|---|---|---|---|---|---|---|---|---|
| po | 50 | 1333 | 0.83 | 0.51 | 2197 | 2198 | 1.36 | | 13.1 |
| iv | 5 | | | 0.33 | 1667 | 1672 | 0.28 | 51.4 | |

[1] Plasma elimination half-life. [2] Plasma exposure. [3] Mean residence time. [4] Oral bioavailability.

*2.4. Docking studies with **6ab** as a potential ATP synthase inhibitor*

In order to investigate the binding mode of the squaramide in the active site of *Mtb* ATP synthase, the most promising compound **6ab** was docked into the active site of the enzyme using the Discovery Studio 2018 according to the reported homology model of *Mtb* ATP synthase and the receptor cavity (active site of the protein) [21]. The conformation corresponding to the highest docking score with CDOCKER protocol was selected as the best binding pose. As exhibited in Fig. 3, the pyridine moiety of compound **6ab** formed a hydrogen bond interaction with Arg186, which is consistent with the proposed binding mode of the pyridine in compound **2**. Furthermore, the introduction of nitrogen atom formed a key hydrogen bond with Gly62, which could provide an additional binding affinity. In addition, the benzene moiety formed a Pi-anion interaction with Glu65, which plays a crucial role in the interaction of bedaquiline with *Mtb* ATP synthase [15]. The dioxolane moiety could form a water hydrogen bond as compound **2**. Compound **6ab** could also form the non-conventional interaction with Ala66, Leu183, Ile59 and Ser182 to improve the affinity.

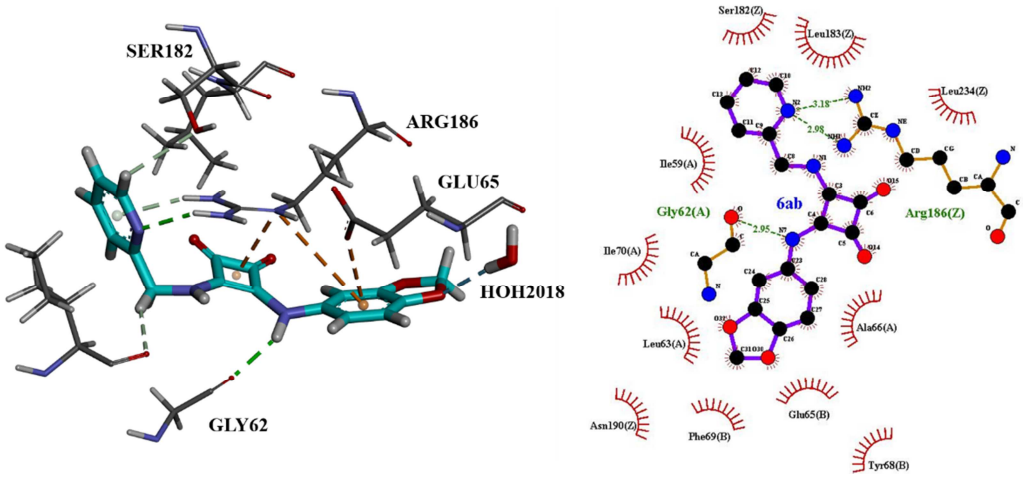


**Fig. 3**. Predicted binding mode of compound **6ab** with *Mtb* ATP synthase.

To further confirm the anti-TB mechanism of squaramides, we measured the activities of the selected squaramides against a bedaquiline-resistant strain. As shown in **Table 7**, compounds **6t** and **6ab** showed potent activities against the drug-sensitive H37Rv strain as well as compound **2** and bedaquiline, but they all demonstrated decreased potency against the bedaquiline-resistant strain. The MIC fold shifts of compounds **6t**, **6ab**, **2** and bedaquiline were 5.7, 7.5, 14.9 and 62, respectively. In this assay, the known drug INH showed the same sensitivity to H37Rv and the bedaquiline-resistant strain. Combined with the result that compounds **6t** and **6ab** displayed potent activity against two MDR (XDR)-TB clinical isolated strains (excluding the resistance strain to bedaquline) as compound **2**, an inhibitor of the *Mtb* ATP synthase. It suggested that squaramides **6t**, **6ab** may exert anti-TB activity through the inhibition of *Mtb* ATP synthase.

**Table 7** Activity of selected compounds against bedaquiline-resistant strain

| Compds. | MIC (μg/mL) | | MIC |
|---|---|---|---|
| | H37Rv[1] | resistant strain[2] | Foldshift[3] |
| **2** | 0.202 | 3.016 | 14.9 |
| **6t** | 0.845 | 4.851 | 5.7 |
| **6ab** | 0.500 | 3.758 | 7.5 |
| **Bedaquiline** | 0.036 | 2.235 | 62 |
| **INH** | 0.038 | 0.052 | - |

[1] Retested. [2] Resistance to bedaquiline.[3] Ratio of MIC values against bedaquiline-resistant strain and H37Rv.

## 3. Conclusion

In summary, we have identified a class of novel squaramide derivatives (**6**) as anti-TB agents through hydrogen-bond increasing strategy from cyclobut-3-ene-1,2-diones compound **2**. Compound **6ab** exhibited potent *in vitro* activity against H37Rv as well as two MDR (XDR)-TB clinical isolated strains. Preliminary druggability evaluation of compound **6ab** displayed good metabolic stability, low cytotoxicity, and acceptable oral bioavailability. The docking study revealed the key interactions of compound **6ab** with Arg186, Glu65 and Gly62

residues of *Mtb* ATP synthase, similar to compound **2**. The result of *in vitro* anti-bedaquiline-resistant strain assay further supported that compound **6ab** may exhibit anti-TB activity via the inhibition of ATP synthase. Hence, compound **6ab** is a promising anti-TB lead compound warranting further optimization, which will be reported in due course.

## 4. Experimental

### *4.1. General experimental information*

All the solvents and chemicals were obtained from commercial sources and used without further purification. TLC was performed on silica gel plates (GF254) with visualization of components by UV light (254 nm). Column chromatography was carried out on silica gel (200-300 mesh). The structural identities of the prepared compounds were confirmed by $^{1}$H NMR and $^{13}$C NMR spectroscopy and high-resolution mass spectrometry (HRMS). $^{1}$H NMR spectra were obtained on Varian Mercury-400 at 400 MHz or Varian Mercury-500 at 500 MHz. $^{13}$C NMR spectra were obtained on Bruker-400 at 100 MHz. Chemical shifts values were referenced to the residual solvent peak and reported in ppm (δ scale) and all coupling constant (*J*) values were given in Hz. $CDCl_3$ or DMSO-$d_6$ were used as the standard NMR solvents. The following multiplicity abbreviations are used: (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet and (br) broad. ESI-HRMS data were measured on Thermo Exactive Orbitrap plus spectrometer. Melting points were determined on Yanaco MP-J3 microscope melting point apparatus.

### *4.2. Experimental procedures*

#### *4.2.1. General procedure for the synthesis of compound **8***

To a magnetically stirred solution of intermediate **7** (5.0 mmol, 1.0 equiv.) in dry THF (50 mL) was added *t*-BuOK (10.0 mmol, 2.0 equiv.). The reaction mixture was stirred under argon atmosphere at room temperature for 12 h. The reaction was quenched with 10 mL $H_2O$ and the pH was adjusted to 1 with HCl (1N). The solvent was concentrated under reduced pressure, the residue was purified by column chromatography (1~3 % MeOH/$CH_2Cl_2$) to afford compound **8**.

*4.2.1.1. 4-Hydroxy-3-(4-morpholinophenyl)furan-2(5H)-one (**8a**).* Yield 61.6%. $^{1}$H

NMR (500 MHz, DMSO□$d_6$) δ 12.44 (s, 1H), 7.79 (d, *J* = 8.5 Hz, 2H), 6.95 (d, *J* = 8.6 Hz, 2H), 4.73 (s, 2H), 3.73 (d, *J* = 4.0 Hz, 4H), 3.10 (s, 4H).

*4.2.1.2. 4-Hydroxy-3-(4-thiomorpholinophenyl)furan-2(5H)-one (**8b**).* Yield 51.5%. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 8.12 (d, *J* = 8.8 Hz, 2H), 6.74 (d, *J* = 8.8 Hz, 2H), 3.94 (s, 2H), 3.35 – 3.30 (m, 4H), 2.72 – 2.66 (m, 4H).

*4.2.2 General procedure for the synthesis of compound **9***

DIPEA (1.5 mmol, 1.5 equiv.) was added to a stirred solution of compound **8** (1.0 mmol, 1.0 equiv.) in dry $CH_2Cl_2$ (20 mL) at room temperature. The solution was cooled to -78 °C and $Tf_2O$ (1.2 mmol, 1.2 equiv.) in 10 mL dry $CH_2Cl_2$ was slowly added under argon atmosphere. The reaction mixture was slowly risen to 0 °C and stirred for additional 2 h. The reaction mixture was diluted with $CH_2Cl_2$ (20 mL) and washed with $NaHCO_3$ (20 mL), brine (20 mL), dried over $Na_2SO_4$. The filtrate was removed under reduced pressure, the residue was purified by column chromatography (1% MeOH/$CH_2Cl_2$) to afford compound **9**.

*4.2.2.1. 4-(4-Morpholinophenyl)-5-oxo-2,5-dihydrofuran-3-yl trifluoromethanesulfonate (**9a**).* Yield 62.6%. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, *J* = 9.0 Hz, 2H), 6.98 (d, *J* = 9.0 Hz, 2H), 5.05 (s, 2H), 3.96 – 3.80 (m, 4H), 3.34 – 3.19 (m, 4H).

*4.2.2.2. 4-(4-Thiomorpholinophenyl)-5-oxo-2,5-dihydrofuran-3-yl trifluoromethanesulfonate (**9b**).* Yield 53.9%. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.87 – 7.70 (m, 2H), 6.96 (d, *J* = 8.9 Hz, 2H), 5.04 (s, 2H), 3.76 – 3.68 (m, 4H), 2.79 – 2.72 (m, 4H).

*4.2.3. General procedure for the synthesis of the target compound **3***

A capped tube was charged with compound **9** (0.2 mmol, 1.0 equiv.) and corresponding amine (0.4 mmol, 2.0 equiv.) in isopropanol (5 mL). The tube was flushed with argon, capped, and heated at 85 ℃ for 3 h. After cooling to room temperature, the solvent was removed under reduced pressure, the residue was purified by column chromatography (2% MeOH/$CH_2Cl_2$) to afford the target compound **3**.

*4.2.3.1. 3-(4-Morpholinophenyl)-4-((pyridin-2-ylmethyl)amino)furan-2(5H)-one (**3a**).*

Brown solid, yield 34.1%. mp 159-161 °C. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 8.58 (d, *J* = 3.5 Hz, 1H), 7.70 (s, 1H), 7.48 (d, *J* = 8.0 Hz, 2H), 7.21 (d, *J* = 7.6 Hz, 1H), 6.98 (d, *J* = 8.1 Hz, 2H), 6.49 (s, 1H), 4.83 (s, 2H), 4.44 (d, *J* = 4.9 Hz, 2H), 3.87 (brs, 4H), 3.18 (brs, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.1, 161.1, 155.0, 149.9, 149.5, 137.1, 128.6, 123.0, 122.5, 121.4, 116.0, 96.0, 66.9, 65.4, 49.2, 48.1. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{22}N_3O_3$, 352.1656; found, 352.1652.

*4.2.3.2. 3-(4-Morpholinophenyl)-4-((pyridin-3-ylmethyl)amino)furan-2(5H)-one (**3b**).* Brown solid, yield 39.8%. mp 246-248 °C. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 8.57 (s, 1H), 8.54 (s, 1H), 7.61 (d, *J* = 7.3 Hz, 1H), 7.39 – 7.30 (m, 3H), 6.93 (d, *J* = 8.3 Hz, 2H), 5.59 (brs, 1H), 4.76 (s, 2H), 4.36 (d, *J* = 5.7 Hz, 2H), 3.85 (s, 4H), 3.15 (s, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 173.8, 161.0, 150.2, 149.6, 148.5, 134.9, 132.6, 128.8, 124.0, 121.7, 116.0, 97.0, 66.8, 65.2, 49.1, 45.6. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{22}N_3O_3$, 352.1656; found, 352.1650.

*4.2.3.3. 3-(4-Thiomorpholinophenyl)-4-((pyridin-2-ylmethyl)amino)furan-2(5H)-one (**3c**).* Brown solid, yield 29.5%. mp 146-148 °C. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.58 (d, *J* = 4.6 Hz, 1H), 7.71 (td, *J* = 7.7, 1.7 Hz, 1H), 7.47 (d, *J* = 8.7 Hz, 2H), 7.28 – 7.24 (m, 1H), 7.22 (d, *J* = 7.8 Hz, 1H), 6.98 (d, *J* = 7.4 Hz, 2H), 6.51 (brs, 1H), 4.84 (s, 2H), 4.44 (d, *J* = 5.4 Hz, 2H), 3.60 – 3.56 (m, 4H), 2.76 (brs, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.1, 161.2, 155.0, 149.5, 137.1, 128.7, 123.0, 121.5, 117.4, 95.8, 65.4, 52.1, 48.1, 26.6. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{22}N_3O_2S$, 368.1427; found, 368.1426.

*4.2.4. Synthesis of 3-amino-4-(4-morpholinophenyl)furan-2(5H)-one (**11a**)*

To a mixture of 3-amino-4-bromofuran-2(5*H*)-one **10** (1.0 mmol, 1.0 equiv.), (4-morpholinophenyl)boronic acid (1.2 mmol, 1.2 equiv.) and $Na_2CO_3$ (1.2 mmol, 1.2 equiv.) in dioxane (5 mL) and $H_2O$ (1 mL) was added $Pd(PPh_3)_4$ (0.05 mmol, 0.05 equiv.). The mixture was degassed and stirred at 70 °C under argon atmosphere for 12 h. The reaction mixture was cooled to room temperature, diluted with $CH_2Cl_2$ (20 mL), dried over $Na_2SO_4$ and filtrated. The solvent was removed under reduced pressure, the residue was purified by column chromatography (1% MeOH/$CH_2Cl_2$) to afford compound **11a**. Yellow solid, Yield 61.0%. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.32

(d, *J* = 8.3 Hz, 2H), 6.97 (d, *J* = 8.3 Hz, 2H), 5.00 (s, 2H), 3.94 (brs, 2H), 3.92 – 3.82 (m, 4H), 3.30 – 3.16 (m, 4H).

*4.2.5. General procedure for the synthesis of compound **12***

To a solution of compound **11** (0.2 mmol, 1.0 equiv.) and the corresponding aldehyde (0.3 mmol, 1.5 equiv.) in dry MeOH (5 mL) was added catalytic amount of acetic acid (2 μL). The mixture was heated in MW at 100 °C under argon atmosphere for 1 h. After cooling to room temperature, the precipitate was filtered and washed with methanol to afford compound **12**.

*4.2.5.1. 4-(4-Morpholinophenyl)-3-((pyridin-2-ylmethylene)amino)furan-2(5H)-one (**12a**).* Yellow solid, yield 57.2%. $^1$H NMR (500 MHz, $CDCl_3$) δ 9.44 (s, 1H), 8.75 (d, *J* = 3.3 Hz, 1H), 8.20 (d, *J* = 7.7 Hz, 1H), 7.87 (d, *J* = 8.4 Hz, 2H), 7.82 (t, *J* = 7.6 Hz, 1H), 7.42 – 7.34 (m, 1H), 6.95 (d, *J* = 8.4 Hz, 2H), 5.21 (s, 2H), 3.87 (brs, 4H), 3.30 (brs, 4H).

*4.2.5.2. 4-(4-Morpholinophenyl)-3-((pyridin-3-ylmethylene)amino)furan-2(5H)-one (**12b**).* Yellow solid, yield 88.7%. $^1$H NMR (400 MHz, $CDCl_3$) δ 9.52 (s, 1H), 9.17 (s, 1H), 8.72 (d, *J* = 4.2 Hz, 1H), 8.26 (d, *J* = 7.8 Hz, 1H), 7.91 (d, *J* = 8.7 Hz, 2H), 7.46 (dd, *J* = 7.7, 4.8 Hz, 1H), 6.96 (d, *J* = 8.8 Hz, 2H), 5.23 (s, 2H), 3.90 – 3.86 (m, 4H), 3.34 – 3.30 (m, 4H).

*4.2.6. General procedure for the synthesis of the target compound **4***

To a solution of compound **12** (0.1 mmol, 1.0 equiv.) in dry 1,2-dichloroethane (5 mL) was added $NaBH_3CN$ (0.11 mmol, 1.1 equiv.). The mixture was refluxed under argon atmosphere for 6 h. The solvent was removed under reduced pressure, the residue was purified by column chromatography (1~3% MeOH/$CH_2Cl_2$) to afford the target compound **4**.

*4.2.6.1. 4-(4-Morpholinophenyl)-3-((pyridin-2-ylmethyl)amino)furan-2(5H)-one (**4a**).* Yellow solid, yield 65.5%. mp 73-75 °C. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.54 (d, *J* = 4.4 Hz, 1H), 7.62 (td, *J* = 7.7, 1.6 Hz, 1H), 7.33 (d, *J* = 8.7 Hz, 2H), 7.20 – 7.13 (m, 2H), 6.92 (d, *J* = 8.8 Hz, 2H), 5.05 (brs, 1H), 4.93 (s, 2H), 4.44 (s, 2H), 3.93 – 3.82 (m, 4H), 3.26 – 3.18 (m, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 172.0, 157.8, 150.9, 149.2, 136.7, 128.3, 128.1, 125.9, 122.8, 122.2, 121.6, 115.0, 69.8, 66.7, 49.6, 48.5.

HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{22}N_3O_3$, 352.1656; found, 352.1643.

*4.2.6.2. 4-(4-Morpholinophenyl)-3-((pyridin-3-ylmethyl)amino)furan-2(5H)-one (**4b**).* Yellow solid, yield 37.0%. mp 85-87 °C. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.50 (s, 1H), 8.32 (s, 1H), 7.56 (d, *J* = 7.5 Hz, 1H), 7.32 – 7.18 (m, 3H), 6.92 (d, *J* = 8.4 Hz, 2H), 4.91 (s, 2H), 4.29 (brs, 3H), 3.96 – 3.76 (m, 4H), 3.34 – 3.09 (m, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 172.0, 151.2, 148.7, 148.4, 135.5, 134.8, 128.4, 127.5, 127.3, 123.7, 122.3, 114.9, 70.0, 66.7, 48.3, 45.9. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{22}N_3O_3$, 352.1656; found, 352.1644.

*4.2.7. Synthesis of 3-hydroxy-4-(4-morpholinophenyl)-1H-pyrrole-2,5-dione (**14a**)*

To a solution of compound **13** (10.0 mmol, 1.0 equiv.) and diethyl oxalate (15.0 mmol, 1.5 equiv.) in dry DMF (50 mL) was added *t*-BuOK (30.0 mmol, 3.0 equiv.) in portion at 0 □, the reaction mixture was stirred at room temperature under argon atmosphere overnight. The reaction mixture was diluted with water (100 mL) and acidified with 6N HCl to pH = 1. The precipitate was filtered, washed with $H_2O$ and dried to afford compound **14a**. Yellow solid, yield 93.0%. $^1$H NMR (400 MHz, DMSO□*d*$_6$) δ 9.65 (brs, 1H), 8.02 (s, 2H), 6.86 (s, 2H), 3.82 – 3.64 (m, 4H), 3.13 – 2.94 (m, 4H).

*4.2.8. Synthesis of 3-chloro-4-(4-morpholinophenyl)-1H-pyrrole-2,5-dione (**15a**)*

A solution of oxalyl chloride (7.7 mmol, 1.1 equiv.) in dry $CH_2Cl_2$ (10 mL) was added dropwise to a solution of intermediate **14a** (7.0 mmol, 1.0 equiv.) in dry DMF (5 mL) and $CH_2Cl_2$ (5 mL) at 0 □. After stirring at room temperature for 4 h, the reaction mixture was poured into cold water (50 mL) and extracted with $CH_2Cl_2$ (50 mL), washed with $NaHCO_3$, brine, and dried over $Na_2SO_4$. The solvent was removed under reduced pressure and the residue was purified by column chromatography (1~20% ethyl acetate/petroleum) to afford the compound **15a**. Yellow solid, yield 75.2%. $^1$H NMR (400 MHz, DMSO□*d*$_6$) δ 11.33 (brs, 1H), 7.88 (d, *J* = 9.1 Hz, 2H), 7.07 (d, *J* = 9.2 Hz, 2H), 3.77 – 3.71 (m, 4H), 3.30 – 3.25 (m, 4H).

*4.2.9. General procedure for the synthesis of the target compound **5***

To a solution of compound **15a** (0.2 mmol, 1.0 equiv.) in DMF (1 mL) was

added corresponding amine (0.4 mmol, 2.0 equiv.). The reaction mixture was heated in MW at 150 °C for 0.5 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (1~3 %MeOH/$CH_2Cl_2$) to afford the target compound **5**.

*4.2.9.1. 3-(4-Morpholinophenyl)-4-((pyridin-2-ylmethyl)amino)-1H-pyrrole-2,5-dione (**5a**).* Yellow solid, yield 33.8%. mp 231-233 □. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.57 (d, *J* = 4.3 Hz, 1H), 7.65 (td, *J* = 7.7, 1.7 Hz, 1H), 7.31 (d, *J* = 8.7 Hz, 2H), 7.25 – 7.17 (m, 2H), 7.05 (d, *J* = 7.9 Hz, 1H), 6.95 (d, *J* = 8.5 Hz, 2H), 6.74 (brs, 1H), 4.47 (d, *J* = 5.2 Hz, 2H), 3.92 – 3.86 (m, 4H), 3.24 – 3.19 (m, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 171.9, 167.7, 155.0, 150.5, 149.2, 141.7, 136.7, 131.0, 122.7, 121.7, 120.8, 115.1, 102.0, 66.9, 49.0, 48.2. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{21}N_4O_3$, 365.1608; found, 365.1596.

*4.2.9.2. 3-(4-Morpholinophenyl)-4-((pyridin-3-ylmethyl)amino)-1H-pyrrole-2,5-dione (**5b**).* Yellow solid, yield 27.4%. mp 219-221 °C. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.53 (brs, 1H), 8.30 (s, 1H), 7.40 (d, *J* = 7.2 Hz, 1H), 7.33 (s, 1H), 7.26 – 7.21 (m, 1H), 7.18 (d, *J* = 8.3 Hz, 2H), 6.89 (d, *J* = 8.3 Hz, 2H), 5.57 (brs, 1H), 4.39 (d, *J* = 5.8 Hz, 2H), 3.89 – 3.83 (m, 4H), 3.22 – 3.16 (m, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 171.5, 167.6, 150.8, 149.3, 149.0, 140.9, 135.4, 135.1, 131.2, 123.6, 120.0, 115.0, 103.1, 66.8, 48.8, 45.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{21}N_4O_3$, 365.1608; found, 365.1596.

*4.2.9.3. 3-(4-Morpholinophenyl)-4-((pyridin-4-ylmethyl)amino)-1H-pyrrole-2,5-dione (**5c**).* Yellow solid, yield 37.0%. mp 245-247 °C. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.55 (d, *J* = 5.2 Hz, 2H), 7.68 (brs, 1H), 7.50 (d, *J* = 8.7 Hz, 2H), 7.21 (d, *J* = 4.2 Hz, 2H), 6.91 (d, *J* = 8.7 Hz, 2H), 3.98 (s, 2H), 3.89 – 3.83 (m, 4H), 3.29 – 3.24 (m, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 171.3, 170.7, 152.3, 149.3, 147.6, 140.2, 132.8, 130.9, 123.9, 118.6, 114.4, 66.6, 47.7, 29.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{21}N_4O_3$, 365.1608; found, 365.1593.

*4.2.10. General procedure for the synthesis of the target compound **6***

To a solution of 3,4-dimethoxy-3-cyclobutene-1,2-dione **16** (1.0 mmol, 1.0

equiv.) in ethanol (10 mL) was added substituted aniline (1.0 mmol, 1.0 equiv.). The reaction mixture was stirred at room temperature overnight. The precipitate was filtered, washed with cooled ethanol and dried to afford the intermediate **17**. To suspended solution of **17** in ethanol (10 mL) was added the corresponding amines (1.2 mmol, 1.2 equiv.). The reaction mixture was stirred at room temperature overnight. The precipitate was filtered, wash with cooled ethanol and dried to afford the target compound **6**.

*4.2.10.1.*

*3-((4-Morpholinophenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6a**).* Yellow solid, yield 89.3% (two steps). mp > 250 □. $^{1}$H NMR (400 MHz, DMSO□$d_6$) δ 9.70 (brs, 1H), 8.60 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.09 (brs, 1H), 7.83 (td, *J* = 7.7, 1.8 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.36 (dd, *J* = 6.1, 1.3 Hz, 1H), 7.32 (d, *J* = 9.2 Hz, 2H), 6.94 (d, *J* = 9.0 Hz, 2H), 4.92 (d, *J* = 5.7 Hz, 2H), 3.77 – 3.70 (m, 4H), 3.08 – 3.02 (m, 4H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.2, 180.7, 168.5, 163.7, 157.0, 149.1, 147.0, 137.1, 131.1, 122.6, 121.6, 119.2, 116.0, 66.0, 48.8, 48.3. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{21}N_4O_3$, 365.1608; found, 365.1604.

*4.2.10.2.*

*3-((Pyridin-2-ylmethyl)amino)-4-((4-thiomorpholinophenyl)amino)cyclobut-3-ene-1,2-dione (**6b**).* Off-white solid, yield 48.9% (two steps). mp > 250 °C. $^{1}$H NMR (400 MHz, DMSO□$d_6$) δ 9.69 (brs, 1H), 8.60 (ddd, *J* = 4.9, 1.7, 0.9 Hz, 1H), 8.08 (brs, 1H), 7.83 (td, *J* = 7.7, 1.8 Hz, 1H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.35 (ddd, *J* = 7.5, 4.8, 1.0 Hz, 1H), 7.31 (d, *J* = 8.9 Hz, 2H), 6.92 (d, *J* = 9.0 Hz, 2H), 4.92 (d, *J* = 5.5 Hz, 2H), 3.46 – 3.41 (m, 4H), 2.69 – 2.64 (m, 4H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.2, 180.7, 168.5, 163.7, 157.0, 149.1, 146.8, 137.1, 131.0, 122.7, 121.6, 119.3, 117.4, 51.5, 48.3, 25.6. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{21}N_4O_2S$, 381.1380; found, 381.1372.

*4.2.10.3.*

*3-((Pyridin-3-ylmethyl)amino)-4-((4-thiomorpholinophenyl)amino)cyclobut-3-ene-1,2-dione (**6c**).* White solid, yield 93.7% (two steps). mp > 250 ℃. $^{1}$H NMR (400 MHz,

DMSO□$d_6$) δ 9.53 (brs, 1H), 8.61 (d, *J* = 1.7 Hz, 1H), 8.53 (dd, *J* = 4.8, 1.7 Hz, 1H), 7.88 (brs, 1H), 7.82 – 7.78 (m, 1H), 7.42 (ddd, *J* = 7.9, 4.8, 0.8 Hz, 1H), 7.27 (d, *J* = 8.9 Hz, 2H), 6.91 (d, *J* = 9.1 Hz, 2H), 4.84 (d, *J* = 6.0 Hz, 2H), 3.46 – 3.41 (m, 4H), 2.68 – 2.64 (m, 4H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.2, 180.7, 168.2, 163.9, 148.9, 148.7, 146.8, 135.4, 134.2, 130.8, 123.7, 119.5, 117.3, 51.4, 44.6, 25.6. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{21}N_4O_2S$, 381.1380; found, 381.1377.

*4.2.10.4.*

*3-(((6-Methylpyridin-2-yl)methyl)amino)-4-((4-thiomorpholinophenyl)amino)cyclobut-3-ene-1,2-dione (**6d**).* Yellow solid, yield 65.1% (two steps). mp > 250 °C. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.66 (brs, 1H), 8.01 (brs, 1H), 7.71 (t, *J* = 7.7 Hz, 1H), 7.30 (d, *J* = 8.9 Hz, 2H), 7.20 (d, *J* = 7.7 Hz, 2H), 6.92 (d, *J* = 9.0 Hz, 2H), 4.85 (d, *J* = 4.9 Hz, 2H), 3.46 – 3.42 (m, 4H), 2.69 – 2.64 (m, 4H), 2.49 (s, 3H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.2, 180.6, 168.4, 163.7, 157.7, 156.5, 146.8, 137.4, 130.9, 122.0, 119.4, 118.5, 117.4, 51.5, 48.5, 25.6, 23.9. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{23}N_4O_2S$, 395.1536; found, 395.1531.

*4.2.10.5.*

*3-(((5-Methylpyridin-2-yl)methyl)amino)-4-((4-thiomorpholinophenyl)amino)cyclobut-3-ene-1,2-dione (**6e**).* Off-white solid, yield 44.8% (two steps). mp > 250 °C. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.36 (s, 1H), 7.60 (d, *J* = 7.7 Hz, 1H), 7.45 – 7.36 (m, 1H), 7.31 (d, *J* = 7.8 Hz, 2H), 6.91 (d, *J* = 7.0 Hz, 2H), 4.88 (s, 2H), 3.54 – 3.41 (m, 4H), 2.83 – 2.70 (m, 4H), 2.37 (s, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 183.7, 181.8, 168.6, 164.8, 153.8, 149.4, 148.5, 138.8, 133.3, 131.5, 122.9, 120.5, 118.7, 52.8, 48.8, 27.0, 18.2. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{23}N_4O_2S$, 395.1536; found, 395.1538.

*4.2.10.6.*

*3-((4-(Piperidin-1-yl)phenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6f**).* Off-white solid, yield 55.2% (two steps). mp > 250 °C. $^1$H NMR (500 MHz, DMSO□$d_6$) δ 9.66 (brs, 1H), 8.59 (s, 1H), 8.05 (brs, 1H), 7.83 (t, *J* = 7.7 Hz, 1H), 7.42 (d, *J* = 7.2 Hz, 1H), 7.35 (s, 1H), 7.28 (d, *J* = 7.2 Hz, 2H), 6.91 (d, *J* = 8.0 Hz, 2H), 4.92 (s, 2H), 3.06 (s, 4H), 1.61 (s, 4H), 1.51 (s, 2H). $^{13}$C NMR (100 MHz,

DMSO□$d_6$) δ 183.1, 180.7, 168.5, 163.7, 157.0, 149.1, 147.8, 137.1, 130.5, 122.7, 121.6, 119.2, 116.7, 50.0, 48.3, 25.2, 23.7. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{23}N_4O_2$, 363.1816; found, 363.1808.

*4.2.10.7.*

*3-((4-(Dimethylamino)phenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1, 2-dione (**6g**).* Yellow solid, yield 46.7% (two steps). mp 248-250 °C. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.63 (brs, 1H), 8.61 – 8.58 (m, 1H), 8.03 (brs, 1H), 7.83 (td, *J* = 7.7, 1.7 Hz, 1H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.35 (dd, *J* = 7.3, 5.0 Hz, 1H), 7.27 (d, *J* = 8.8 Hz, 2H), 6.73 (d, *J* = 9.0 Hz, 2H), 4.92 (d, *J* = 5.5 Hz, 2H), 2.85 (s, 6H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 182.9, 180.8, 168.3, 163.7, 157.1, 149.1, 146.9, 137.1, 128.7, 122.6, 121.6, 119.5, 113.1, 48.3, 40.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{18}H_{19}N_4O_2$, 323.1503; found, 323.1497.

*4.2.10.8.*

*3-((4-Methoxyphenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6h**).* Off-white solid, yield 61.3% (two steps). mp 206-208 □. $^1$H NMR (500 MHz, DMSO□$d_6$) δ 9.72 (brs, 1H), 8.60 (d, *J* = 4.0 Hz, 1H), 8.08 (brs, 1H), 7.83 (t, *J* = 7.5 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.39 – 7.33 (m, 3H), 6.92 (d, *J* = 8.5 Hz, 2H), 4.92 (s, 2H), 3.73 (s, 3H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.4, 180.6, 168.6, 163.7, 157.0, 155.2, 149.1, 137.1, 132.1, 122.7, 121.6, 119.6, 114.4, 55.2, 48.3. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{17}H_{16}N_3O_3$, 310.1186; found, 310.1177.

*4.2.10.9.*

*3-((3-Methoxyphenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6i**).* Off-white solid, yield 52.4% (two steps). mp 197-199 °C. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.84 (brs, 1H), 8.60 (d, *J* = 4.2 Hz, 1H), 8.20 (brs, 1H), 7.83 (td, *J* = 7.7, 1.8 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.35 (dd, *J* = 7.1, 5.2 Hz, 1H), 7.23 (t, *J* = 8.1 Hz, 2H), 6.92 (dd, *J* = 7.9, 1.7 Hz, 1H), 6.60 (dd, *J* = 8.1, 2.3 Hz, 1H), 4.94 (d, *J* = 5.5 Hz, 2H), 3.75 (s, 3H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 184.0, 180.5, 169.0, 163.6, 160.1, 156.8, 149.1, 140.2, 137.1, 130.1, 122.7, 121.7, 110.1, 108.3, 103.7, 55.0, 48.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{17}H_{16}N_3O_3$, 310.1186; found, 310.1183.

*4.2.10.10.*

*3-((2-Methoxyphenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione* ***(6j)***. Off-white solid, yield 45.3% (two steps). mp 126-128 °C. $^{1}$H NMR (400 MHz, DMSO□$d_6$) δ 9.37 (brs, 1H), 8.81 (brs, 1H), 8.60 (d, *J* = 4.5 Hz, 1H), 7.83 (t, *J* = 7.3 Hz, 1H), 7.78 (d, *J* = 7.8 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.40 – 7.29 (m, 1H), 7.06 – 6.99 (m, 2H), 6.95 – 6.90 (m, 1H), 4.95 (d, *J* = 5.7 Hz, 2H), 3.88 (s, 3H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 184.9, 180.9, 169.7, 164.1, 157.7, 149.8, 149.2, 137.7, 128.2, 124.1, 123.3, 122.3, 121.1, 120.4, 111.7, 56.3, 49.0. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{17}H_{16}N_3O_3$, 310.1186; found, 310.1182.

*4.2.10.11*.

*3-((4-Chlorophenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione* ***(6k)***. Off-white solid, yield 54.2% (two steps). mp 240-242 □. $^{1}$H NMR (400 MHz, DMSO□$d_6$) δ 9.91 (brs, 1H), 8.60 (d, *J* = 4.3 Hz, 1H), 8.21 (brs, 1H), 7.83 (td, *J* = 7.7, 1.7 Hz, 1H), 7.51 – 7.41 (m, 3H), 7.37 (d, *J* = 8.9 Hz, 2H), 7.36 – 7.32 (m, 1H), 4.93 (d, *J* = 4.8 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 184.6, 181.0, 169.7, 163.9, 157.3, 149.7, 138.5, 137.7, 129.7, 127.0, 123.3, 122.2, 120.1, 49.0. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{16}H_{13}ClN_3O_2$, 314.0691; found, 314.0690.

*4.2.10.12*.

*3-((Pyridin-2-ylmethyl)amino)-4-((4-(trifluoromethyl)phenyl)amino)cyclobut-3-ene-1, 2-dione* ***(6l)***. Yellow solid, yield 65.1% (two steps). mp 246-248 □. $^{1}$H NMR (400 MHz, DMSO□$d_6$) δ 10.00 (brs, 1H), 8.60 (d, *J* = 4.2 Hz, 1H), 8.25 (brs, 1H), 7.84 (td, *J* = 7.6, 1.5 Hz, 1H), 7.53 (d, *J* = 8.8 Hz, 2H), 7.44 (d, *J* = 7.8 Hz, 1H), 7.38 – 7.33 (m, 3H), 4.94 (d, *J* = 4.7 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 184.2, 180.4, 169.2, 163.4, 156.8, 149.1, 143.2, 138.3, 137.1, 122.7, 122.3, 121.7, 120.0 (q, $^{1}J_{F,C}$ = 254 Hz), 119.3, 48.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{17}H_{13}F_3N_3O_2$, 347.0882; found, 347.0890.

*4.2.10.13*.

*3-((6-Morpholinopyridin-3-yl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1, 2-dione* ***(6m)***. Light green solid, yield 71.7% (two steps). mp > 250 °C. $^{1}$H NMR (500 MHz, DMSO□$d_6$) δ 9.75 (brs, 1H), 8.60 (s, 1H), 8.17 – 8.08 (m, 2H), 7.83 (s, 1H), 7.77 (s, 1H), 7.42 (d, *J* = 7.1 Hz, 1H), 7.35 (s, 1H), 6.87 (d, *J* = 8.6 Hz, 1H), 4.92 (s,

2H), 3.69 (brs, 4H), 3.38 (brs, 4H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.4, 180.8, 168.7, 163.7, 157.0, 155.7, 149.1, 137.9, 137.1, 129.0, 127.0, 122.6, 121.6, 107.2, 65.8, 48.3, 45.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{19}H_{20}N_5O_3$, 366.1561; found, 366.1553.

*4.2.10.14.*

*3-((5-Morpholinopyridin-2-yl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1, 2-dione (**6n**).* Yellow solid, yield 55.6% (two steps). mp > 250 □. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.78 (brs, 1H), 8.59 (d, *J* = 4.1 Hz, 1H), 8.15 (d, *J* = 2.8 Hz, 2H), 7.83 (td, *J* = 7.7, 1.8 Hz, 1H), 7.76 (d, *J* = 7.9 Hz, 1H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.34 (ddd, *J* = 7.5, 4.9, 1.0 Hz, 1H), 6.86 (d, *J* = 9.1 Hz, 1H), 4.91 (d, *J* = 4.6 Hz, 2H), 3.71 – 3.67 (m, 4H), 3.41 – 3.35 (m, 4H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.5, 180.9, 168.8, 163.8, 157.1, 155.8, 149.2, 138.0, 137.2, 129.1, 127.2, 122.7, 121.7, 107.3, 65.9, 48.4, 45.6. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{19}H_{20}N_5O_3$, 366.1561; found, 366.1562.

*4.2.10.15.*

*3-((4-(1,4-Oxazepan-4-yl)phenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-en e-1,2-dione (**6o**).* Yellow solid, yield 56.7% (two steps). mp > 250 °C. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.62 (brs, 1H), 8.60 (d, *J* = 4.3 Hz, 1H), 8.03 (brs, 1H), 7.83 (t, *J* = 7.6 Hz, 1H), 7.42 (d, *J* = 7.7 Hz, 1H), 7.37 – 7.32 (m, 1H), 7.26 (d, *J* = 8.0 Hz, 2H), 6.73 (d, *J* = 7.8 Hz, 2H), 4.98 – 4.83 (m, 2H), 3.74 – 3.63 (m, 2H), 3.59 – 3.42 (m, 6H), 1.92 – 1.82 (m, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 182.8, 180.7, 168.2, 163.6, 157.1, 149.1, 144.3, 137.1, 128.0, 122.6, 121.6, 119.9, 112.1, 68.7, 68.4, 51.2, 48.3, 47.2, 28.5. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{23}N_4O_3$, 379.1765; found, 379.1759.

*4.2.10.16.*

*3-((4-(3-Oxomorpholino)phenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene -1,2-dione (**6p**).* Yellow solid, yield 40.4% (two steps). mp 250-252 °C. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.90 (brs, 1H), 8.61 (d, *J* = 4.4 Hz, 1H), 8.22 (brs, 1H), 7.84 (t, *J* = 7.6 Hz, 1H), 7.45 (t, *J* = 7.7 Hz, 3H), 7.38 – 7.33 (m, 3H), 4.94 (d, *J* = 4.5 Hz, 2H),

4.19 (s, 2H), 3.99 – 3.93 (m, 2H), 3.73 – 3.68 (m, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.9, 180.4, 169.0, 165.8, 163.5, 156.8, 149.1, 137.1, 137.1, 136.3, 126.4, 122.7, 121.7, 118.2, 67.6, 63.4, 48.9, 48.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{19}N_4O_4$, 379.1401; found, 379.1402.

*4.2.10.17.*

*3-((4-(1-Oxidothiomorpholino)phenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6q**).* Off-white solid, yield 55.6% (two steps). mp > 250 □. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.73 (brs, 1H), 8.60 (d, *J* = 4.3 Hz, 1H), 8.11 (brs, 1H), 7.83 (td, *J* = 7.7, 1.7 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.37 – 7.31 (m, 3H), 7.01 (d, *J* = 9.0 Hz, 2H), 4.92 (d, *J* = 5.1 Hz, 2H), 3.76 – 3.66 (m, 2H), 3.57 – 3.48 (m, 2H), 2.97 – 2.88 (m, 2H), 2.73 – 2.65 (m, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.5, 180.9, 168.8, 163.9, 157.3, 149.4, 145.4, 137.4, 131.3, 122.9, 121.9, 119.7, 117.0, 48.6, 43.9, 40.6. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{21}N_4O_3S$, 397.1329; found, 397.1327.

*4.2.10.18.*

*3-((3-Thiomorpholinophenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1, 2-dione (**6r**).* White solid, yield 82.5% (two steps). mp 204-206 °C. $^1$H NMR (500 MHz, DMSO□$d_6$) δ 9.74 (brs, 1H), 8.60 (s, 1H), 8.18 (brs, 1H), 7.83 (s, 1H), 7.43 (d, *J* = 7.1 Hz, 1H), 7.35 (s, 1H), 7.16 (dd, *J* = 17.6, 9.5 Hz, 2H), 6.67 (d, *J* = 6.0 Hz, 1H), 6.59 (d, *J* = 6.2 Hz, 1H), 4.93 (s, 2H), 3.62 – 3.54 (m, 4H), 2.68 – 2.60 (m, 4H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.7, 180.6, 168.9, 163.7, 156.9, 150.9, 149.1, 139.8, 137.1, 129.9, 122.7, 121.6, 110.2, 107.9, 105.3, 50.5, 48.4, 24.8. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{21}N_4O_2S$, 381.1380; found, 381.1380.

*4.2.10.19.*

*3-((3-Methyl-4-morpholinophenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6s**).* Off-white solid, yield 81.0% (two steps). mp 240-242 °C. $^1$H NMR (500 MHz, DMSO□$d_6$) δ 9.73 (brs, 1H), 8.60 (s, 1H), 8.10 (brs, 1H), 7.83 (s, 1H), 7.43 (d, *J* = 7.2 Hz, 1H), 7.35 (s, 1H), 7.24 (s, 2H), 7.02 (d, *J* = 8.6 Hz, 1H), 4.93 (s, 2H), 3.72 (s, 4H), 2.79 (s, 4H), 2.25 (s, 3H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.6, 180.6, 168.7, 163.7, 156.9, 149.1, 146.5, 137.1, 134.2, 133.0, 122.7, 121.6, 120.7, 119.7, 116.4, 66.5, 52.0, 48.3, 17.6. HRMS (ESI): m/z $[M+H]^+$ calcd for

$C_{21}H_{23}N_4O_3$, 379.1765; found, 379.1765.

*4.2.10.20.*

*3-((3-Methyl-4-thiomorpholinophenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6t**).* Off-white solid, yield 84.5% (two steps). mp 235-237 □. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.72 (brs, 1H), 8.66 – 8.52 (m, 1H), 8.10 (brs, 1H), 7.83 (td, *J* = 7.7, 1.7 Hz, 1H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.35 (dd, *J* = 7.3, 5.0 Hz, 1H), 7.27 – 7.20 (m, 2H), 7.01 (d, *J* = 9.3 Hz, 1H), 4.92 (d, *J* = 5.0 Hz, 2H), 3.04 – 2.99 (m, 4H), 2.76 – 2.71 (m, 4H), 2.21 (s, 3H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.6, 180.6, 168.7, 163.7, 156.9, 149.1, 147.8, 137.1, 134.3, 133.4, 122.7, 121.6, 120.6, 120.5, 116.4, 54.1, 48.3, 27.7, 17.5. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{23}N_4O_2S$, 395.1534; found, 395.1536.

*4.2.10.21.*

*3-((3-Cyano-4-morpholinophenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6u**).* Off-white solid, yield 86.3% (two steps). mp > 250 □. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.94 (brs, 1H), 8.60 (d, *J* = 4.5 Hz, 1H), 8.20 (brs, 1H), 7.86 – 7.79 (m, 2H), 7.55 (d, *J* = 8.9 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.35 (dd, *J* = 7.4, 4.9 Hz, 1H), 7.20 (dd, *J* = 8.8, 2.5 Hz, 1H), 4.93 (d, *J* = 5.8 Hz, 2H), 3.32 – 3.26 (m, 4H), 2.80 – 2.74 (m, 4H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.9, 180.6, 168.9, 163.1, 156.7, 151.4, 149.1, 137.1, 134.0, 124.0, 122.7, 121.7, 121.3, 117.5, 106.7, 54.2, 48.4, 27.3. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{20}N_5O_2S$, 406.1332; found, 406.1330.

*4.2.10.22.*

*3-((Pyridin-2-ylmethyl)amino)-4-((4-thiomorpholino-3-(trifluoromethyl)phenyl)amino)cyclobut-3-ene-1,2-dione (**6v**).* Off-white solid, yield 72.8% (two steps). mp 145-147 □. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 10.03 (brs, 1H), 8.60 (d, *J* = 4.1 Hz, 1H), 8.20 (brs, 1H), 7.84 (td, *J* = 7.7, 1.6 Hz, 1H), 7.79 (s, 1H), 7.63 (dd, *J* = 8.7, 2.4 Hz, 1H), 7.54 (d, *J* = 8.6 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.35 (dd, *J* = 7.4, 4.9 Hz, 1H), 4.94 (d, *J* = 4.4 Hz, 2H), 3.06 – 2.99 (m, 4H), 2.73 – 2.66 (m, 4H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 184.1, 180.5, 169.1, 163.2, 156.7, 149.1, 147.2, 137.1,

136.5, 126.8, 126.6, 123.5 (q, $^{1}J_{F, C}$ = 272 Hz), 122.7, 122.5, 121.7, 116.0, 55.1, 48.4, 27.6, 18.5. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{20}F_3N_4O_2S$, 449.1254; found, 449.1249.

*4.2.10.23.*

*3-((3-Methoxy-4-thiomorpholinophenyl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6w**).* Off-white solid, yield 44.3% (two steps). mp 250-252 °C. $^{1}$H NMR (500 MHz, DMSO□$d_6$) δ 9.78 (brs, 1H), 8.60 (d, *J* = 4.2 Hz, 1H), 8.13 (brs, 1H), 7.83 (t, *J* = 7.3 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.37 – 7.33 (m, 1H), 7.28 (s, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 6.81 (d, *J* = 10.1 Hz, 1H), 4.93 (s, 2H), 3.78 (s, 3H), 3.12 (brs, 4H), 2.71 (brs, 4H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.4, 180.7, 168.7, 163.5, 156.9, 152.8, 149.1, 137.4, 137.1, 134.6, 122.7, 121.6, 119.7, 109.7, 103.0, 55.3, 53.0, 48.4, 27.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{23}N_4O_3S$, 411.1485; found, 411.1480.

*4.2.10.24.*

*3-((2,3-Dihydro-1H-inden-5-yl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6aa**).* Off-white solid, yield 81.4% (two steps). mp 236-238 □. $^{1}$H NMR (400 MHz, DMSO□$d_6$) δ 9.76 (brs, 1H), 8.60 (d, *J* = 4.8 Hz, 1H), 8.13 (brs, 1H), 7.83 (td, *J* = 7.7, 1.6 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.35 (dd, *J* = 7.6, 5.0 Hz, 2H), 7.16 (s, 2H), 4.93 (d, *J* = 5.5 Hz, 2H), 2.81 (dt, *J* = 14.9, 7.4 Hz, 4H), 2.00 (p, *J* = 7.3 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.7, 180.6, 168.7, 163.8, 157.0, 149.1, 144.9, 138.1, 137.1, 124.6, 122.7, 121.6, 116.2, 114.3, 109.4, 48.4, 32.4, 31.5, 25.1. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{19}H_{18}N_3O_2$, 320.1393; found, 320.1391.

*4.2.10.25.*

*3-(Benzo[d][1,3]dioxol-5-ylamino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6ab**).* Brown solid, yield 77.3% (two steps). mp 218-220 □. $^{1}$H NMR (400 MHz, DMSO□$d_6$) δ 9.77 (brs, 1H), 8.60 (d, *J* = 4.7 Hz, 1H), 8.12 (brs, 1H), 7.83 (t, *J* = 7.6 Hz, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.35 (t, *J* = 6.4 Hz, 1H), 7.25 (s, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 6.75 (d, *J* = 8.3 Hz, 1H), 6.00 (s, 2H), 4.92 (d, *J* = 4.0 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.4, 180.6, 168.6, 163.5, 156.9, 149.1, 147.8, 142.9,

137.1, 133.5, 122.7, 121.6, 110.7, 108.4, 101.1, 100.5, 48.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{17}H_{14}N_3O_4$, 324.0979; found, 324.0983.

*4.2.10.26.*

*3-((2,3-Dihydrobenzo[b][1,4]dioxin-6-yl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6ac**).* Off-white solid, yield 53.5% (two steps). mp 254-256 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.68 (brs, 1H), 8.59 (d, *J* = 4.8 Hz, 1H), 8.11 (brs, 1H), 7.83 (td, *J* = 7.7, 1.8 Hz, 1H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.35 (dd, *J* = 7.0, 5.1 Hz, 1H), 7.12 (s, 1H), 6.81 (s, 2H), 4.92 (s, 2H), 4.25 – 4.18 (m, 4H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 183.6, 180.7, 168.8, 163.8, 157.1, 149.3, 143.7, 139.4, 137.3, 132.9, 122.9, 121.8, 117.6, 111.4, 107.4, 64.3, 64.0, 48.5. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{18}H_{16}N_3O_4$, 338.1135; found, 338.1126.

*4.2.10.27.*

*3-((3,4-Dihydro-2H-benzo[b][1,4]dioxepin-7-yl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6ad**).* Off-white solid, yield 85.4% (two steps). mp 231-233 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) δ 9.73 (brs, 1H), 8.60 (d, *J* = 2.3 Hz, 1H), 8.12 (brs, 1H), 7.83 (s, 1H), 7.42 (d, *J* = 7.2 Hz, 1H), 7.35 (s, 1H), 7.19 (s, 1H), 6.93 (s, 2H), 4.92 (s, 2H), 4.12 (s, 2H), 4.06 (s, 2H), 2.08 (s, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 183.6, 180.5, 168.7, 163.6, 156.9, 151.5, 149.1, 146.6, 137.1, 134.6, 122.7, 122.1, 121.6, 112.9, 111.4, 70.5, 48.4, 31.6. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{19}H_{18}N_3O_4$, 352.1292; found, 352.1296.

*4.2.10.28.*

*3-((1-Oxo-2,3-dihydro-1H-inden-5-yl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6ae**).* Off-white solid, yield 43.0% (two steps). mp > 250 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) δ 10.27 (brs, 1H), 8.61 (s, 1H), 8.42 (brs, 1H), 7.84 (s, 1H), 7.66 (s, 1H), 7.58 (d, *J* = 7.9 Hz, 1H), 7.45 (d, *J* = 7.1 Hz, 1H), 7.40 (d, *J* = 7.0 Hz, 1H), 7.36 (s, 1H), 4.95 (s, 2H), 3.06 (s, 2H), 2.59 (s, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 204.8, 185.4, 180.9, 170.3, 163.5, 157.9, 157.2, 149.7, 145.3, 137.7, 131.3, 125.0, 123.3, 122.2, 117.9, 115.0, 49.0, 36.5, 26.0. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{19}H_{16}N_3O_3$, 334.1186; found, 334.1188.

*4.2.10.29.*

*3-((3-Oxo-2,3-dihydro-1H-inden-5-yl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6af**).* Off-white solid, yield 65.0% (two steps). mp > 250 °C. $^{1}$H NMR (500 MHz, DMSO□$d_6$) δ 9.99 (brs, 1H), 8.60 (s, 1H), 8.20 (brs, 1H), 7.84 (s, 1H), 7.74 – 7.63 (m, 2H), 7.55 (d, *J* = 7.4 Hz, 1H), 7.45 (d, *J* = 7.2 Hz, 1H), 7.36 (s, 1H), 4.94 (s, 2H), 3.05 (s, 2H), 2.65 (s, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 206.4, 184.7, 181.1, 169.6, 164.0, 157.4, 149.8, 149.7, 139.0, 138.2, 137.7, 128.2, 125.6, 123.3, 122.2, 111.8, 49.0, 36.9, 25.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{19}H_{16}N_3O_3$, 334.1186; found, 334.1188.

*4.2.10.30.*

*3-((5-Oxo-5,6,7,8-tetrahydronaphthalen-2-yl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6ag**).* Yellow solid, yield 69.1% (two steps). mp 242-244 °C. $^{1}$H NMR (400 MHz, DMSO□$d_6$) δ 10.13 (brs, 1H), 8.60 (s, 1H), 8.33 (brs, 1H), 7.82 (d, *J* = 7.7 Hz, 2H), 7.48 – 7.38 (m, 2H), 7.38 – 7.30 (m, 2H), 4.94 (s, 2H), 2.89 (s, 2H), 2.53 (s, 2H), 2.01 (s, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 196.5, 185.4, 180.9, 170.1, 163.5, 157.2, 149.7, 147.0, 143.8, 137.7, 128.8, 127.1, 123.3, 122.2, 117.2, 116.5, 49.0, 38.9, 29.8, 23.3. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{20}H_{18}N_3O_3$, 348.1343; found, 348.1342.

*4.2.10.31.*

*3-(Benzo[d]thiazol-5-ylamino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6ah**).* Yellow solid, yield 87.2% (two steps). mp > 250 °C. $^{1}$H NMR (500 MHz, DMSO□$d_6$) δ 10.04 (brs, 1H), 9.39 (s, 1H), 8.61 (brs, 1H), 8.26 (s, 2H), 8.10 (d, *J* = 7.7 Hz, 1H), 7.84 (s, 1H), 7.51 (d, *J* = 6.8 Hz, 1H), 7.45 (d, *J* = 7.1 Hz, 1H), 7.36 (s, 1H), 4.96 (s, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 184.0, 180.6, 169.1, 163.6, 157.4, 156.8, 154.0, 149.1, 137.8, 137.2, 127.5, 122.9, 122.7, 121.7, 117.0, 111.5, 48.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{17}H_{13}N_4O_2S$, 337.0754; found, 337.0753.

*4.2.10.32.*

*3-(Benzo[d]thiazol-6-ylamino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6ai**).* Yellow solid, yield 71.4% (two steps). mp > 250 °C. $^{1}$H NMR (500 MHz,

DMSO□$d_6$) δ 10.07 (brs, 1H), 9.24 (d, *J* = 12.0 Hz, 1H), 8.60 (brs, 1H), 8.34 – 8.13 (m, 2H), 8.08-7.94 (m, 1H), 7.83 (s, 1H), 7.56 (s, 1H), 7.40 (d, *J* = 43.8 Hz, 2H), 4.95 (s, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 184.0, 180.6, 169.2, 163.4, 156.8, 154.3, 149.1, 148.8, 137.1, 136.9, 134.8, 123.4, 122.7, 121.7, 117.8, 110.5, 48.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{17}H_{13}N_4O_2S$, 337.0754; found, 337.0753.

*4.2.10.33.*

*3-(Benzo[b]thiophen-5-ylamino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-di one (**6aj**).* Off-white solid, yield 55.3% (two steps). mp 235-237 °C. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 9.91 (brs, 1H), 8.61 (d, *J* = 4.4 Hz, 1H), 8.22 (brs, 1H), 7.95 (d, *J* = 8.8 Hz, 2H), 7.84 (td, *J* = 7.7, 1.8 Hz, 1H), 7.78 (d, *J* = 5.4 Hz, 1H), 7.45 (t, *J* = 6.8 Hz, 2H), 7.40 (d, *J* = 5.4 Hz, 1H), 7.35 (dd, *J* = 7.1, 5.2 Hz, 1H), 4.95 (s, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 184.0, 180.9, 169.1, 164.0, 157.1, 149.3, 140.4, 137.4, 136.2, 133.7, 129.0, 124.0, 123.4, 122.9, 121.9, 116.4, 112.3, 48.6. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{18}H_{14}N_3O_2S$, 336.0801; found, 336.0800.

*4.2.10.34.*

*3-((1H-Indol-5-yl)amino)-4-((pyridin-2-ylmethyl)amino)cyclobut-3-ene-1,2-dione (**6ak**).* Off-white solid, yield 81.3% (two steps). mp > 250 °C. $^1$H NMR (400 MHz, DMSO□$d_6$) δ 11.06 (brs, 1H), 9.76 (brs, 1H), 8.60 (d, *J* = 4.1 Hz, 1H), 8.08 (brs, 1H), 7.83 (td, *J* = 7.7, 1.7 Hz, 1H), 7.63 (s, 1H), 7.44 (d, *J* = 7.8 Hz, 1H), 7.38 – 7.32 (m, 3H), 7.16 (d, *J* = 6.7 Hz, 1H), 6.38 (s, 1H), 4.94 (s, 2H). $^{13}$C NMR (100 MHz, DMSO□$d_6$) δ 183.1, 180.8, 168.4, 164.2, 157.1, 149.1, 137.1, 132.6, 131.0, 127.8, 126.4, 122.6, 121.6, 113.6, 111.8, 109.2, 101.0, 48.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{18}H_{15}N_4O_2$, 319.1190; found, 319.1191.

### *4.3. Biological evaluation*

#### *4.3.1. Minimum inhibitory concentration assay (MIC)*

MICs against replicating *M. tuberculosis* were determined by the microplate alamar blue assay (MABA) [31]. RFP, INH and **2** were included as positive controls. *M. tuberculosis* H37Rv, clinical isolates (14826, 13946 strain) or bedaquiline-resistant

strains were grown to late log phase (70 to 100 Klett units) in Difco Middlebrook 7H9 Broth (Seebio) supplemented with 0.2% (vol/vol) glycerol, 0.05% Tween 80, and 10% (vol/vol) albumin-dextrose-catalase (Seebio) (7H9-ADC-TG). Cultures were centrifuged, washed twice, and then suspended in phosphate-buffered saline. Suspensions were passed through an 8 μm-pore-size filter to remove clumps, and aliquots were frozen at -80 °C. Twofold dilutions of compounds were prepared in 7H9-ADC-TG in a volume of 100 μL in 96-well clear-bottom microplates (BD). *M. tuberculosis* (100 μL containing $2 \times 10^5$ CFU) was added to yield a final testing volume of 200 μL. The plates were incubated at 37 °C; on day 7 of incubation, 12.5 μL of 20% Tween 80 and 20 μL of alamar blue were added to all wells. After incubation at 37 °C for 16 to 24 h, the fluorescence was read at an excitation of 530 nm and an emission of 590 nm. The MIC was defined as the lowest concentration effecting a reduction in fluorescence of ≥ 90% relative to the mean of replicate bacterium-only controls.

*4.3.2. Cytotoxicity assay*

Vero cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were incubated in a humidified atmosphere of 5% $CO_2$ at 37 °C. Stocks of cells were cultured in 25-$cm^2$ tissue culture flasks and subcultured two to three times per week. Cytotoxicity testing was performed in a transparent 96-well microplate. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. The cells were incubated at 37 °C under 5% $CO_2$ until confluent and then diluted with culture medium to $4 \times 10^5$ cells/mL. Threefold serial dilutions of the stock solutions resulted in final concentrations of 64 to 0.26 μg/mL in a final volume of 100 μL. After incubation at 37 °C for 48 h, the medium was removed, and the monolayers were washed twice with 100 μL of warm Hanks balanced salt solution (HBSS). Warm medium (100 μL) and 10 μL of freshly made methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) were added to each well, and then the plates were incubated for 4 h, after which the absorbance was determined at 492 nm.

*4.3.3. Hepatocyte stability assay*

The assay was performed with hepatocytes from pooled male CD-1 mouse (Bioreclamation IVT) and pooled human (Bioreclamation IVT). Pre-incubate the hepatocyte suspensions (2 million cells/mL) in the 37 °C $CO_2$ incubator for 20 min. Add 400 μL of test compound solution (2 μM) into wells on a 24-well plate. Start the reaction by adding 400 μL of hepatocytes suspensions into wells on a 24-well plate. Compounds were tested at 1 μM with a final hepatocyte concentration of 1 million cells/mL. Gently shake the plate to mix. Incubate the plates in the $CO_2$ incubator. At each time point (0, 30 min), remove 30 μL of the reaction mixture from the plate. Stop the reaction by adding 300 μL of quenching solution to the 30 μL reaction mixtures. Samples were mixed well and then were centrifuged at 4,000 rpm at 4 °C for 15 min. Remove 100 μL of supernatant for LC-MS/MS analysis. The assay evaluated the metabolic stability of compounds in hepatocytes by measuring amount of parent remaining of the test compounds.

*4.3.4. hERG Inhibition assay*

The electrophysiology recording of hERG channel current was carried out following the standard protocol as described previously [32]. HEK 293 cells were stably transfected with human Ether-à-go-go related gene (hERG) channel. The voltage-gated hERG potassium channel current was recorded at room temperature (25 °C) from randomly selected transfected cells under whole-cell manual patch clamp systems equipped with EPC10 USB (HEKA) or Multiclamp 700B amplifier (Molecular Devices), while electrical data was digitalized by Digidata1440A with sampling frequency at 10 kHz using Patchmaster or pClamp10 respectively. hERG current inhibition in presence of 5 concentrations, including 30, 10, 3.0, 1.0 and 0.3 μM, was tested for $IC_{50}$ determination. Dofetilide was also included as a positive control to ensure the accuracy and sensitivity of the test system. All experiments were performed in duplicate for $IC_{50}$ determination. The compound with $IC_{50} > 30$ μM was generally considered to have a lower potential for hERG $K^+$ channel inhibition.

*4.3.5. Pharmacokinetic studies in mouse*

All animal protocols were approved by Institute Animal Care and Use Committee. The selected compound **6ab** was subjected to pharmacokinetic studies in Balb/c mouse (male) weighing 26 to 27 g with three mice in oral administration group and three mice in intravenous injection group. The tested compound was formulated at a concentration of 5 mg/mL for a dose of 50 mg/kg given orally (p.o.) and at 1 mg/mL for a dose of 5 mg/kg given intravenously (i.v.). The tested compound was formulated with 0.5% carboxymethyl cellulose for p.o. administration and with 10%DMSO/50%PEG400/40%water for i.v. administration. Blood samples were collected at 5, 15, 30 min, 1, 2, 4, 7, 24 h after oral dosing and i.v. administration. Plasma was harvested and stored at -80 °C until analysed. Plasma samples were extracted with acetonitrile containing terfenadine as an internal standard using a 20:1 extractant-to-plasma ratio. Analyte quantitation was performed by a LC/TSQ Quantum Access mass spectrometer (AB Sciex 5500). Chromatographic separation was performed on a Kinetex C18 100A column (30 mm × 3 mm, 2.6 μm) with an isocratic mobile phase of acetonitrile/water containing 0.1% formic acid at 0.7 mL/min flow rate. Compound detection on the mass spectrometer was performed in electrospray positive ionization mode. The selected reaction monitoring transition was m/z 324.19/92.20. The pharmacokinetic parameters were calculated using WinNonlin software version 6.3 based on non-compartmental analysis (Pharsight Corporation, Mountain View, USA). The oral bioavailability was calculated as the ratio between the area under the curve (AUC) following intravenous administration corrected for dose ($F = (AUC_{p.o.} \times dose_{i.v.})/(AUC_{i.v.} \times dose_{p.o.}) \times 100\%$).

## Notes

The authors declare no competing financial interest.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at

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- Novel series of furan-2(5*H*)-ketone, maleimide and squaramide derivatives were synthesized.
- The squaramide series displayed excellent anti-TB activity and low cytotoxicity.
- Compound **6ab** exhibited potent *in vitro* activity against both drug-sensitive and drug-resistant *M. tuberculosis*.
- Compound **6ab** demonstrated good druggability profiles.
- Compound **6ab** was a promising lead as a potential *Mtb* ATP synthase inhibitor.

**Declaration of interests**

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: