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Discovery of a Novel, Highly Potent, and Selective Thieno[3,2-*d*]pyrimidinone-Based Cdc7 inhibitor with a Quinuclidine Moiety (TAK-931) as an Orally Active Investigational Anti-Tumor Agent

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ABSTRACT: In our pursuit of developing a novel, potent, and selective cell division cycle 7 (Cdc7) inhibitor, we optimized the previously-reported thieno[3,2-*d*]pyrimidinone analogue **I** showing time-dependent Cdc7 kinase inhibition and slow dissociation kinetics. These medicinal chemistry efforts led to the identification of compound **3d** which exhibited potent cellular activity, excellent kinase selectivity, and anti-tumor efficacy in a COLO205 xenograft mouse model. However, the issue of formaldehyde adduct formation emerged during a detailed study of **3d**, which was deemed an obstacle to

further development. A structure-based approach to circumvent the adduct formation culminated in the discovery of compound **11b** (**TAK-931**) possessing a quinuclidine moiety as a preclinical candidate. In this paper, the design, synthesis, and biological evaluation of this series of compounds will be presented.

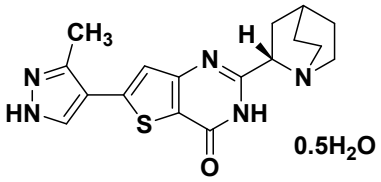
INTRODUCTION

DNA replication, a fundamental process for cell proliferation, begins from the origin finding points which consist of pre-replicative complexes formed during the previous G1 phase of the cell cycle. Mechanisms that control entry in the S phase and proper execution of DNA synthesis are often impaired in malignant cells. Thus, targeting the aberrant mechanisms is a potential strategy for cancer therapy.

The serine/threonine kinase, cell division cycle 7 (Cdc7) has emerged as an attractive target for the treatment of cancer. Cdc7 plays a crucial role in the initiation and maintenance of DNA replication in eukaryotic cells.[1-3] Phosphorylation of one or more residues of minichromosome maintenance 2 (MCM2) by the Cdc7 kinase induces loading of other accessory factors and subsequent generation of active replication forks, thereby initiating DNA replication. Depletion of Cdc7 using small interfering RNA leads to induction of apoptosis in cancer cells, whereas normal cells are spared from knockdown of the Cdc7 protein.[4]

However, the proof of concept (POC) of Cdc7 inhibitor[5-12] in clinical trials has not been reported so far. We envisioned that one of the reasons is difficulty in identifying potent and selective Cdc7 inhibitor. There are multiple reports of potent Cdc7 inhibitors with low nanomolar activities, but most of them showed weak pharmacodynamic (PD) effects in cells (IC_{50} >1 μM). The finding suggests that a

significant gap between cell-free and cell-based activities still remains as a challenge for discovering a desirable clinical candidate. Recently, we have overcome the issues and disclosed preclinical pharmacological profiles of TAK-931 (Figure 1), novel, potent Cdc7-selective inhibitor possessing strong cellular activities, currently being investigated under clinical trials.[13] In this report, we describe lead optimization to identify TAK-931 as follows.



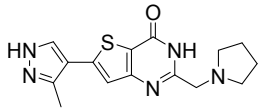
TAK-931

Figure 1. Chemical structure of TAK-931.[13]

We previously described a new class of thieno[3,2-*d*]pyrimidin-4(3*H*)-one-based Cdc7 inhibitors, represented by compound **I**, showing time-dependent Cdc7 kinase inhibition with slow dissociation kinetics (Figure 2).[14] The property implies that the compounds have inherent nature to exert effective pharmacological effects at high concentration of ATP in cells and/or in vivo efficacy. In order to develop further optimization strategy, docking model of compound **I** with Cdc7 protein structure (4F9C) was analyzed. The docking study suggested that the space accommodating substituents at α-positions of the pyrrolidine nitrogen is directed to the solvent-exposed region (Figure 3). Thus, a cyclic amine moiety whose α-carbon directly binds to the 2-position of the thienopyrimidinone scaffold was expected to be tolerated with maintaining the hydrogen bonding between the amine nitrogen and Asp196 residue with

reduced entropy loss by reduction of rotatable bond, which encouraged us to design compound **II** (Figure 4).

Herein, we report synthesis, structure-activity relationships (SARs), and biological evaluation of this series. Furthermore, the strategy will be verified by molecular modeling studies utilizing the reported Cdc7 crystal structure and analysis of co-crystal with ROCK2 for the optimized compound.



I

Cdc7: IC_{50} = 0.70 nM
Cdk2/cycE: IC_{50} = >10000 nM
ROCK1: IC_{50} = 140 nM
pMCM2: IC_{50} = 250 nM
COLO205: EC_{50} = 1100 nM

Figure 2. Previously-identified thieno[3,2-*d*]pyrimidin-4(3*H*)-one-based Cdc7 inhibitor **I**.

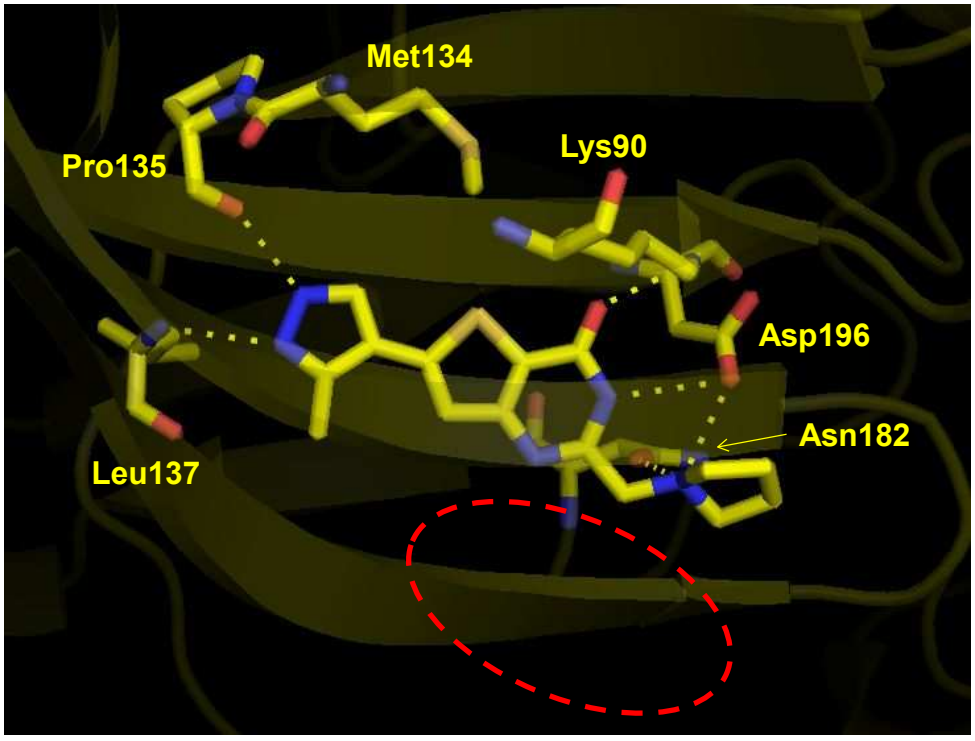


Figure 3. Docking model of compound **I** with Cdc7 crystal structure (4F9C).

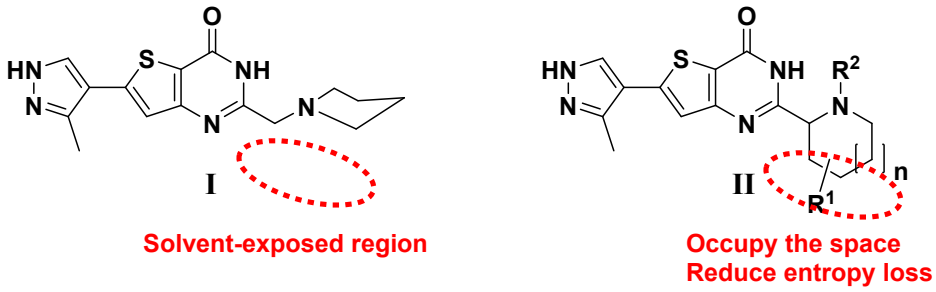


Figure 4. Design of compound **II** possessing a cyclic amine moiety at the 2-position.

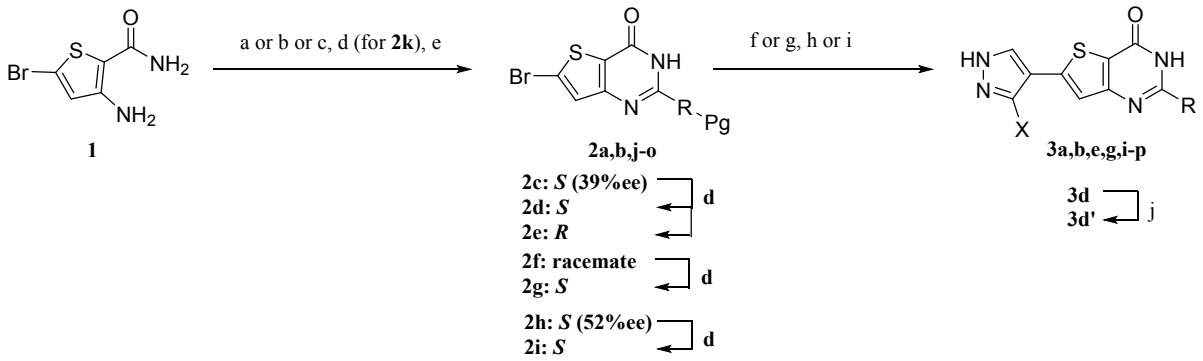
CHEMISTRY

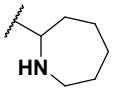
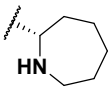
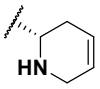
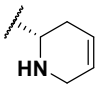
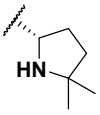
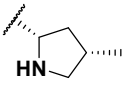
The general synthetic route of 6-(5-substituted-lpyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-ones **3a,b,d,e,g,i-p** possessing various cyclic amines at the 2-position is shown in Scheme 1. As mentioned later in Table 1, the *2*(*S*)-enantiomer **3d** showed a more favorable profile than the antipode **3e**; therefore, compounds having *2*(*S*)-cyclic amino groups were mainly prepared. Condensation of 3-amino-5-bromothiophene-2-carboxamide **1**[15] with *N*-protected cyclic amino acid, followed by cyclization under basic conditions provided 2-substituted 6-bromothieno[3,2-*d*]pyrimidine-4(3*H*)-ones **2a-c,f,h,j-o**. Mixed anhydride, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU), or acid chloride was properly used by considering the reactivity of the amines. In the condensation step, mono-acylated intermediate was generally detected by LC-MS, even when excess amount of the carboxylic acid was used, suggesting poor reactivity of the amino group of **1**. However, racemization was observed in the reaction with 6-membered cyclic amino acids, providing **2c** in 39% ee and **2h** in 52% ee. An azepane derivative **2f** was also prepared as a racemate because racemic

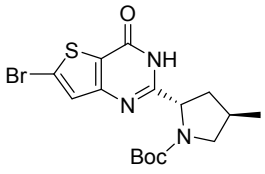
azepane 2-carboxylic acid was used for the condensation reaction. Thus, compounds **2d**,**e,g,i** were obtained by chiral resolution of **2c,f,h**. By contrast, **2a**,**j,l** gave a single peak in chiral HPLC analysis under multiple conditions, suggesting that no racemization occurred in the reaction with 4- or 5-membered cyclic amino acids. In the case of **2k**, a minor peak found by chiral HPLC analysis was determined as a (2*S*,4*R*)-enantiomer **2k'** by X-ray analysis, indicating contamination of the undesirable diastereomer in the purchased (2*S*,4*S*)-*N*-Boc-4-methylproline. Achiral compound **2n** was successfully prepared under typical conditions of mixed anhydride method (room temperature~60 °C), despite having the bulky bicyclo ring. However, condensation reaction of **1** with (*S*)-2-methylproline didn't proceed due to steric hindrance. Condensation under microwave irradiation at 120 °C was used, finally affording the crude cyclized product **2m** in 14% yield. In the case of **2o**, even condensation under microwave irradiation didn't proceed efficiently. Therefore, the bicyclo-amino acid **19** was converted to the corresponding acid chloride **20** (see Scheme 4), which was reacted with **1** at room temperature to give the achiral compound **2o** in 40% yield.

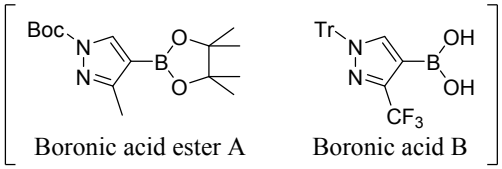
The optically-pure **2a**,**b**,**d**,**e**,**g**,**i-m** and achiral **2n**,**o** were subjected to Suzuki coupling reaction with the corresponding protected pyrazolylboronic acids A–B, and subsequent deprotection afforded the desired compounds **3a**,**b**,**d,e**,**g**,**i-p**. As for **3d**, the corresponding free amine **3d'** was obtained by the treatment with triethylamine. The precise optical purities of compounds **3d**,**e** showing a good in vitro profile described later were determined to be more than 98% ee by chiral HPLC analysis. The results suggested that racemization occurred only in the activation step of the carboxylic acid.

Scheme 1[a]



	X	R	*N*-Protective group (2)	Salt (3)
a	Me		Boc	free
b	Me		Boc	2HCl
c	Me		Boc (39%ee)	-
d	Me		Boc	2HCl
d'	Me		—	free
e	Me		Boc	2HCl
f	Me		Boc (racemate)	-
g	Me		Boc	free
h	Me		Boc (52%ee)	-
i	Me		Boc	2HCl
j	Me		Boc	free
k	Me		Boc	2HCl
l	Me		Boc	2HCl
m	Me		Boc (crude)	2HCl
n	Me		Boc	2HCl
o	Me		Cbz	HCl
p	CF_3		—	HCl





[a]Reagents and conditions: (a) *N*-Boc-amino acid, *i*-BuOCOCl, Et_3N, THF for **2a,c,f,h,j-n**; (b) *N*-Boc-*L*-proline, HATU, DIEA, DMF for **2b**; (c) benzyl

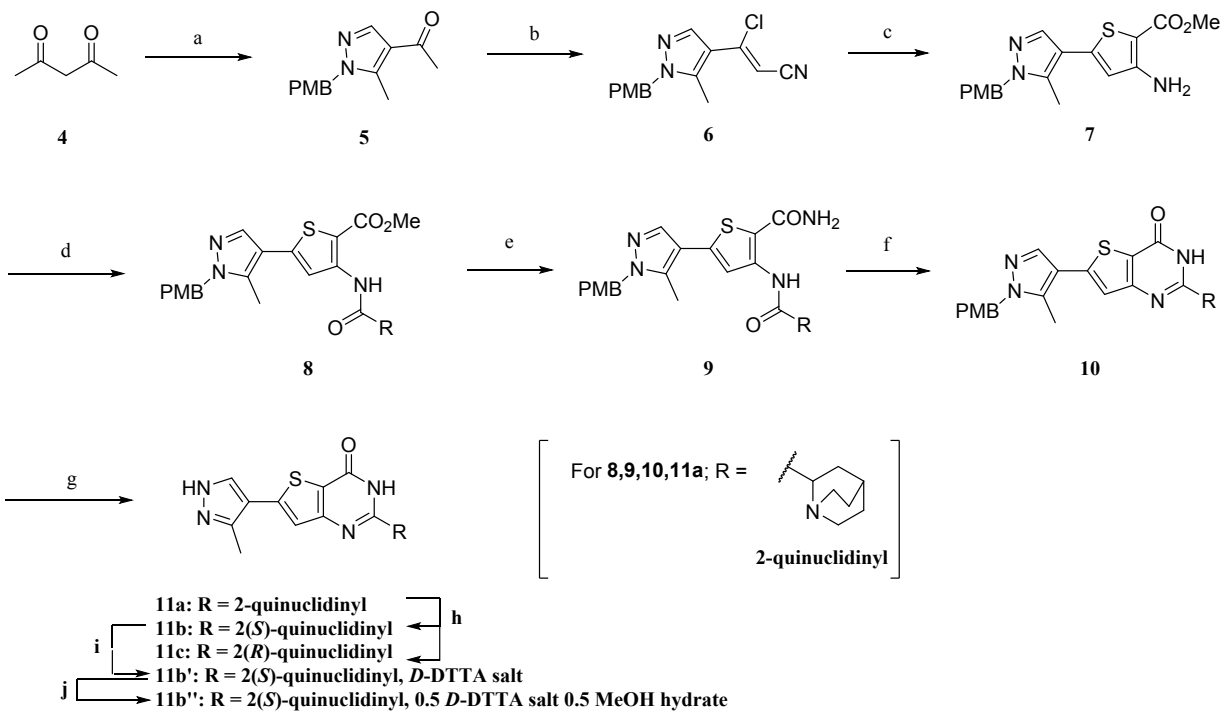
1-(chlorocarbonyl)-7-azabiciclo[2.2.1]heptane-7-carboxylate (**20**), DIEA, THF for **2o**; (d) chiral HPLC separation for **2d,e,g,i,k**; (e) NaOH, EtOH, water, 14%–quant. (2 steps); (f) **boronic acid ester A**, $PdCl_2$(dppf), Cs_2CO_3, DME–water for **3a,b,d,e,g,i-o**; (g) **boronic acid B**, $Pd(PPh_3)_4$, Na_2CO_3, EtOH–water for **3p**; (h) 4 M HCl in EtOAc for **3a,b,d,e,g,i-n,p**, 17–83% (2 steps); (i) 1) Pd/C, HCO_2H. 2) HCl in MeOH for **3o**, 24% (2 steps); (j) Et_3N, MeOH, 88%.

An alternative synthetic route without Suzuki coupling reaction was examined for the preparation of **11b,c**. More precisely, thiophene-2-carboxamides having a substituted pyrazole were prepared prior to construction of thienopyrimidine scaffold as described in Scheme 2.

1-(1-(4-Methoxybenzyl)-5-methyl-1*H*-pyrazol-4-yl)ethanone **5** was synthesized from 1,3-pentanedione **4** by reaction with *N,N*-dimethylformamide dimethyl acetal and subsequent cyclization with *p*-methoxybenzyl(PMB)-hydrazine. The acetyl pyrazole **5** was chloroformylated under Vilsmeier conditions, and the resulting intermediate was then treated with hydroxylamine to provide the corresponding chloroacrylonitril **6**. Compound **6** was reacted with methyl thioglycolate under basic conditions to afford aminothiophene derivative **7**. Condensation of **7** with quinuclidine carbonyl chloride prepared from the corresponding carboxylic acid **27** (see Scheme 4) in situ was carried out to give carboxamide **8**. The ester group of **8** was converted to diamide **9** by saponification and subsequent condensation with ammonium chloride. Construction of thienopyrimidine scaffold was done in the same manner as described in the preparation of **2** (Scheme 1), and the ring-closure product **10** was treated with trifluoroacetic acid (TFA) in the presence of anisole to afford the target compound **11a**. Racemate **11a**

was subjected to preparative chiral HPLC to provide both enantiomers **11b,c**. To confirm absolute configuration at the chiral center, X-ray crystallography analysis of **11b** was attempted. After multiple experiments of recrystallization from free amine and the various salts, single crystal **11b''** was successfully obtained from the corresponding di-*p*-toluoyl-*D*-tartaric acid (*D*-DTTA) salt **11b'** and MeOH–methyl ethyl ketone. As a consequence, compound **11b** was found to be an *S*-isomer by single crystal X-ray analysis as shown in Figure 5.

Scheme 2 [a]



4 5 6 7 8 9 10

[a]Reagents and conditions: (a) 1) *N,N*-dimethylformamide dimethyl acetal; 2) EtOH, Et_3N, *p*-methoxybenzyl(PMB)-hydrazine hydrochloride, 62%; (b) 1) DMF, $POCl_3$; 2) hydroxylamine hydrochloride, 71%; (c) methyl thioglycolate, NaH, DMF, 83%; (d) 2-quinuclidinecarboxylic acid

hydrochloride (**27**), $SOCl_2$, DIEA, THF, 78%; (e) 1) NaOH, MeOH; 2) EDCI, HOBt, Et_3N, NH_4Cl, DMF, 90%; (f) NaOH, EtOH, 99%; (g) TFA, anisole, 78%; (h) chiral HPLC separation and recrystallization, 37% for both **11b** and **11c**; (i) *D*-DTTA, MeOH, 72%; (j) single crystal preparation from MeOH–methyl ethyl ketone.

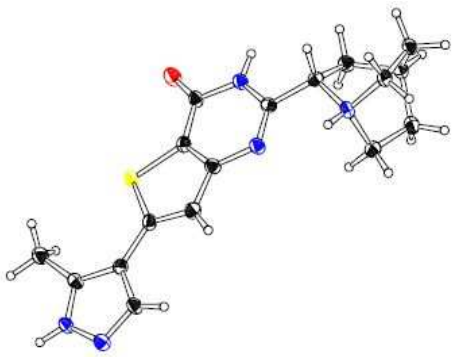


Figure 5. ORTEP of **11b''** (CCDC 1918344, only host ion is displayed). Thermal ellipsoids are drawn at 30% probability.

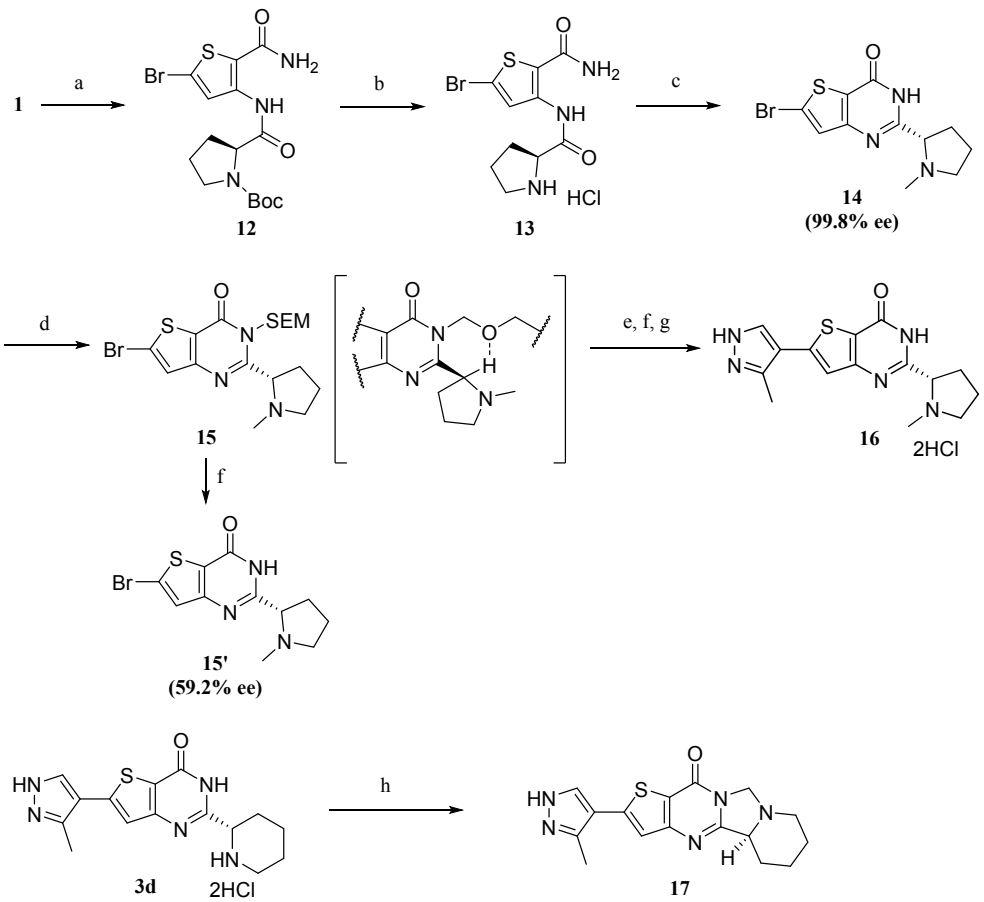
N-Methylpyrrolidin-2-yl derivative **16** and tetracyclic compound **17** were synthesized by the procedure presented in Scheme 3. Condensation of the aminothiophene **1**, with *N*-Boc-*L*-proline, followed by removal of the Boc group provided diamide **12**. Reductive amination with formaldehyde and ring closure under basic conditions furnished thienopyrimidinone **14** with high optical purity. Suzuki coupling of **14** with *N*-protected pyrazolylboronic acid was unsuccessful, presumably due to inactivation of the palladium catalyst by coordination with the pyrimidine and pyrrolidine nitrogen atoms. The speculation led us to protect the pyrimidine nitrogen of **14**. After protection with 2-(trimethylsilyl)ethoxymethyl (SEM) group, Suzuki coupling of **15** proceeded smoothly to produce the

corresponding coupling product, which was successively treated with tetra-*n*-butylammonium fluoride (TBAF) and HCl–EtOAc to provide the desired compound **16**.

To investigate whether racemization occurs in the additional reaction process as observed in Scheme 1, we checked optical purity of **15'** that was prepared from the SEM-protected **15**. High optical purity of **14** (99.8% ee) proved that racemization did not occur in the two-step procedure, i.e. the removal of the Boc group and the reductive amination. However, relatively low optical purity of **15'** (59.2% ee) was observed, indicating that protection and/or deprotection with SEM group caused racemization. We speculated that abstracting the hydrogen atom on the chiral center by a strong base such as sodium hydride was facilitated by neighboring-group participation of the oxygen atom of the SEM group as shown in Scheme 3. Thus, optical purity of **16** might not be high despite obtaining no experimental data.

As mentioned later, thienopyrimidinones having an *N*-nonsubstituted cyclic amine at the 2-position were found to form a formaldehyde adduct. The adduct **17** was synthesized from **3d** by treatment with formaldehyde. The precise chemical structure of **17** was confirmed by a detailed NMR analysis.

Scheme 3[a]



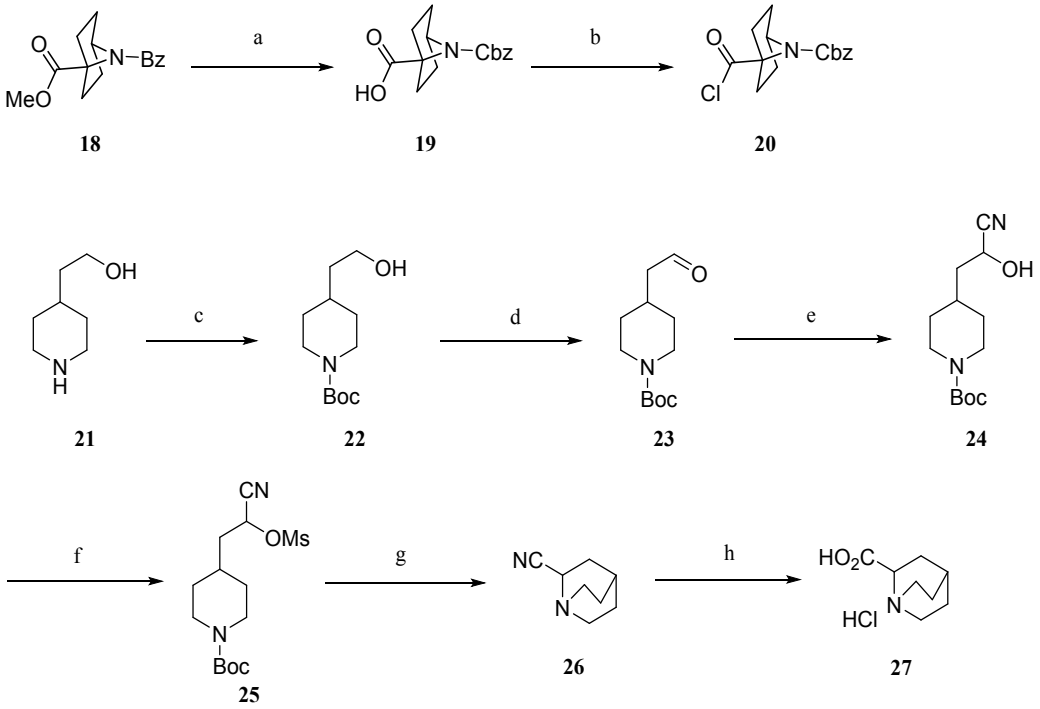
[a]Reagents and conditions: (a) *N*-Boc-*L*-proline, *i*-BuOCOCl, Et_3N, THF, 88%; (b) 4 M HCl in EtOAc, MeOH, THF, 89%; (c) 1) HCHO, $NaBH_3CN$, MeOH. 2) NaOH, 96% (2 steps); (d) NaH, SEMCl, THF, 51%; (e) **boronic acid ester A** (see Scheme 1), $PdCl_2$(dppf), Cs_2CO_3, DME-water; (f) TBAF, THF; (g) 4 M HCl in EtOAc, 11% (3 steps); (h) HCHO, Et_3N, MeOH, 81%.

The requisite carboxylic acid derivatives **20**, **27** for the synthesis of **2o**, **8** were prepared as shown in Scheme 4. Although the synthesis of **20** was already reported in the literature[16], we prepared the compound by the alternative route. Starting from 7-azabicyclo[2.2.1]heptane derivative **18**[17], simultaneous acidic hydrolysis of the ester and amide, followed by protection of the secondary amine

gave Cbz-protected amino acid **19** successfully. Compound **19** was converted to the corresponding acid chloride **20**, which was used for the synthesis of **2o** shown in Scheme 1.

Quinuclidine-carboxylic acid hydrochloride **27**, which was used for the synthesis of **8** shown in Scheme 2, was prepared according to the modified procedure similar to that described in the literature[18] to remove ammonium chloride contaminated in the final step (see Experimental Section).

Scheme 4[a]



[a]Reagents and conditions: (a) 1) conc. HCl. 2) Cbz-Cl, Na_2CO_3, 1,4-dioxane, water, 27% (2 steps); (b) oxalyl chloride, DMF (cat.), THF, quant; (c) $(Boc)_2O$, *t*-BuOH, water; (d) pyridine sulfur trioxide, Et_3N, DMSO, 81% (2steps); (e) NaCN, HCl, Et_2O, water; (f) MsCl, Et_3N, THF; (g) 1) TFA, CH_2Cl_2. 2) Et_3N, MeCN, 40% (from **23**, 4 steps); (h) 1) conc. HCl, 2) 2 M NaOH, 3) 6 M HCl, 65% (3 steps).

RESULTS AND DISCUSSION

Structure activity relationships (SARs) of 6-(pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-ones

In order to clarify SARs of this chemical series, all synthesized compounds were evaluated for the ability to inhibit Cdc7 kinase activity. Kinase selectivity was assessed by measuring inhibitory activities against Cdk2/cyclinE and ROCK1.

Firstly, suitable ring size and chirality of cyclic amine groups at the 2-position were investigated and the results are presented in Table 1. (*S)*-Piperidinyl derivative **3d** showed subnanomolar inhibitory activity for Cdc7 kinase, which was about 2-fold more potent than the corresponding *R*-enantiomer **3e**. From the point of view of kinase selectivity, **3d** was significantly superior to the counterpart **3e**. Introduction of a double bond into the piperidine ring of **3d** (**3i**) gave an inferior in vitro profile to those of **3d**, demonstrating that **3d** is the best among the 6-membered cyclic amine compounds. Reduction or expansion of the ring size (**3a,b,g**) resulted in decreased activity and selectivity, clearly indicating that the most preferable ring size is 6-membered.

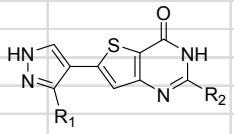
As previously reported,[14] the 3-methylpyrazol-4-yl group was found to be a favorable hinge binder of the thienopyrimidinones series in terms of both Cdc7 inhibitory activity and kinase selectivity. Moreover, the 3-methyl group on the pyrazole ring significantly contributes to the time dependency on Cdc7 kinase inhibition and slow dissociation property, presumably leading to potent growth inhibition of cancer cells *in vitro*. In order to confirm the finding, 3-substituent on the pyrazole ring of **3d** was re-investigated. To be precise, an electron-withdrawing group of similar size such as a trifluoromethyl group was incorporated, providing compound **3p** showing almost equivalent potency and selectivity

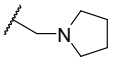
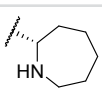
compared to **3d**. Compounds **3d,p** were found to exhibit an improved *in vitro* profile relative to the lead compound **I**.

The selected compounds **3d,p** were subsequently evaluated for time dependency on Cdc7 kinase inhibition, phosphorylation status of Ser40 of MCM2 in Hela cells (cervix adenocarcinoma cell line), and growth inhibition of COLO205 cells (colorectal adenocarcinoma cell line), and the profiles were compared with those of compound **I** (Table 2). To assess time dependency, selected compounds were assayed with varied ATP concentrations and pre-incubation time. Increased ATP concentrations (50 μM) without pre-incubation reduced Cdc7 inhibitory activities of all the compounds by more than 160-fold when compared with the data obtained under standard conditions (ATP concentration: 1 μM, pre-incubation time: 10 min). By contrast, longer pre-incubation time (60 min) significantly enhanced the Cdc7 kinase inhibition with IC_{50} values ranging from 0.41 to 1.7 nM, despite high ATP concentrations. Further kinetics analysis by the Proteros reporter displacement assay[19-20] revealed that **3d** has about 4-fold weaker binding affinity, but almost equivalent values of K_{off} and residence time compared to compound **I**. These results demonstrate that all the compounds are ATP-competitive inhibitors of Cdc7 kinase with slow dissociation kinetics. The characteristics contribute to competing with higher ATP concentrations (~mM) under physiological conditions, suggesting that the inhibitors can display potent cellular activities. In fact, **3d,p** exhibited greater reduction of Ser40 phosphorylation of MCM2 in Hela cells when compared with compound **I**, probably due to more optimal occupation of the ATP binding pocket. A same tendency was observed for growth inhibition of COLO205 cells. Compounds **3d**,**p** proved to show higher growth inhibition potency relative to compound **I** by reflecting the stronger blockade of Ser40

phosphorylation. Based on the overall profile, compound **3d** was chosen for further evaluation. The kinase selectivity of **3d** was further assessed in the broad kinase panel. Of 317 kinases, only 15 kinases were inhibited more than 80% by **3d** at 1000 nM. IC_{50} value for inhibition of DYRK1A (top 3 kinase) was 59 nM, 134-fold higher than IC_{50} value for inhibition of Cdc7 (IC_{50} = 0.44 nM). The results confirmed excellent kinase selectivity of **3d** as a Cdc7 kinase inhibitor (Table 3).

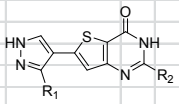
Table 1. Effects of ring size and chirality of the 2-cyclic amine group on Cdc7 inhibitory activity and kinase selectivity



Compound	R_1	R_2	Enzyme inhibition: IC_{50} (nM)[a]			Selectivity	
			Cdc7	Cdk2/cycE	ROCK1	Cdk2/Cdc7	ROCK1/Cdc7
I			0.70 (0.51-0.96)	>10000	140 (130-160)	x14000	x200
3a			2.1 (1.8-2.5)	6900 (5800-8300)	760 (640-910)	x3300	x360
3b			0.71 (0.60-0.84)	3900 (3500-4400)	360 (330-400)	x5500	x510
3d			0.44 (0.35-0.55)	6500 (5800-7400)	420 (360-500)	x15000	x950
3e			0.91 (0.38-2.2)	4800 (4300-5300)	370 (320-420)	x5300	x410
3i			0.53 (0.40-0.70)	4700 (4200-5300)	340 (300-390)	x8900	x640
3g			1.2 (0.94-1.6)	2800 (2200-3700)	450 (390-530)	x2300	x380
3p			0.43 (0.29-0.64)	9500 (8500-11000)	530 (470-590)	x22000	x1200

[a]Numbers in parentheses represent 95% confidence interval.

Table 2. Effect of 2-substituent of the thienopyrimidinone scaffold and 3-substituent of the pyrazole moiety on time-dependency of Cdc7 inhibition, MCM2 phosphorylation, and COLO205 cell growth



Compound	R_1	R_2	Cdc7 inhibition: IC_{50} (nM)			Dissociation Kinetics			pMCM2	COLO205
			ATP 1 μM[a]	ATP 50 μM[b]						
			10 min[c]	0 min[c]	60 min[c]	K_D[d]	K_{off}[e] (sec^{-1})	residence time (min)	IC_{50} (nM)	EC_{50} (nM)
I			0.70	190	1.7	5.41×10^{-10}	4.94×10^{-4}	34	250	1100
3d			0.44	120	0.41	1.96×10^{-9}	6.30×10^{-4}	26	36	130
3p			0.43	72	0.72	NT	NT	NT	41	230

[a]ATP concentration (*Km*) in the standard cell-free assay conditions.

[b]ATP concentration (x50 *Km*).

[c]Pre-incubation time with a tested compound.

[d]Equilibrium dissociation constant.

[e]Dissociation rate constant.

Table 3. Kinase selectivity data of **3d**.

Enzyme	IC_{50} (nM)	% inhibition at 1000 nM
CLK4	NT	100
STK17A (DRAK1)	NT	99
DYRK1A	59.0	98
DYRK1B	78.6	96
DMPK	64.6	95
DAPK3 (ZIPK)	NT	92
CDK9/cyclin T1	NT	90
GSK3A (GSK3 alpha)	NT	89
GSK3B (GSK3 beta)	NT	89
CLK2	NT	88
HIPK4	176	87
CSNK1G2 (CK1 gamma 2)	NT	87
CDK8/cyclin C	351	87
DAPK1	203	85
CLK1	310	81
302 kinase assays	NT	<80

Concentration producing 50% inhibition (IC_{50}) values and percent inhibition at 1000 nM of **3d** against 317 kinases are reported by Invitrogen Corp.

In vivo evaluation of compound 3d

In vivo efficacy of compound **3d** was next investigated. Prior to the in vivo efficacy studies, a preliminary pharmacokinetic (PK) profile of the inhibitor was obtained by mouse cassette-dosing test, indicating that **3d** showed acceptable PK profile (Cmax = 0.978 μg/mL, $AUC_{0\text{-}8h}$ = 1.35 μg/mL·h at 10 mg/kg, po). In vivo pharmacodynamic (PD) effects and anti-tumor efficacy of **3d** were examined in a COLO205 xenograft mouse model. Oral administration of 100 mg/kg of **3d** significantly reduced phosphorylated Ser40/41 of MCM2 (72% reduction at 4 h) in the in vivo PD assay, while the phosphorylation level of Ser41, which was not a substrate of Cdc7 but Cdk2, was not changed significantly (Figure 6). Following the decrease in the phosphorylation level of MCM2, the protein level

of Cyclin B1, which is a marker of late S or G2/M phase, was increased. Cleaved poly (ADP-ribose) polymerase (PARP) did not increase after the single administration, suggesting multiple dosing is necessary to induce tumor apoptosis. Oral administration of **3d** for 14 days significantly inhibited tumor growth in the xenograft model at doses of 50 and 100 mg/kg twice daily (T/C = 6%) without substantial body weight loss (Figure 7).

Consequently, compound **3d** with the notable in vitro profiles both in cell-free and cell-based assays was found to produce significant anti-tumor effects. High selectivity for Cdc7 over other target classes was confirmed (data not shown), which led us to conduct an in-depth examination of compound **3d** in a preclinical study.

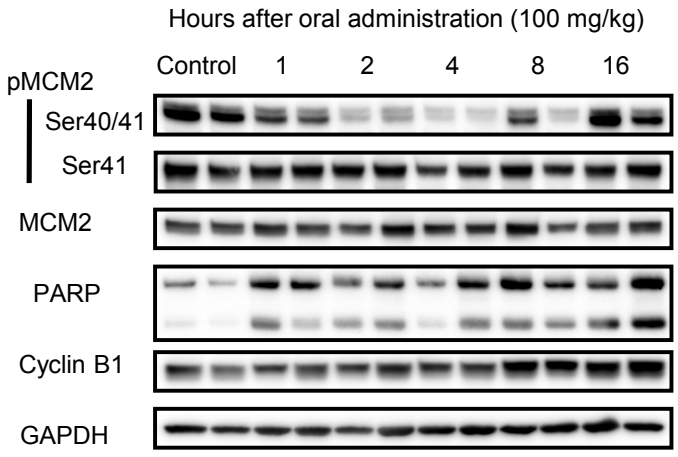


Figure 6. In vivo pharmacodynamic (PD) effects in a COLO205 xenograft mouse model. Compound **3d** (100 mg/kg) was orally administrated to mice bearing COLO205 xenografted tumor. At each time point, xenografted tumor was removed from the mice and homogenized. Protein level or phosphorylation level of each sample was determined by western blotting analysis. Band intensities of phosphorylated

Ser40/41, phosphorylated Ser41 MCM2, PARP, and cyclin B1 were measured and normalized with GAPDH band intensity.

(a) (b)

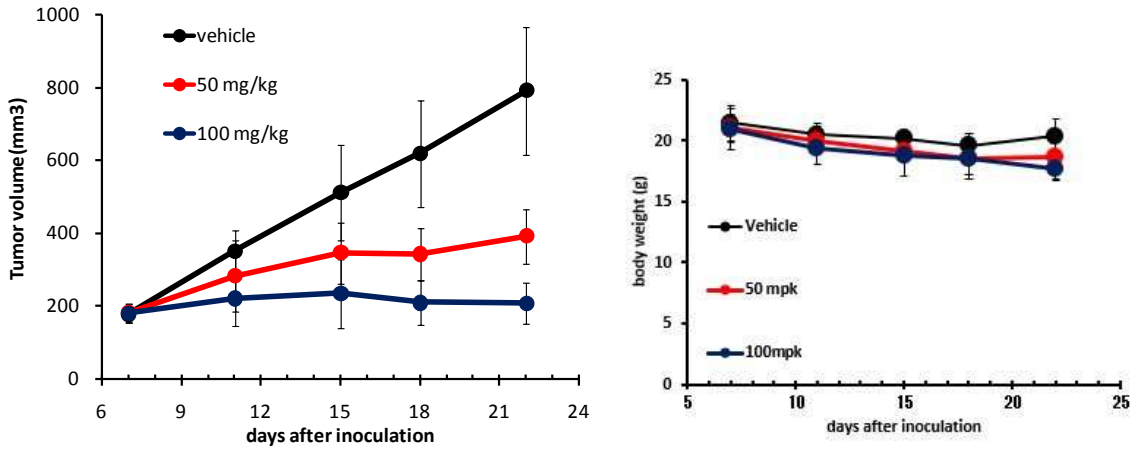


Figure 7. (a) Anti-tumor effects of **3d** in a COLO205 xenograft mouse model. Compound **3d** (50 or 100 mg/kg) was orally administrated twice daily to mice bearing COLO205 xenografted tumor for 14 days (n = 5). Tumor size and body weight mass were measured twice weekly. (b) Body weight measured during the anti-tumor efficacy study.

Formaldehyde adduct issue of compound 3d

In the course of examining ADME-Tox profiles, dosage form, and suitable salts, compound **3d** (both its free amine and the corresponding salts) were found to be transformed to a mixture of **3d** and an unknown compound having 328 mass (M+H, data not shown). The unknown compound was determined as a formaldehyde adduct whose physicochemical data were identical to those of the authentic sample **17** prepared in Scheme 3. Although the source of formaldehyde wasn't obvious, it was suggested that **3d**

promptly reacts with formaldehyde present in the assay system to give the formaldehyde adduct **17**. Conversely, **17** proved to be easily converted to **3d** by reaction with H_2O (Figure 8).

Further investigation of **17** revealed that the in vitro activities of **17** were essentially equipotent to those of **3d**. Considering the fact that **3d** or **17** is transformed to a mixture of **3d** and **17** in aqueous media (data not shown), it is unclear which compound mainly contributes to exerting the in vitro and in vivo effects. Additionally, it also indicates that exact concentrations of **3d** and **17** can't be determined both in vitro and in vivo. Therefore, we decided to discontinue **3d** as a preclinical candidate because the instability is considered to be a major obstacle to further development.

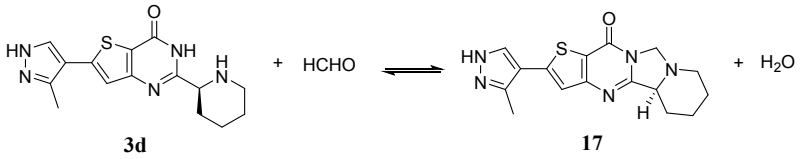


Figure 8. Interconversion of **3d** and its formaldehyde adduct **17** in aqueous media

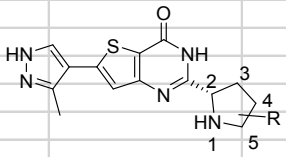
Further optimization to mitigate the risk of formaldehyde adduct

To avoid the formaldehyde adduct formation with maintaining the other in vitro/in vivo profiles of **3d**, a further optimization study was performed. Preliminarily, we carried out methyl scan of the 2-pyrrolidinyl moiety of **3b** to circumvent the adduct formation by steric hindrance and examined how an additionally-incorporated methyl group affects Cdc7 inhibitory activity and kinase selectivity (Table 4). Except for the 2-methyl group (**3m**), the 1-, 3(*S*)-, 4(*S*)-methyl, and 5,5-dimethyl groups were well

tolerated (**16**, **3l,k,j**), resulting in less than 3-fold drop in Cdc7 inhibitory activity. As for kinase selectivity, no obvious trend was observed. To further examine the 2-substituent of the pyrrolidine moiety, we then attempted bicyclization of **3m** to produce **3n**. Surprisingly, compound **3n** proved to exhibit improved potency as well as improved selectivity relative to **3m**. It is speculated that bicyclization of the cyclic amine moiety can compromise potency, selectivity, and steric hindrance, which encouraged us to subsequently investigate azabicycloalkane analogs (Table 5). As in the case of **3n**, compound **3o** showed superior Cdc7 inhibitory activity compared to **3d**; however, the selectivity of **3o** did not exceed that of **3d**. (*S*)-Enantiomer **11b** with an alternate bicyclic system was found to be more potent Cdc7 inhibitor with improved selectivity than the antipode **11c** and the lead compound **3d**. Moreover, the in vitro profile of **11b** is comparable to that of **3n** (see Table 4); therefore, **3n, 11b** were assessed in detailed assays (Table 6). Although **11b** exhibited slightly weaker Cdc7 inhibitory activity compared to **3n** under standard conditions, **11b** was approximately 4-fold more potent than **3n** after being pre-incubated with 50 μM of ATP for 60 min. Kinetics analysis by the Proteros reporter displacement assay[19-20] indicated **11b** has about 5-fold greater binding affinity and equivalent values of K_{off} and residence time relative to **3d**. The results demonstrate that **3n, 11b** are considered to be ATP-competitive inhibitors with slow dissociation kinetics in a similar fashion to **3d**. By reflecting the time-dependency in Cdc7 inhibition and slow dissociation kinetics, **11b** displayed the greatest reduction of phosphorylated MCM2 and COLO205 growth inhibition among the three inhibitors.

Table 4. Effect of introduction of a methyl group into the 2(*S*)-pyrrolidinyl moiety of **3b** on Cdc7

inhibitory activity and kinase selectivity

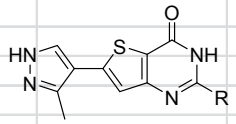


Compound	R	Enzyme inhibition: IC_{50} (nM)[a]			Selectivity	
		Cdc7	Cdk2/cycE	ROCK1	Cdk2/Cdc7	ROCK1/Cdc7
3b	-	0.71 (0.60-0.84)	3900 (3500-4400)	360 (330-400)	x5500	x510
16	1-Me	1.9 (1.6-2.2)	>10000	160 (140-180)	x5300	x84
3m	2-Me	9.8 (0.44-220)	9900 (9100-11000)	270 (230-320)	x1000	x28
3l	3(*S*)-Me	0.61 (0.48-0.78)	620 (540-700)	130 (110-160)	x1000	x210
3k	4(*S*)-Me	1.3 (0.97-1.8)	3700 (3400-4100)	630 (580-690)	x2800	x480
3j	5,5-di-Me	1.9 (1.5-2.3)	>10000	1500 (1200-1700)	x5300	x790
3n	2,4-methylene	0.16 (1.2E-30-2.1E+10	2700 (2300-3200)	220 (200-250)	x17000	x1400

[a]Numbers in parentheses represent 95% confidence interval.

[b]The optical purity is inconclusive.

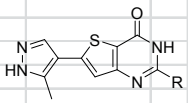
Table 5. Effect of introduction of azabicycloalkane into the 2-position on Cdc7 inhibitory activity and kinase selectivity



Compound	R	Enzyme inhibition: IC_{50} (nM)[a]			Selectivity	
		Cdc7	Cdk2/cycE	ROCK1	Cdk2/Cdc7	ROCK1/Cdc7
3d		0.44 (0.35-0.55)	6500 (5800-7400)	420 (360-500)	x15000	x950
3o		0.16 (0.11-0.22)	1500 (1400-1600)	96 (87-110)	x9400	x600
11b		0.26 (0.20-0.32)	6300 (5600-7000)	430 (390-470)	x24000	x1700
11c		1.0 (0.84-1.3)	>10000	460 (400-540)	x10000	x460

[a]Numbers in parentheses represent 95% confidence interval.

Table 6. Effect of 2-substituent of the thienopyrimidinone scaffold on time-dependency of Cdc7 inhibition, MCM2 phosphorylation, and COLO205 cell growth



Compound	R	Cdc7 inhibition: IC_{50} (nM)			Dissociation Kinetics			pMCM2	COLO205
		ATP 1 μM[a]	ATP 50 μM[b]		K_D[d]	K_{off}[e] (sec^{-1})	residence time (min)	IC_{50} (nM)	EC_{50} (nM)
		10 min[c]	0 min[c]	60 min[c]					
3d		0.44	120	0.41	1.96×10^{-9}	6.30×10^{-4}	26	36	130
3n		0.16	77	2.1	NT	NT	NT	130	750
11b		0.26	43	0.54	4.24×10^{-10}[f]	6.30×10^{-4}[f]	26[f]	17	81

[a]ATP concentration (*Km*) in the standard cell-free assay conditions.

[b]ATP concentration (x50 *Km*).

[c]Pre-incubation time with a tested compound.

[d]Equilibrium dissociation constant.

[e]Dissociation rate constant.

[f]Reported values.[13]

Study on formaldehyde adduct formation of 11b

In order to examine if **11b** can form the formaldehyde adduct and to understand how structural features of the 2-substituent affect the adduct formation, reactivity of **11b**, **3d'** (free amine of **3d**), and **3o** in aqueous media was evaluated by LC-MS, and the results are shown in Figure 9. As described earlier, incubation of **3d'** for 30 min in the presence of 12 equivalent of formaldehyde in aqueous solution afforded the formaldehyde adduct **17** (retention time: 4.31 min), but no formaldehyde adduct was detected in the cases of **11b** and **3o**. Indeed, no adduct formation of **11b** and **3o** was observed in the biological assay systems and during the formulation study (data not shown). The result clearly demonstrated that only secondary amine without steric hindrance and/or conformational constraint can form the formaldehyde adduct.

On the basis of the results, compound **11b** showing the most desirable in vitro profile without the risk of the adduct formation was selected for further evaluation. By preclinical evaluation including detailed pharmacological studies, **11b** was nominated as a clinical candidate (**TAK-931**) (Figure 1), currently being investigated under clinical trials.[13]

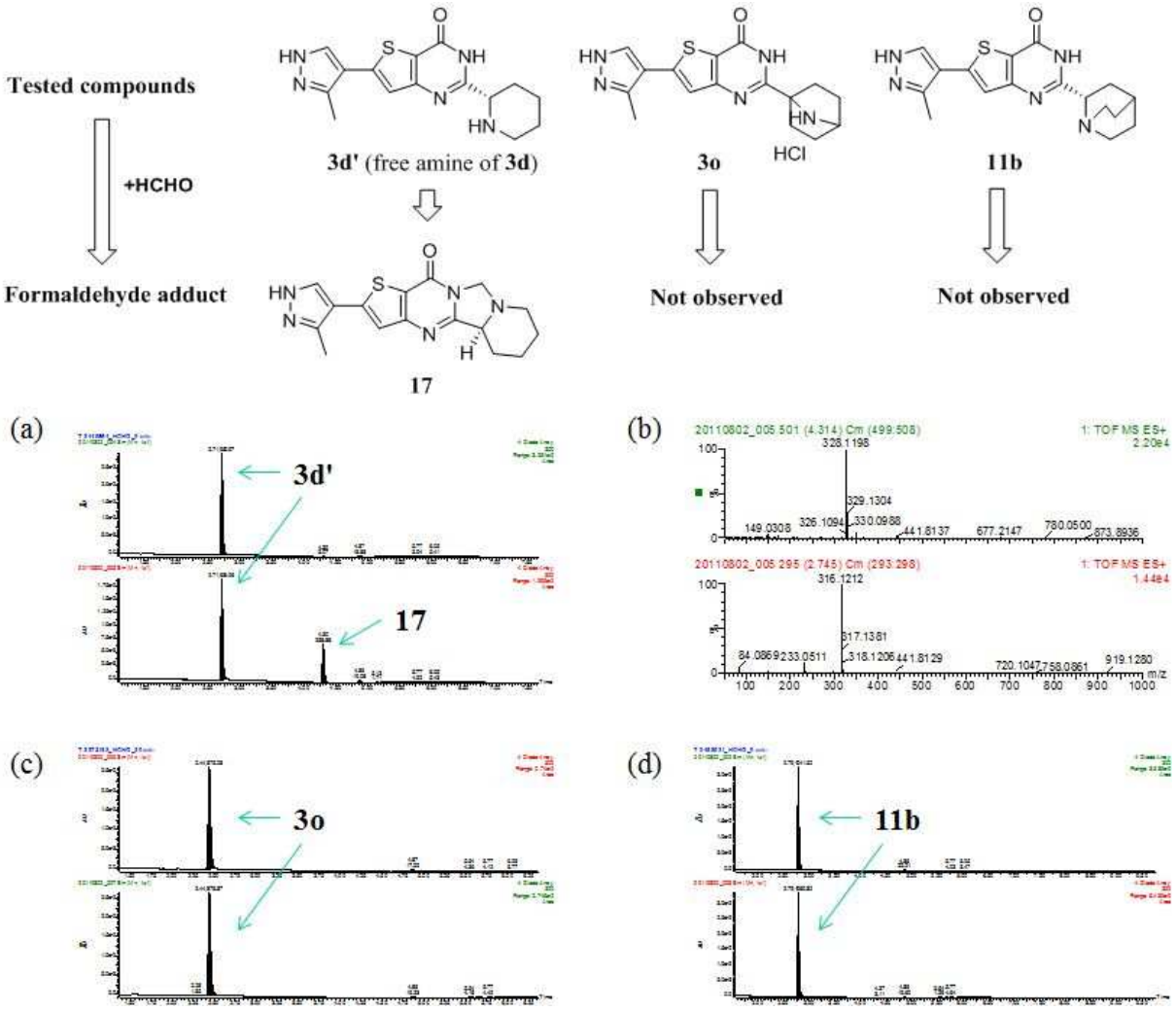


Figure 9. Results of formaldehyde adduct formation study. (a) UV chromatogram of **3d'** at initial (upper) and after 30 min incubation (lower). (b) MS spectra for the peaks of **17** (RT = 4.31 min, upper) and **3d'** (RT = 2.75 min, lower). (c) UV chromatogram of **3o** at initial (upper) and after 30 min incubation (lower). (d) UV chromatogram of **11b** at initial (upper) and after 30 min incubation (lower).

Molecular modeling and X-ray co-crystallization studies

To obtain further insight on the molecular basis of the high potency and kinase selectivity of **11b**, docking study of **11b** was carried out by using the Cdc7 crystal structure (4F9C)[21], and X-ray

co-crystallization of **11b** and the lead compound **I** with ROCK2, of which all the 34 amino acid residues in the ATP binding pocket are identical to those of ROCK1, was attempted. The docking study suggested that **11b** binds to the Cdc7 kinase in a similar manner to compound **I**, i.e. (i) hydrogen bond network formed by Asp196, the pyrrolidinyl nitrogen, and the neighboring lactam NH, (ii) hydrogen bond between Lys90 and the carbonyl group, (iii) hydrogen bond between Pro135/Lys137 and the pyrazole nitrogens (Figure 10(a)). Co-crystallization of **11b** and the lead compound **I** with ROCK2 was successful and the superposition of the binding modes indicated a marked difference from each other as shown in Figure 10(b). In the co-crystal structure of **I**, a hydrogen bond interaction between Asp232 and the pyrrolidine nitrogen was detected, suggesting tight binding to ROCK2. By contrast, in the co-crystal structure of **11b**, only weak electron density around quinuclidine moiety was observed, indicating no obvious interaction between Asp 232 and the quinuclidine nitrogen. It is presumably due to the steric bulkiness of the tertiary amine moiety at the 2-position. ROCK2 and ROCK1 proteins have not only the identical ATP binding pocket in terms of the amino acid sequence, but also 86% sequence homology when comparing the ROCK2 protein in the co-crystals (6P5M, 6P5P) and the corresponding amino acid sequence of the ROCK1 protein (Uniplot ID: Q13464). Therefore, we speculate that the binding modes of **11b** and the compound **I** to ROCK2 are essentially similar to those to ROCK1, respectively. The difference in the predicted binding modes of the inhibitors to the Cdc7 and ROCK1/2 proteins could, at least in part, elucidate the high potency and kinase selectivity of **11b**, which supported the above-mentioned optimization strategy.

(a) (b)

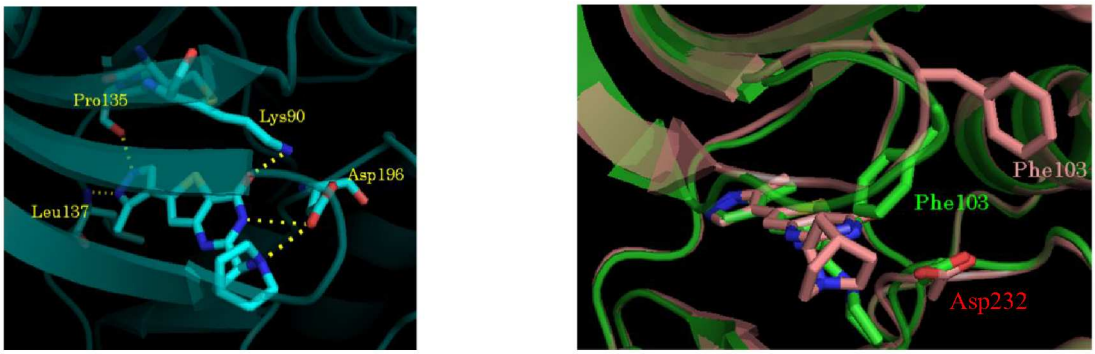


Figure 10. (a) Docking study on compound **11b** with the Cdc7 crystal structure (PDB: 4F9C). (b) Superposition of X-ray co-crystallographic data of compounds **I** (green, PDB: 6P5M) and **11b** (beige, PDB: 6P5P) bound to the ROCK2 protein.

CONCLUSION

We have successfully discovered the novel, highly potent, and selective thienopyrimidinone-based Cdc7 inhibitor **11b** (**TAK-931**) possessing a quinuclidine moiety. Starting from the lead compound **I**, optimization of this chemical series was carried out, resulting in the identification of the (*S*)-piperidin-2-yl analog **3d** with time-dependent kinase inhibition and slow dissociation kinetics. However, an issue of the formaldehyde adduct formation of **3d**, which is considered to be a major obstacle to further development, was found. To circumvent the risk, we employed structure-based approach for further optimization, and the medicinal chemistry efforts culminated in the discovery of the time-dependent inhibitor **11b** showing the most desirable in vitro profile without the risk of the adduct formation. Currently, **11b** (**TAK-931**) is under clinical trials (NCT02699749 and NCT03261947) as a novel anti-tumor agent.

EXPERIMENTAL SECTION

Chemistry

General

Starting materials, reagents, and solvents for reactions were reagent grade and used as purchased. Thin layer chromatography (TLC) analyses were carried out using Merck Kieselgel 60 F254 plates or Fuji Silysia Chemical Ltd. TLC plate NH. Chromatographic purification was carried out using silica gel (Merck, 70–230 mesh) or amino silica gel (Fuji Silysia, aminopropyl-coated, 100–200 mesh) or Purif-Pack (SI 60 μM or NH 60 μM, Fuji Silysia Chemical, Ltd.) or Combi-Flash. The proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Bruker AVANCE II (300 MHz), Bruker AV 300 (300 MHz), or Bruker AV (500 MHz) instruments. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard. Abbreviations are used as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublets of doublet, br = broad, br s = broad singlet. Coupling constants (J values) are given in hertz (Hz). HPLC with Corona charged aerosol detector (CAD) was used to confirm > 95% purity of each compound. The column used was Capcell Pak C18AQ (3.0 mm i.d. × 50 mm, Shiseido, Japan) or L-column 2 ODS (2.0 mm i.d. × 30 mm, CERI, Japan) with a temperature of 50 ^{o}C and a flow rate of 0.5 mL/min. Mobile phase A and B under neutral conditions were a mixture of 50 mmol/L ammonium acetate, water, and MeCN (1:8:1, v/v/v) and a mixture of 50 mmol/L ammonium acetate and MeCN (1:9, v/v), respectively. The ratio of mobile phase B was increased linearly from 5% to 95% over 3 min, 95% over the next 1 min. Mobile phase A and B under acidic conditions were a mixture

of 0.2% formic acid in 10 mmol/L ammonium formate and 0.2% formic acid in MeCN, respectively. The ratio of mobile phase B was increased linearly from 14% to 86% over 3 min, 86% over the next 1 min. MS spectra were recorded using a Shimadzu LCMS-2020 or Agilent 6130 Quadrupole LCMS with electrospray ionization (ESI or APCI). Elemental analysis and high resolution mass spectrometry (HRMS) were measured by Takeda Analytical Research Laboratories, Ltd.

***tert*-Butyl (2*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)azetidine-1-carboxylate (2a).** To a solution of (*S*)-*N*-Boc-azetidine-2-carboxylic acid (510 mg, 2.53 mmol) and Et_3N (0.419 mL, 3.03 mmol) in THF (5 mL) was added isobutyl chloroformate (0.346 mL, 2.66 mmol) at 0 °C. The mixture was stirred at room temperature for 30 min. To the resulting mixture was added 3-amino-5-bromothiophene-2-carboxamide[15] (267 mg, 1.21 mmol). The mixture was stirred at 60 °C for 19 h, then diluted with saturated $NaHCO_3$ aq., and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo. The residue was dissolved with EtOH (5 mL), and 2 M NaOH (2.83 mL, 5.65 mmol) was added. The mixture was stirred at 70 °C for 3 h, then cooled to room temperature. The mixture was neutralized by addition of 6 M HCl (1 mL), and water (6 mL) was added. The precipitate was collected by filtration to give **2a** (335 mg, 71%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.04–1.51 (9H, m), 2.20–2.35 (1H, m), 2.44–2.57 (1H, m), 3.84 (1H, br s), 3.91–4.02 (1H, m), 5.01 (1H, dd, J = 8.6, 5.6 Hz), 7.64 (1H, s), 12.74 (1H, br s). Single peak was detected by chiral HPLC analysis [column: CHIRALPAK AD-3 4.6 mm i.d. × 250 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/EtOH/Et_2NH (700:300:1, v/v/v), flow rate: 1 mL/min, column

temperature: 30 °C, detection: 220 nM].

***tert*-Butyl (2*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)pyrrolidine-1-carboxylate (2b).** A mixture of (*S*)-*N*-Boc-proline (8.78 g, 40.8 mmol), HATU (15.5 g, 40.8 mmol) and DIEA (8.31 mL, 47.6 mmol) in DMF (45 mL) was stirred at room temperature for 30 min. To the resulting mixture was added 3-amino-5-bromothiophene-2-carboxamide (3.00 g, 13.6 mmol). The mixture was stirred at 90 °C for 3.5 h, and cooled to 60 °C, then diluted with saturated $NaHCO_3$ aq., and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 9:1 to 4:6, v/v) to give acyl intermediate (6.04 g). A mixture of this material and 2 M NaOH (20.4 mL, 40.8 mmol) in EtOH (40 mL) was stirred at 70 °C for 2 h, and cooled to room temperature. The mixture was neutralized by addition of 6 M HCl (7 mL), and water (80 mL) was added. The precipitate was collected by filtration, and washed with Et_2O–*n*-hexane (1:4, v/v) to give **2b** (2.35 g, 43%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.11 (9H of major, s), 1.37 (9H of minor, s), 1.74–2.02 (3H, m), 2.18–2.33 (1H, m), 3.36–3.42 (1H, m), 3.47–3.59 (1H, m), 4.55 (1H of major, dd, J = 7.8, 5.0 Hz), 4.58–4.65 (1H of minor, m), 7.57 (1H of minor, s), 7.60 (1H of major, s), 12.71 (1H, br s). This material was observed as a 2:1 mixture of rotamers by ^{1}H NMR analysis.

***tert*-Butyl (2*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)piperidine-1-carboxylate (2c).** Compound **2c** (24.8 g) was prepared from 3-amino-5-bromothiophene-2-carboxamide (16.6 g, 75.0

mmol) and (*S*)-*N*-Boc-piperidine-2-carboxylic acid (37.8 g, 165 mmol) in 80% yield by a procedure similar to that described for **2a** as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.17–1.86 (14H, m), 1.98–2.10 (1H, m), 3.38–3.52 (1H, m), 3.76–3.88 (1H, m), 4.93–5.05 (1H, m), 7.59 (1H, s), 12.63 (1H, br s). 38.7% ee {determined by chiral HPLC analysis [column: CHIRALPAK ADH DJ153 4.6 mm i.d. × 250 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/IPA/Et_2NH (700:300:1, v/v/v), flow rate: 1 mL/min, column temperature: 30 °C, detection: 220 nM]}.

***tert*-Butyl (2*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)piperidine-1-carboxylate (2d) and *tert*-butyl (2*R*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)piperidine-1-carboxylate (2e).** **2c** (20.0 g) was purified by preparative chiral HPLC [column: CHIRALPAK AD JG001 50 mm i.d. × 500 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/IPA/Et_2NH (700:300:1, v/v/v), flow rate: 80 mL/min, column temperature: 30 °C, detection: 220 nM, loading: 150 mg/load] to give **2d** (13.1 g, 66%, 99.9% ee) and **2e** (5.34 g, 27%, 99.9% ee) as a white solid.

***tert*-Butyl 2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)azepane-1-carboxylate (2f).** Compound **2f** (474 mg) was prepared from 3-amino-5-bromothiophene-2-carboxamide (238 mg, 1.08 mmol) and *N*-Boc-azepane-2-carboxylic acid (550 mg, 2.26 mmol) in quantitative yield by a procedure similar to that described for **2a** as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.12–1.46 (12H, m), 1.58–1.99 (4H, m), 2.11–2.35 (1H, m), 3.16–3.29 (1H, m), 3.77–3.88 (1H of minor, m), 3.97 (1H of

major, dd, J = 14.8, 5.2 Hz), 4.65 (1H of major, dd, J = 12.0, 4.8 Hz), 4.83 (1H of minor, dd, J = 12.1, 5.9 Hz), 7.58 (1H of minor, s), 7.60 (1H of major, s), 12.61 (1H, br s). This material was observed as a 5:4 mixture of rotamers by ^{1}H NMR analysis.

***tert*-Butyl (2*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)azepane-1-carboxylate (2g). 2f** (772 mg) was purified by preparative chiral HPLC [column: CHIRALPAK AD NF001 50 mm i.d. × 500 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/EtOH (1:1, v/v), flow rate: 60 mL/min, column temperature: 30 °C, detection: 220 nM, loading: 260 mg/load] to give **2g** (tR2, 326 mg) as a white solid. 99.9% ee {determined by chiral HPLC analysis [column: CHIRALPAK AD KF054 4.6 mm i.d. × 250 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/EtOH (1:1, v/v), flow rate: 0.5 mL/min, column temperature: 30 °C, detection: 220 nM]}. Absolute structure was determined by X-ray crystallography analysis (Figure 11)

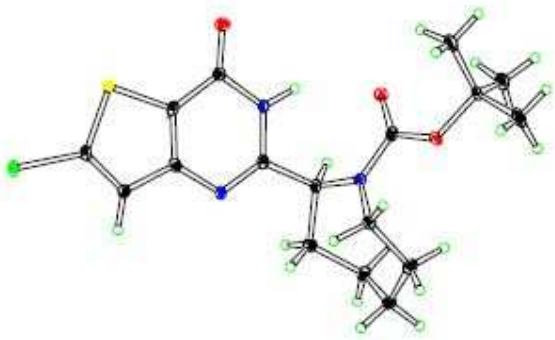


Figure 11. ORTEP of **2g** (CCDC 1918343). Thermal ellipsoids are drawn at 30% probability.

X-ray structure analysis of 2g

A single crystal was obtained from a EtOAc solution, and analyzed as follows:

Crystal data for **2g**: $C_{17}H_{22}BrN_3O_3S$, *MW* = 428.34; crystal size, 0.30 × 0.09 × 0.04 mm; colorless, platelet; monoclinic, space group $P2_1$, a = 10.9062(2) Å, b = 6.64467(12) Å, c = 12.8662(2) Å, $\alpha = \gamma$ = 90°, β = 98.3804(7)°, V = 922.43(3) Å^3, Z = 2, Dx = 1.542 g/cm^3, T = 100 K, μ = 4.283 mm^{-1}, λ = 1.54187 Å, R_1 = 0.032, wR_2 = 0.095, Flack Parameter[22] = –0.03(2).

All measurements were made on a Rigaku R-AXIS RAPID diffractometer using graphite monochromated Cu-Kα radiation. The structure was solved by direct methods with SIR92[23] and was refined using full-matrix least-squares on F^2 with SHELXL-97.[24] All non-H atoms were refined with anisotropic displacement parameters. The coordinates of the structure were deposited in the CCDC under the accession code CCDC 1918343.

***tert*-Butyl**

(2*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)-3,6-dihydropyridine-1(2*H*)-carboxy late (2h). Compound **2h** (723 mg) was prepared from 3-amino-5-bromothiophene-2-carboxamide (674 mg, 3.05 mmol) and (*S*)-1-(*tert*-butoxycarbonyl)-1,2,3,6-tetrahydropyridine-2-carboxylic acid (1.04 g, 4.58 mmol) in 58% yield by a procedure similar to that described for **2a** as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.19–1.48 (9H, m), 2.53–2.67 (2H, m), 3.92–4.19 (2H, m), 5.09–5.32 (1H, m), 5.60–5.82 (2H, m), 7.55 (1H, s), 12.66 (1H, br s). 52.0% ee {determined by chiral HPLC analysis [column: CHIRALPAK IC MD026 4.6 mm i.d. × 250 mm, mobile phase: *n*-hexane/EtOH (9:1, v/v), flow rate: 1 mL/min, column temperature: 30 °C, detection: 220 nM]}.

***tert*-Butyl**

(2*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)-3,6-dihydropyridine-1(2*H*)-carboxylate (2i). 2h (720 mg) was purified by preparative chiral HPLC [column: CHIRALPAK IC ME001 50 mm i.d. × 500 mm, mobile phase: *n*-hexane/EtOH (9:1, v/v), flow rate: 80 mL/min, column temperature: 30 °C, detection: 220 nM, loading: 360 mg/load] to give **2i** (tR2, 461 mg) as a white solid. 99.9% ee {determined by chiral HPLC analysis [column: CHIRALPAK IC MD026 4.6 mm i.d. × 250 mm, mobile phase: *n*-hexane/EtOH (9/1, v/v), flow rate: 1 mL/min, column temperature: 30 °C, detection: 220 nM]}.

***tert*-Butyl**

(5*S*)-5-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)-2,2-dimethylpyrrolidine-1-carboxylate (2j). Compound **2j** (431 mg) was prepared from 3-amino-5-bromothiophene-2-carboxamide (250 mg, 1.13 mmol) and (*S*)-1-(*tert*-butoxycarbonyl)-5,5-dimethylpyrrolidine-2-carboxylic acid (577 mg, 2.37 mmol) in 89% yield by a procedure similar to that described for **2a** as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.11 (9H of major, s), 1.30–1.43 (6H, m), 1.57 (9H of minor, s), 1.66–1.88 (2H, m), 1.90–2.25 (2H, m), 4.67 (1H of major, dd, J = 8.3, 3.6 Hz), 4.70–4.77 (1H of minor, m), 7.54 (1H of minor, s), 7.57 (1H of major, s), 12.68 (1H, br s). This material was observed as a 7:4 mixture of rotamers by ^{1}H NMR analysis. Only single peak was detected by chiral HPLC analysis [column: CHIRALPAK AD-H CG075 4.6 mm i.d. × 250 mm, mobile phase: *n*-hexane/IPA/Et_2NH (800:200:1, v/v/v), flow rate: 1 mL/min, column temperature: 30 °C, detection: 220 nM]}.

***tert*-Butyl**

(2*S*,4*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)-4-methylpyrrolidine-1-carboxylate (2k). To a mixture of (2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-methylpyrrolidine-2-carboxylic acid (492 mg, 2.14 mmol) and Et_3N (0.353 mL, 2.55 mmol) in THF (5 mL) was added dropwise isobutyl chloroformate (0.292 mL, 2.24 mmol) at 0 °C. The mixture was stirred at room temperature for 30 min, then 3-amino-5-bromothiophene-2-carboxamide (225 mg, 1.02 mmol) was added. The mixture was stirred at 60 °C for 24 h, and cooled to room temperature, then diluted with saturated $NaHCO_3$ aq., and extracted with EtOAc. The organic layer was collected, washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo. The residue was purified by preparative chiral HPLC [column: CHIRALPAK AD NF001 50 mm i.d. × 500 mm, mobile phase: *n*-hexane/EtOH (85:15, v/v), flow rate: 80 mL/min, column temperature: 30 °C, detection: 220 nM, loading: 90 mg/load] to remove (2*S*,4*R*)-derivative **2k'** (42 mg), determined by X-ray crystallography analysis (Figure 12), which is considered to be derived from a contaminated isomer of the starting material. Other significant peak could not be detected, and (2*S*, 4*S*)-acyl intermediate (tR2, 302 mg) was obtained as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.94–1.03 (3H, m), 1.22 (9H of major, s), 1.39 (9H of minor, s), 1.41–1.56 (1H, m), 2.13–2.33 (1H, m), 2.35–2.48 (1H, m), 2.82–3.00 (1H, m), 3.70 (1H, dd, J = 10.1, 7.5 Hz), 4.13 (1H, t, J = 8.1 Hz), 7.71 (2H, br s), 8.03 (1H of minor, s), 8.05 (1H of major, s), 11.65 (1H, s). This material was observed as a 3:2 mixture of rotamers by ^{1}H NMR analysis. To a suspension of this material (302 mg) in EtOH (5 mL) was added 2 M NaOH (1.73 mL, 3.47 mmol). The mixture was stirred at 70 °C for 4

h, and cooled to room temperature. The mixture was neutralized by addition of 6 M HCl (0.6 mL), and water (8 mL) was added. The precipitate was collected by filtration to give **2k** (240 mg, 57%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.98–1.05 (3H, m), 1.08 (9H of major, s), 1.35 (9H of minor, s), 1.45–1.66 (1H, m), 2.18–2.33 (1H, m), 2.34–2.46 (1H, m), 2.97–3.13 (1H, m), 3.57–3.71 (1H, m), 4.49–4.60 (1H, m), 7.61 (1H of minor, s), 7.64 (1H of major, s), 12.73 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (2H) were not observed. This material was observed as a 2:1 mixture of rotamers by ^{1}H NMR analysis.

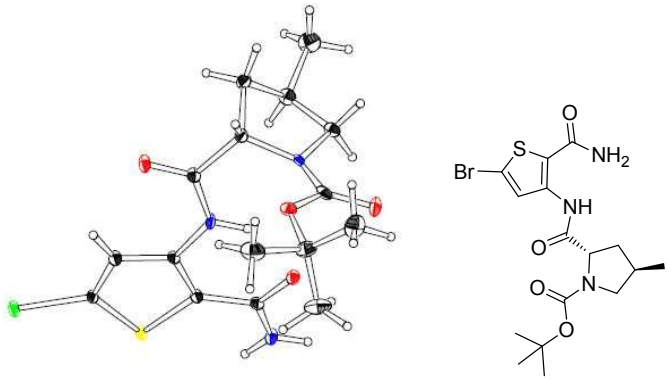


Figure 12. ORTEP of (2*S*,4*R*)-derivative **2k'** (CCDC 1918342), minor byproduct in the synthesis of **2k**. Thermal ellipsoids are drawn at 20% probability.

X-ray structure analysis of (2*S*,4*R*)-derivative 2k', minor byproduct in the synthesis of 2k

A single crystal was obtained from a mixture of IPA and THF, and analyzed as follows:

Crystal data for **2k**: $C_{16}H_{22}BrN_3O_4S$, *MW* = 432.33; crystal size, 0.36 × 0.21 × 0.11 mm; colorless, block; triclinic, space group P1, *a* = 9.22876(17) Å, *b* = 10.8877(2) Å, *c* = 12.6333(2) Å, *α* = 94.4434(7)°,

β = 91.0322(7)°, γ = 109.2418(7)°, V = 1193.60(4) Å^3, Z = 2, Dx = 1.203 g/cm^3, T = 100 K, μ = 3.346 mm^{-1}, λ = 1.54187 Å, R_1 = 0.042, wR_2 = 0.118, Flack Parameter[22] = 0.003(19).

All measurements were made on a Rigaku R-AXIS RAPID diffractometer using graphite monochromated Cu-Kα radiation. The structure was solved by direct methods with SIR92[23] and was refined using full-matrix least-squares on F^2 with SHELXL-97.[24] All non-H atoms were refined with anisotropic displacement parameters. The coordinates of the structure were deposited in the CCDC under the accession code CCDC 1918342.

***tert*-Butyl**

(2*S*,3*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)-3-methylpyrrolidine-1-carboxylate (2l). Compound **2l** (276 mg) was prepared from 3-amino-5-bromothiophene-2-carboxamide (208 mg, 0.94 mmol) and (2*S*,3*S*)-1-(*tert*-butoxycarbonyl)-3-methylpyrrolidine-2-carboxylic acid (492 mg, 2.14 mmol) in 71% yield by a procedure similar to that described for **2a** as a pale yellow oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.03–1.07 (3H, m), 1.09 (9H of major, s), 1.35 (9H of minor, m), 1.47–1.62 (1H, m), 1.99–2.11 (1H, m), 2.24–2.38 (1H, m), 3.42–3.57 (2H, m), 4.05–4.15 (1H, m), 7.61 (1H of minor, s), 7.63 (1H of major, s), 12.77 (1H, br s). This material was observed as a 5:2 mixture of rotamers by ^{1}H NMR analysis.

***tert*-Butyl**

(2*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)-2-methylpyrrolidine-1-carboxylate

(2m). To a mixture of (*S*)-1-(*tert*-butoxycarbonyl)-2-methylpyrrolidine-2-carboxylic acid (1.20 g, 5.23 mmol) and Et_3N (1.22 mL, 8.72 mmol) in THF (20 mL) was added dropwise isobutyl chloroformate (0.566 mL, 4.36 mmol) at 0 °C. The mixture was stirred at room temperature for 1 h, then 3-amino-5-bromothiophene-2-carboxamide (964 mg, 4.36 mmol) was added. The mixture was reacted under microwave irradiation to 120 °C for 3 h, and cooled to room temperature, then diluted with saturated $NaHCO_3$ aq., and extracted with EtOAc. The organic layer was collected, washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo. To a suspension of this material in EtOH (20 mL) was added 2 M NaOH (10.9 mL, 21.8 mmol). The mixture was stirred at 100 °C overnight, and cooled to room temperature. The mixture was diluted with saturated $NaHCO_3$ aq., and extracted with EtOAc. The organic layer was collected, washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo. The residue was purified by column chromatography on amino silica gel (*n*-hexane/EtOAc, 100:0 to 0:100, v/v) to give crude **2m** (244 mg, 14%) as a pale yellow solid. This material was used in the next reaction without further purification.

***tert*-Butyl**

1-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)-2-azabicyclo[2.1.1]hexane-2-carboxylate

(2n). Compound **2n** (180 mg) was prepared in 29% yield from 3-amino-5-bromothiophene-2-carboxamide (327 mg, 1.48 mmol) and 2-(*tert*-butoxycarbonyl)-2-azabiciclo[2.1.1]hexane-1-carboxylic acid (455 mg, 2.00 mmol) by a procedure similar to that described for **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.02 (9H, br s), 1.70 (2H, dd, J = 4.5,

1.7 Hz), 1.97 (2H, br s), 2.64 (1H, t, *J* = 2.9 Hz), 3.34–3.38 (2H, m), 7.19 (1H, s), the exchangeable hydrogen attached to the hetero atom (1H) was not observed.

Benzyl 1-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)-7-azabicyclo[2.2.1]heptane-7-carboxylate (2o). To a mixture of 3-amino-5-bromothiophene-2-carboxamide (921 mg, 4.17 mmol) and benzyl 1-(chlorocarbonyl)-7-azabiciclo[2.2.1]heptanes-7-carboxylate **20** (5.00 mmol) in THF (25 mL) was added DIEA (2.91 mL, 16.7 mmol) at room temperature. After 1.5 h, the mixture was diluted with saturated $NaHCO_3$ aq., and extracted with EtOAc. The organic layer was collected, washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo. To a suspension of this material in EtOH (25 mL) was added 2 M NaOH (10.4 mL, 20.8 mmol). The mixture was stirred at 100 °C overnight, and cooled to room temperature. The mixture was diluted with saturated $NaHCO_3$ aq., and extracted with EtOAc. The organic layer was collected, washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo. The residue was purified by column chromatography on amino silica gel (*n*-hexane/EtOAc, 100:0 to 0:100, then EtOAc/MeOH, 100:0 to 80:20, v/v) to give **2o** (763 mg, 40%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.51–1.64 (2H, m), 1.77–1.93 (4H, m), 2.16–2.29 (2H, m), 4.36–4.43 (1H, m), 4.90 (2H, s), 7.07–7.27 (5H, m), 7.56 (1H, s), 12.52 (1H, br s).

2-((2*S*)-Azetidin-2-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (3a). A mixture of **2a** (328 mg, 0.849 mmol), *tert*-butyl

3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate (626 mg, 1.70 mmol), Cs_2CO_3 (554 mg, 1.70 mmol) and $PdCl_2$(dppf) (139 mg, 0.17 mmol) in DME (10 mL)–water (1 mL) was degassed and stirred under Ar at 80 °C for 2 h, then diluted with water, and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 6:4 to 3:7, v/v) to give di-Boc intermediate (291 mg). This material was dissolved with MeOH (5 mL), and 4 M HCl in EtOAc (1 mL) was added. The mixture was stirred at 50 °C for 1.5 h. To the mixture was added EtOAc (4 mL), and the precipitate was collected by filtration. This material was treated with Et_3N (1 mL) in MeOH (5 mL) at room temperature for 1 h, then diluted with brine, and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo, and triturated with MeOH (0.5 mL)–EtOAc (2.0 mL). The precipitate was collected by filtration to give **3a** (42.1 mg, 17%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.41–2.61 (2H, m), 2.45 (3H, s), 3.30–3.38 (1H, m), 3.61 (1H, q, J = 7.9 Hz), 4.73 (1H, t, J = 7.8 Hz), 7.38 (1H, s), 8.03 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (3H) were not observed. HRMS: Calcd for $C_{13}H_{14}N_5OS$ $[M+H]^+$: 288.0914. Found: 288.0907. Anal. Calcd for $C_{13}H_{13}N_5OS \cdot 0.5H_2O$: C, 52.69; H, 4.76; N, 23.63. Found: C, 52.89; H, 4.55; N, 23.36.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-((2*S*)-pyrrolidin-2-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (3b). Compound **3b** (1.39 g) was prepared from **2b** (2.27 g, 5.67 mmol) in 66% yield by a procedure similar to that described for **3a** as a white solid. Mp 278–280 °C. ^{1}H NMR (300 MHz,

DMSO-d_6) δ 1.94–2.16 (3H, m), 2.39–2.47 (1H, m), 2.46 (3H, s), 3.23–3.49 (2H, m), 4.61–4.74 (1H, m), 7.37 (1H, s), 8.10 (1H, s), 8.98 (1H, br s), 10.07 (1H, br s), 12.87 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (2H) were not observed. Anal. Calcd for $C_{14}H_{15}N_5OS \cdot 2HCl$: C, 44.93; H, 4.58; N, 18.71. Found: C, 44.86; H, 4.61; N, 18.66.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-((2*S*)-piperidin-2-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (3d). Compound **3d** (1.77 g) was prepared from **2d** (3.25 g, 7.84 mmol) in 58% yield by a procedure similar to that described for **3a** as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.48–1.91 (5H, m), 2.24–2.32 (1H, m), 2.46 (3H, s), 2.97–3.12 (1H, m), 3.29–3.41 (1H, m), 4.14–4.29 (1H, m), 7.34 (1H, s), 8.11 (1H, s), 9.07–9.23 (1H, m), 9.36–9.48 (1H, m), 12.81 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (2H) were not observed. Anal. Calcd for $C_{15}H_{17}N_5OS \cdot 2HCl \cdot 0.5H_2O$: C, 45.34; H, 5.07; N, 17.63; Cl, 17.85. Found: C, 45.61; H, 5.07; N, 17.57; Cl, 17.73. 99.8% ee {determined by chiral HPLC analysis [column: SUMICHIRAL ADH DJ153 4.6 mm i.d. × 250 mm, Sumika Chemical Analysis Service Co. Ltd., mobile phase: *n*-hexane/EtOH/Et_3N (600:400:5, v/v/v), flow rate: 1 mL/min, column temperature: 30 °C, detection: 254 nM]}.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-((2*S*)-piperidin-2-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (3d'). To a suspension of **3d** (255 mg, 0.66 mmol) in MeOH (7 mL) was added Et_3N (0.279 mL, 2.00 mmol). Then amino silica gel (5 g) was added, and the mixture was triturated. The mixture was concentrated in vacuo, and the residue was purified by column chromatography on amino silica gel (EtOAc/MeOH, 100:0 to

70:30, v/v) to give **3d'** (183 mg, 88 %) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.36–1.62 (4H, m), 1.77–1.94 (2H, m), 2.44 (3H, s), 2.59–2.69 (1H, m), 2.98–3.08 (1H, m), 3.60–3.68 (1H, m), 7.31 (1H, s), 8.00 (1H, br s), the exchangeable hydrogen attached to the hetero atom (3H) was not observed.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-((2*R*)-piperidin-2-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (3e). Compound **3e** (60.8 mg) was prepared from **2e** (120 mg, 0.290 mmol) in 54% yield by a procedure similar to that described for **3a** as a white solid. Mp 252–255 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.48–1.94 (5H, m), 2.24–2.35 (1H, m), 2.46 (3H, s), 2.96–3.13 (1H, m), 3.29–3.41 (1H, m), 4.16–4.27 (1H, m), 7.34 (1H, s), 8.12 (1H, s), 9.07–9.25 (1H, m), 9.35–9.50 (1H, m), 12.82 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (2H) were not observed. Anal. Calcd for $C_{15}H_{17}N_5OS \cdot 2HCl \cdot 0.3H_2O$: C, 45.76; H, 5.02; N, 18.01; Cl. Found: C, 45.86; H, 5.03; N, 17.79. 98.7% ee {determined by chiral HPLC analysis [column: SUMICHIRAL ADH DJ153 4.6 mm i.d. × 250 mm, Sumika Chemical Analysis Service Co. Ltd., mobile phase: *n*-hexane/EtOH/Et_3N (600:400:5, v/v/v), flow rate: 1 mL/min, column temperature: 30 °C, detection: 254 nM]}.

2-((2*S*)-Azepan-2-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (3g). Compound **3g** (92.7 mg) was prepared from **2g** (310 mg, 0.724 mmol) in 39% yield by a procedure similar to that described for **3a** as a white solid. Mp 187–192 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.40–1.89 (7H, m), 2.05–2.21 (1H, m), 2.45 (3H, s), 2.74–2.97 (2H, m), 3.76–3.86 (1H, m), 7.34 (1H, s), 8.01 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (3H) were not observed. Anal. Calcd

for $C_{16}H_{19}N_5OS \cdot 0.2H_2O$: C, 57.71; H, 5.87; N, 21.03. Found: C, 57.57; H, 5.78; N, 21.03.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-((2*S*)-1,2,3,6-tetrahydropyridin-2-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (3i). Compound **3i** (620 mg) was prepared from **2i** (900 mg, 2.18 mmol) in 74% yield by a procedure similar to that described for **3a** as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.35-2.47 (4H, m), 2.70–2.88 (1H, m), 3.62–3.85 (2H, m), 4.34–4.49 (1H, m), 5.76–6.04 (2H, m), 7.37 (1H, s), 8.12 (1H, s), 9.74 (2H, br s), 12.86 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (2H) were not observed. Anal. Calcd for $C_{15}H_{15}N_5OS \cdot 2HCl \cdot 0.2H_2O$: C, 46.21; H, 4.50; N, 17.96. Found: C, 46.07; H, 4.68; N, 17.69.

2-((2*S*)-5,5-Dimethylpyrrolidin-2-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (3j). Compound **3j** (176 mg) was prepared from **2j** (423 mg, 0.988 mmol) in 54% yield by a procedure similar to that described for **3a** as a white solid. Mp 190–191 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.17 (3H, s), 1.19 (3H, s), 1.60 (2H, t, J = 7.41 Hz), 1.92–2.05 (1H, m), 2.23–2.38 (1H, m), 2.45 (3H, s), 4.24 (1H, dd, J = 8.6, 6.3 Hz), 7.37 (1H, s), 8.02 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (3H) were not observed. Anal. Calcd for $C_{16}H_{19}N_5OS \cdot 0.5H_2O$: C, 56.78; H, 5.96; N, 20.69. Found: C, 56.73; H, 5.83; N, 20.58.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-((2*S*,4*S*)-4-methylpyrrolidin-2-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (3k). Compound **3k** (137 mg) was prepared from **2k** (323 mg, 1.41 mmol) in 61% yield

by a procedure similar to that described for **3a** as a white solid. Mp 270–275 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.07 (3H, d, J = 6.6 Hz), 1.59–1.75 (1H, m), 2.34–2.50 (1H, m), 2.46 (3H, s), 2.60–2.76 (1H, m), 2.82–2.99 (1H, m), 3.40–3.53 (1H, m), 4.59–4.76 (1H, m), 7.37 (1H, s), 8.10 (1H, s), 9.04 (1H, br s), 12.85 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (3H) were not observed. Anal. Calcd for $C_{15}H_{17}N_5OS \cdot 2HCl$: C, 46.40; H, 4.93; N, 18.04. Found: C, 46.45; H, 4.96; N, 18.04.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-((2*S*,3*S*)-3-methylpyrrolidin-2-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (3l). Compound **3l** (127 mg) was prepared from **2l** (272 mg, 0.66 mmol) in 50% yield by a procedure similar to that described for **3a** as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.17 (3H, d, J = 6.8 Hz), 1.60–1.75 (1H, m), 2.14–2.28 (1H, m), 2.46 (3H, s), 2.50–2.59 (1H, m), 3.36–3.48 (2H, m), 4.17–4.25 (1H, m), 7.37 (1H, s), 8.09 (1H, s), 9.00 (1H, br s), 10.25 (1H, br s), 12.85 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (2H) were not observed. Anal. Calcd for $C_{15}H_{17}N_5OS \cdot 2HCl \cdot H_2O$: C, 44.34; H, 5.21; N, 17.24. Found: C, 44.62; H, 5.26; N, 17.00.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-((2*S*)-2-methylpyrrolidin-2-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (3m). Compound **3m** (54 mg) was prepared from **2m** (239 mg, 0.58 mmol) in 24% yield by a procedure similar to that described for **3a** as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.74 (3H, s), 1.83–2.40 (4H, m), 2.46 (3H, s), 3.27–3.44 (2H, m), 7.37 (1H, s), 8.10 (1H, br s), 9.18 (1H, br s), 9.62 (1H, br s), 12.81 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (2H) were not observed. Anal. Calcd for $C_{15}H_{17}N_5OS \cdot 2HCl \cdot 1.1H_2O$: C, 44.14; H, 5.24; N, 17.16. Found: C,

44.17; H, 5.18; N, 16.92.

2-(2-Azabicyclo[2.1.1]hex-1-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (3n). Compound **3n** (98 mg) was prepared from **2n** (150 mg, 0.364 mmol) in 70% yield by a procedure similar to that described for **3a** as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.80–1.92 (2H, br s), 2.47 (3H, s), 2.68–2.82 (2H, m), 2.92–3.02 (1H, m), 3.28–3.40 (2H, m), 7.42 (1H, s), 8.12 (1H, s), 9.95 (2H, br s), the exchangeable hydrogens attached to the hetero atoms (3H) were not observed. Anal. Calcd for $C_{15}H_{15}N_5OS\cdot 2HCl\cdot 0.2H_2O$: C, 46.21; H, 4.50; N, 17.96. Found: C, 46.25; H, 4.63; N, 17.71.

2-(7-Azabicyclo[2.2.1]hept-1-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one hydrochloride (3o). A mixture of **2o** (708 mg, 1.54 mmol), *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate (948 mg, 3.08 mmol), Cs_2CO_3 (3.07 g, 9.23 mmol) and $PdCl_2$(dppf) (56.3 mg, 0.08 mmol) in DME (12 mL)–water (3 mL) was degassed and stirred under Ar at 90 °C for 1 h, then diluted with water, and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 100:0 to 0:100, v/v) to give intermediate. This material was dissolved with formic acid (15 mL), and 10% Pd/C (50% wet, 300 mg) was added. The mixture was stirred at room temperature for 1 h, then filtered through a pad of Celite, and the pad was washed with formic acid well. The filtrate was

concentrated in vacuo. To the residue was added excess saturated $NaHCO_3$ aq., and extracted with EtOAc–THF (3:1, v/v). The organic layer was collected, washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo. To the residue was added MeOH (5 mL) and 10% HCl in MeOH (2.5 mL). The mixture was concentrated in vacuo, and EtOH (10 mL)–water (1 mL) was added to the residue. The mixture was stirred at 70 °C for 30 min, and cooled to room temperature. The precipitate was collected by filtration to give **3o** (134 mg, 24%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.81–2.09 (6H, m), 2.35–2.47 (5H, m), 4.13–4.21 (1H, m), 7.37 (1H, s), 8.13 (1H, br s), 9.16–9.85 (2H, m), 12.57–13.23 (2H, m). Anal. Calcd for $C_{16}H_{17}N_5OS \cdot HCl \cdot 1.5H_2O$: C, 49.16; H, 5.42; N, 17.92. Found: C, 49.37; H, 5.64; N, 17.56.

2-((2*S*)-Piperidin-2-yl)-6-(3-(trifluoromethyl)-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one hydrochloride (3p). A mixture of **2d** (3.50 g, 8.45 mmol), 3-(trifluoromethyl)-1-trityl-1*H*-pyrazol-4-ylboronic acid (10.7 g, 25.3 mmol), $Pd(PPh_3)_4$ (0.488 g, 0.42 mmol), and Na_2CO_3 (2.24 g, 21.1 mmol) in EtOH (100 mL)–water (10 mL) was stirred at 80 °C under Ar overnight. The mixture was diluted with water and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 9:1 to 5:5, v/v), and further purified by column chromatography on amino silica gel (EtOAc/MeOH, 100:0 to 80:20, v/v). The obtained oil was dissolved in 4 M HCl–EtOAc (15 mL) and MeOH (15 mL), and the solution was stirred at 60 °C overnight. The resulting solid was collected by filtration and washed with EtOAc. The solid was

triturated with EtOH (135 mL)–water (15 mL)–EtOAc (100 mL), and the precipitate was collected by filtration to give **3p** (2.85 g, 83%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.47–1.93 (5H, m), 2.31 (1H, br s), 2.94–3.11 (1H, m), 3.25–3.50 (2H, m), 4.20–4.33 (1H, m), 7.39 (1H, s), 8.61 (1H, s), 9.52 (1H, br s), 12.13–15.00 (1H, m), the exchangeable hydrogen attached to the hetero atom (1H) was not observed. Anal. Calcd for $C_{15}H_{14}N_5OSF_3 \cdot HCl$: C, 44.39; H, 3.73; N, 17.26. Found: C, 44.38; H, 3.79; N, 17.00.

1-(1-(4-Methoxybenzyl)-5-methyl-1*H*-pyrazol-4-yl)ethanone (5). A mixture of pentane-2,4-dione (54.2 g, 541 mmol) and 1,1-dimethoxy-*N*,*N*-dimethylmethanamine (75 mL, 565 mmol) was stirred at 80 °C for 1 h, and cooled to 0 °C. To the mixture was added EtOH (300 mL), Et_3N (137 mL, 983 mmol) and (4-methoxybenzyl)hydrazine hydrochloride (78.0 g, 492 mmol) slowly at 0 °C. The mixture was stirred at room temperature for 18 h, and concentrated in vacuo. The residue was diluted with water (200 mL), and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 100:0 to 50:50, v/v) to give **5** (62.9 g, 52%) as a yellow oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.36 (3H, s), 2.47 (3H, s), 3.72 (3H, s), 5.28 (2H, s), 6.85–6.95 (2H, m), 7.12 (2H, d, J = 8.6 Hz), 8.02 (1H, s).

(2*Z*)-3-Chloro-3-(1-(4-methoxybenzyl)-5-methyl-1*H*-pyrazol-4-yl)acrylonitrile (6). To DMF (20.0 mL, 258 mmol) at 0 °C was added dropwise $POCl_3$ (24.0 mL, 258 mmol), and the mixture was stirred at

0 °C for 15 min. Then, a solution of **5** (31.5 g, 129 mmol) in DMF (100 mL) was added dropwise at 0 °C. The mixture was stirred at 60 °C for 30 min, then hydroxylamine hydrochloride (17.9 g, 258 mmol) was added portionwise at 80 °C (exothermic reaction should be cared), and the mixture was stirred at 80 °C for a further 30 min. The mixture was cooled to room temperature, poured into water, and extracted with EtOAc. The organic layer was dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 100:0 to 50:50, v/v) to give **6** (26.2 g, 71%) as a pale yellow oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.43 (3H, s), 3.72 (3H, s), 5.31 (2H, s), 6.27 (1H, s), 6.90 (2H, d, J = 8.2 Hz), 7.14 (2H, d, J = 8.2 Hz), 7.83 (1H, s).

Methyl 3-amino-5-(1-(4-methoxybenzyl)-5-methyl-1*H*-pyrazol-4-yl)thiophene-2-carboxylate (7). To a solution of methyl thioglycolate (1.87 mL, 20.9 mmol) in MeOH (24 mL) was added 28% NaOMe in MeOH (4.02 g, 20.9 mmol) at 0 °C. After being stirred for 5 min, **6** (4.00 g, 13.9 mmol) was added. The mixture was stirred at 40 °C for 2 h, and cooled to 0 °C. The precipitate was collected by filtration, and washed with MeOH and water to give **7** (4.12 g, 83%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.39 (3H, s), 3.70 (3H, s), 3.72 (3H, s), 5.28 (2H, s), 6.52 (2H, s), 6.64 (1H, s), 6.84–6.95 (2H, m), 7.13 (2H, d, J = 8.8 Hz), 7.72 (1H, s).

Methyl 3-((1-azabicyclo[2.2.2]oct-2-ylcarbonyl)amino)-5-(1-(4-methoxybenzyl)-5-methyl-1*H*-pyrazol-4-yl)thiophene-2-carboxylate (8). A mixture of quinuclidine-2-carboxylic acid **27** (ca. 31% purity, 116 g, 187

mmol), DMF (1.78 g, 24.4 mmol) and thionyl chloride (252 mL, 3.48 mol) was stirred at 30 °C for 18 h. The mixture was concentrated in vacuo, and azeotroped repeatedly with toluene to give a white powder. To the residue was added THF (1 L), **7** (58.1 g, 163 mmol), and DIEA (78.0 mL, 447 mmol). The mixture was stirred at room temperature for 15 min, then at 60 °C for 1 h. The mixture was poured into water and extracted with EtOAc twice. The organic layer was washed with water, brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, and the residue was triturated with MeOH (180 mL). The precipitate was collected by filtration, and washed with MeOH (50 mL x 2) to afford **8** (62.9 g, 78%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.33–1.56 (4H, m), 1.75–1.87 (3H, m), 2.43 (3H, s), 2.54–3.09 (4H, m), 3.61 (1H, t, J = 8.7 Hz), 3.72 (3H, s), 3.81 (3H, s), 5.31 (2H, s), 6.84–6.96 (2H, m), 7.15 (2H, d, J = 8.7 Hz), 7.83 (1H, s), 8.12 (1H, s), 11.25 (1H, s).

***N*-(2-Carbamoyl-5-(1-(4-methoxybenzyl)-5-methyl-1*H*-pyrazol-4-yl)-3-thienyl)quinuclidine-2-carboxamide (9).** A mixture of **8** (80.0 g, 162 mmol), 2 M NaOH (243 mL, 486 mmol), MeOH (436 mL), and THF (364 mL) was stirred at 60 °C for 1.5 h, and then cooled to at 0 °C. To the mixture was added 2 M HCl (243 mL, 486 mmol). The mixture was concentrated in vacuo, and azeotroped repeatedly with toluene to give a beige solid. To the residue was added EDCI (46.5 g, 243 mmol), HOBt (21.9 g, 162 mmol), NH_4Cl (17.3 g, 323 mmol), Et_3N (47.3 mL, 340 mmol) and DMF (720 mL). The mixture was stirred at room temperature overnight, and then water (960 mL) was added dropwise, and cooled to 0 °C. After 1 h, the precipitate was collected by filtration, washed with water and IPE, and dried under vacuum to give **9** (70.0 g, 90%) as a beige solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.32–1.53 (4H, m), 1.70–1.87

(3H, m), 2.42 (3H, s), 2.54–3.06 (4H, m), 3.54 (1H, t, J = 8.5 Hz), 3.72 (3H, s), 5.30 (2H, s), 6.87–6.96 (2H, m), 7.14 (2H, d, J = 8.7 Hz), 7.39 (2H, br s), 7.72 (1H, s), 8.08 (1H, s), 11.85 (1H, s).

2-(1-Azabicyclo[2.2.2]oct-2-yl)-6-(1-(4-methoxybenzyl)-5-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (10). To a suspension of **9** (70.0 g, 146 mmol) in EtOH (700 mL) was added 2 M NaOH (365 mL, 730 mmol). The mixture was stirred at 70 °C for 2 h. The reaction mixture was cooled to room temperature, and 2 M HCl (365 mL, 730 mmol) was added. The resulting solution was evaporated to remove EtOH, and left to stand for 60 h. The precipitate was collected by filtration and washed with water (350 mL × 2), EtOH (70 mL) and IPE (70 mL) to give **10** (66.6 g, 99 %) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.37–1.59 (4H, m), 1.67–1.91 (2H, m), 2.20–2.33 (1H, m), 2.48 (3H, s), 2.59 (2H, d, J = 6.4 Hz), 2.77–2.92 (1H, m), 3.00–3.13 (1H, m), 3.73 (3H, s), 3.84–3.98 (1H, m), 5.32 (2H, s), 6.85–6.95 (2H, m), 7.12–7.21 (2H, m), 7.45 (1H, s), 7.91 (1H, s), 11.54 (1H, br s).

2-(1-Azabicyclo[2.2.2]oct-2-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (11a). A mixture of **10** (50.0 g, 108 mmol), anisole (11.8 ml, 108 mmol) and TFA (417 mL, 5.42 mol) was stirred at 90 °C for 18 h. The mixture was concentrated in vacuo, and the residue (120 g, oil) was dissolved in MeOH (1.5 L). Small amount of undissolved material was removed by decantation. The solution was through a column of Amberlyst A-21 (2.5 kg) (ion-exchange resin) with elution with MeOH (9 L). Then the ion-exchange resin was washed with 2,2,2-trifluoroethanol–MeOH (3L, 1:1, v/v) and MeOH (6 L). All eluant was concentrated in vacuo. The residue was suspended in MeOH (1 L), and the

insoluble sticky gum was removed by filtration. The filtrate was concentrated in vacuo to give a beige solid (101.1 g). The obtained solid was triturated with MeOH (250 mL), and EtOAc (400 mL) was added. After being left to stand at room temperature overnight, the precipitate was collected by filtration and washed with EtOAc (200 mL) and IPE (300 mL) to give **11a** (27.2 g, 74%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.35–1.60 (4H, m), 1.67–1.90 (2H, m), 2.23–2.33 (1H, m), 2.46 (3H, s), 2.55–2.65 (2H, m), 2.78–2.93 (1H, m), 3.00–3.13 (1H, m), 3.90 (1H, t, J = 8.5 Hz), 7.43 (1H, s), 8.04 (1H, br s), 12.28 (1H, br s), the exchangeable hydrogen attached to the hetero atom (1H) was not observed.

2-((2*S*)-1-Azabicyclo[2.2.2]oct-2-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one hemihydrate (11b) and **2-((2*R*)-1-azabicyclo[2.2.2]oct-2-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one hemihydrate (11c). 11a** (20.3 g) was purified by preparative chiral HPLC [column: CHIRALPAK AD 50 mm i.d. × 500 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/IPA/Et_2NH (600:400:1, v/v/v), flow rate: 60 mL/min, column temperature: 30 °C, detection: 220 nM, loading: 1.0 g/load, concentration: 2.5 mg/mL in the mobile phase/MeOH (1:1, v/v), tR1 = **11c**, tR2 = **11b**]. The obtained crude **11b** (9.53 g) was recrystallized from EtOH–water (780 mL, 100/1, v/v) to give **11b** (7.73 g, 37%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.37–1.61 (4H, m), 1.60–1.91 (2H, m), 2.23–2.33 (1H, m), 2.46 (3H, s), 2.54–2.67 (2H, m), 2.77–2.94 (1H, m), 3.00–3.14 (1H, m), 3.91 (1H, t, J = 8.9 Hz), 7.44 (1H, s), 8.03 (1H, br s), 12.24 (1H, br s), the exchangeable hydrogen attached to the hetero atom (1H) was not observed. Anal. Calcd for $C_{17}H_{19}N_5OS·0.5H_2O$: C, 58.26; H, 5.75; N, 19.98. Found: C, 58.25; H, 5.83; N,

19.79. 99.8% ee {determined by chiral HPLC analysis [column: CHIRALPAK AD-H 4.6 mm i.d. × 250 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/IPA/Et_2NH (600:400:1, v/v/v), flow rate: 1 mL/min, column temperature: 30 °C, detection: 254 nM]}. $[\alpha]_D$ –13.6 ° (c = 1.0135, DMSO, 20 °C). The obtained crude **11c** (9.40 g) was recrystallized from EtOH–water (820 mL, 100:1, v/v) to give **11c** (7.66 g, 37%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.39–1.59 (4H, m), 1.68–1.90 (2H, m), 2.23–2.33 (1H, m), 2.46 (3H, s), 2.55–2.65 (2H, m), 2.78–2.92 (1H, m), 3.02–3.13 (1H, m), 3.90 (1H, t, *J* = 8.7 Hz), 7.43 (1H, s), 8.04 (1H, br s), 12.37 (1H, br s), the exchangeable hydrogen attached to the hetero atom (1H) was not observed. Anal. Calcd for $C_{17}H_{19}N_5OS \cdot 0.5H_2O$: C, 58.26; H, 5.75; N, 19.98. Found: C, 58.03; H, 5.81; N, 19.77. 99.7% ee {determined by chiral HPLC analysis [column: CHIRALPAK AD-H 4.6 mm i.d. × 250 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/IPA/Et_2NH (600:400:1, v/v/v), flow rate: 1 mL/min, column temperature: 30 °C, detection: 254 nM]}. $[\alpha]_D$ +15.1 ° (c = 1.0135, DMSO, 20 °C).

2-((2*S*)-1-Azabicyclo[2.2.2]oct-2-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one di-*p*-toluoyl-*D*-tartaric acid (11b')

A mixture of **11b** (171 mg, 0.487 mmol) and (+)-di-*p*-toluoyl-*D*-tartaric acid (193 mg, 0.50 mmol) in MeOH (10 ml) was heated to 70 °C. Once a suspension was dissolved, and a precipitate formed. The mixture was stirred at 70 °C for 10 min, then at room temperature for 2 h. The precipitate was collected by filtration, and washed with MeOH–EtOAc (3:1, v/v) to afford **11b'** (254 mg, 72%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.56–1.74 (4H, m), 2.00–2.29 (3H, m), 2.37 (6H, s), 2.46 (3H, s), 2.89–3.20 (4H, m), 4.25–4.38 (1H, m), 5.69 (2H, s), 7.33 (4H, d, *J* = 8.1 Hz), 7.43 (1H, s), 7.83 (4H, d, *J* = 8.3

Hz), 8.07 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (4H) were not observed. Anal. Calcd for $C_{37}H_{37}N_5O_{10}S$: C, 61.06; H, 5.12; N, 9.62. Found: C, 61.24; H, 5.16; N, 9.65. 99.6% ee {determined by chiral HPLC analysis [column: CHIRALPAK AD3 4.6 mm i.d. × 250 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/IPA/Et_2NH (600:400:3, v/v/v), flow rate: 0.6 mL/min, column temperature: 30 °C, detection: 254 nM]}. Absolute structure was determined by X-ray crystallography analysis of **11b''** as described below (Figure 5, CSD ID: 1918344).

X-ray structure analysis of 11b''

Preparation of single crystal 11b'' A solution of **11b'** (about 0.6 mg) in MeOH (0.15 mL)–methyl ethyl ketone (0.15 mL) was allowed to stand at room temperature under half-open air conditions for 2 days. A colorless single crystal was obtained and analyzed as follows:

Crystal data for **11b''** (Figure 5): $C_{17}H_{20}N_5OS^{+}\cdot$ 0.5 $C_{20}H_{16}O_8^{2-}\cdot$ 0.5 $CH_3OH\cdot H_2O$, MW = 568.64; crystal size, 0.20 × 0.07 × 0.06 mm; colorless, block; monoclinic, space group $P2_1$, a = 9.52273(17) Å, b = 16.7336(3) Å, c = 17.6682(4) Å, β = 100.983(7)°, V = 2763.85(11) Å^3, Z = 4, Dx = 1.366 g/cm^3, T = 100 K, μ = 1.492 mm^{-1}, λ = 1.54187 Å, R_1 = 0.060, wR_2 = 0.130, Flack Parameter[22] = 0.072(18).

All measurements were made on a Rigaku R-AXIS RAPID-191R diffractometer using graphite monochromated Cu-Kα radiation. The structure was solved by direct methods with SIR2008[23] and was refined using full-matrix least-squares on F^2 with SHELXL-97.[24] All non-H atoms were refined with anisotropic displacement parameters. The coordinates of the structure were deposited in the CCDC under the accession code CCDC 1918344.

***tert*-Butyl (2*S*)-2-((5-bromo-2-carbamoyl-3-thienyl)carbamoyl)pyrrolidine-1-carboxylate (12).** To a solution of (*S*)-*N*-Boc-proline (2.04 g, 9.50 mmol) and Et_3N (1.57 mL, 11.3 mmol) in THF (25 mL) was added isobutyl chloroformate (1.29 mL, 9.94 mmol) at 0 °C. The mixture was stirred at room temperature for 30 min. To the resulting mixture was added 3-amino-5-bromothiophene-2-carboxamide **1** (1.00 g, 4.52 mmol). The mixture was stirred at 60 °C for 24 h, then diluted with saturated $NaHCO_3$ aq., and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 60:40 to 30:70, v/v) to give **12** (1.67 g, 88%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.25 (9H of major, s), 1.40 (9H of minor, s), 1.79–1.97 (3H, m), 2.12–2.30 (1H, m), 3.35–3.55 (2H, m), 4.09–4.21 (1H, m), 7.72 (2H, br s), 8.05 (1H, s), 11.66 (1H of major, s), 11.68 (1H of minor, s). This material was observed as an 8:7 mixture of rotamers.

***N*-(5-Bromo-2-carbamoyl-3-thienyl)-L-prolinamide hydrochloride (13).** To a solution of **12** (1.66 g, 3.75 mmol) in MeOH (20 mL)–THF (10 mL) was added 4 M HCl in EtOAc (10 mL), and the mixture was stirred at 50 °C. After being stirred for 1 h, EtOAc (10 mL) was added to the reaction mixture, and the precipitated solid was collected by filtration to give **13** (1.26 g, 95%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.86–2.07 (3H, m), 2.28–2.41 (1H, m), 3.17–3.29 (2H, m), 4.52 (1H, t, J = 7.5 Hz), 7.84 (2H, br s), 7.88 (1H, s), 9.15 (2H, br s), 11.46 (1H, br s).

6-Bromo-2-((2*S*)-1-methylpyrrolidin-2-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (14). To a solution of **13** (1.05 g, 2.96 mmol) in MeOH (25 mL) were added formalin (37% in water, 1.10 mL, 14.8 mmol) and sodium cyanoborohydride (558 mg, 8.88 mmol), and the mixture was stirred at room temperature for 1 h. 2 M NaOH (7.40 mL, 14.8 mmol) was added to the reaction mixture, and the mixture was stirred at 50 °C for a further 5 h. The reaction mixture was neutralized with 6 M HCl (2.5 mL) under ice-cooling, and concentrated under reduced pressure to a half volume. EtOAc (50 mL) and brine (10 mL) were added to the residue, and the separated aqueous layer was extracted with EtOAc (10 mL × 2). The combined organic layers were washed with brine (10 mL) and dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc) to give **14** (892 mg, 96%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.68–1.99 (3H, m), 2.10–2.39 (2H, m), 2.24 (3H, s), 3.08–3.18 (1H, m), 3.25–3.32 (1H, m), 7.57 (1H, s), 11.90 (1H, br s). 99.8% ee {determined by chiral HPLC analysis [column: CHIRALPAK AD-H 4.6 mm i.d. × 250 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/EtOH/Et_2NH (500:500:1, v/v/v), flow rate: 0.5 mL/min, column temperature: 30 °C, detection: 220 nM, racemate was prepared from Boc-DL-proline by the same standard procedure.]}.

6-Bromo-2-[(2*S*)-1-methylpyrrolidin-2-yl]-3-{[2-(trimethylsilyl)ethoxy]methyl}thieno[3,2-*d*]pyrimidin-4(3*H*)-one (15). To a solution of **14** (250 mg, 0.796 mmol) in THF (5 mL) was added sodium hydride (60% in oil, 38.2 mg, 0.955 mmol) under ice-cooling, and the mixture was stirred at 0 °C for 15 min. [2-(Chloromethoxy)ethyl](trimethyl)silane (0.169 mL, 0.955 mmol) was added to the reaction mixture,

and the mixture was stirred at room temperature for 1 h. EtOAc (15 mL) and aq. NH_4Cl (5 mL) were added to the reaction mixture, and the separated aqueous layer was extracted with EtOAc (5 mL). The combined organic layers were washed with brine (10 mL), dried over Na_2SO_4, and filtered. Insoluble material was filtered off, and the filtrate was concentrated under reduced pressure. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc) to give **15** (180 mg, 40%) as a colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ –0.03 (9H, s), 0.82–0.91 (2H, m), 1.73–2.06 (3H, m), 2.17–2.29 (1H, m), 2.21 (3H, s), 2.35 (1H, q, J = 8.4 Hz), 3.05–3.15 (1H, m), 3.64 (2H, t, J = 8.1 Hz), 3.72 (1H, dd, J = 8.4, 7.1 Hz), 5.62 (1H, d, J = 10.5 Hz), 5.72 (1H, d, J = 10.5 Hz), 7.65 (1H, s).

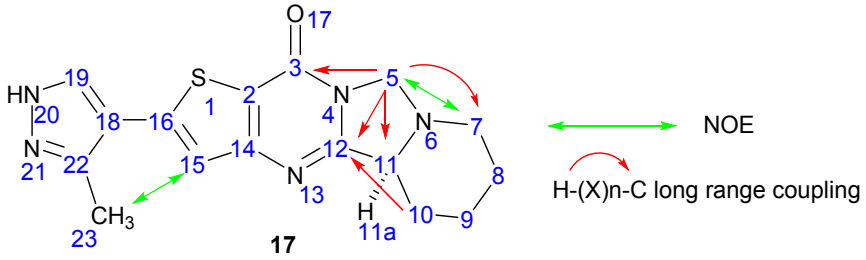
Determination of optical purity of 15' from 15. To a solution of **15** (16.3 mg, 0.0367 mmol) in THF (0.5 mL) was added 1 M TBAF in THF (0.220 mL, 0.220 mmol). The mixture was stirred at 50 °C for 5 days, and directly purified by column chromatography on amino silica gel (EtOAc/MeOH, 100:0 to 80:20, v/v). The obtained crude **15'** was subjected to determination of the optical purity. 59.2% ee {determined by chiral HPLC analysis [column: CHIRALPAK AD-H 4.6 mm i.d. × 250 mm, Daicel Co. Ltd., mobile phase: *n*-hexane/EtOH/Et_2NH (500:500:1, v/v/v), flow rate: 0.5 mL/min, column temperature: 30 °C, detection: 220 nM, racemate was prepared from Boc-DL-proline by the same standard procedure.]}.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-[(2*S*)-1-methylpyrrolidin-2-yl]thieno[3,2-*d*]pyrimidin-4(3*H*)-one

dihydrochloride (16). A mixture of **15** (160 mg, 0.360 mmol), *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate (251 mg, 0.720 mmol), Cs_2CO_3 (234 mg, 0.720 mmol) and $PdCl_2$(dppf) (58.8 mg, 0.0720 mmol) in DME (5 mL)–water (0.5 mL) was degassed and stirred under Ar at 80 °C for 1.5 h, then diluted with water, and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on amino silica gel (*n*-hexane/EtOAc 100:40 to 70:30, v/v) to give crude coupling product. To a solution of the crude coupling product in DMF (2 mL) was added 1 M TBAF in THF (1.44 mL, 1.44 mmol), and the mixture was stirred at 90 °C for 4 h. EtOAc (20 mL) and brine (10 mL) were added to the reaction mixture, and the separated aqueous layer was extracted with EtOAc (10 mL × 2). The combined organic layers were washed with brine (5 mL) and dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on amino silica gel (EtOAc/MeOH 100:0 to 85:15, v/v) to give brown oil. To a solution of this material was added 4 M HCl in EtOAc (2 mL) and EtOAc (1.5 mL), and the precipitate was collected by filtration to give **16** (15.6 mg, 11%) as a pale yellow solid. Mp 191–193 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.92–2.21 (3H, m), 2.46 (3H, s), 2.60–2.71 (1H, m), 2.96 (3H, s), 3.24–3.38 (1H, m), 3.67–3.75 (1H, m), 4.45–4.57 (1H, m), 7.37 (1H, s), 8.10 (1H, br s), 10.08 (1H, br s), 12.92 (1H, br s), the exchangeable hydrogens attached to the hetero atoms (2H) were not observed. Anal. Calcd for $C_{15}H_{17}N_5OS \cdot 2HCl \cdot 0.3H_2O$: C, 45.76; H, 5.02; N, 17.79. Found: C, 45.75; H, 5.00; N, 17.65.

(4b*S*)-2-(3-Methyl-1*H*-pyrazol-4-yl)-5,6,7,8-tetrahydropyrido[1',2':3,4]imidazo[1,5-*a*]thieno[3,2-*d*]pyrimidin-12(4b*H*)-one (17). To a stirred mixture of **3d** (100 mg, 0.26 mmol) in MeOH (5 mL) was added Et_3N (71.8 μL, 0.52 mmol) at room temperature. After being stirred for 5 min, formaldehyde (200 mg, 2.46 mmol) was added to the mixture, which was heated to 50 C° for 1 h. The mixture was poured into aq. $NaHCO_3$, extracted with EtOAc–THF, dried over $MgSO_4$ and filtered. concentrated in vacuo. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (EtOAc/MeOH, 100:0 to 85:15, v/v) to give **17** (68.0 mg, 81 %) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.42–1.66 (4H, m), 1.71–1.84 (1H, m), 1.98–2.10 (1H, m), 2.41 (3H of minor, s), 2.48 (3H of major, s), 2.56–2.66 (1H, m), 2.75–2.84 (1H, m), 3.72–3.79 (1H, m), 4.48 (1H, dd, J = 7.9, 1.9 Hz), 5.00 (1H, d, J = 7.9 Hz), 7.44 (1H, s), 7.89 (1H of major, s), 8.27 (1H of minor, s), 12.94 (1H of minor, br s), 13.01 (1H of major, br s). This material was observed as a 3:2 mixture of rotamers. HRMS: Calcd for $C_{16}H_{18}N_5OS$ $[M+H]^+$: 328.1227. Found: 328.1212.

The chemical structure was determined by HMBC study (Figure 13). Long range coupling was observed between the proton of 5-CH_2 and the carbon of 3-CO but the carbon of 14-C, which supported cyclization manner of compound **17**.



17

Figure 13. Long range coupling and NOE observed in compound **17**

7-[(Benzyloxy)carbonyl]-7-azabiciclo[2.2.1]heptane-1-carboxlic acid (19). A mixture of methyl 7-benzoyl-7-azabiciclo[2.2.1]heptane-1-carboxylate **18**[16] (8.0 g, 32.6 mmol) and concentrated HCl (100 mL) was refluxed for 24 h, and concentrated in vacuo. To the residue was added water (50 mL), and washed with EtOAc twice. The obtained aqueous layer was basified by addition of aqueous Na_2CO_3. To this material was added Na_2CO_3 (9.80 g, 92.5 mmol) and a solution of Cbz chloride (5.40 mL, 37.8 mmol) in 1,4-dioxane (30 mL) was added slowly. The mixture was stirred at room temperature overnight, and washed with EtOAc twice. The obtained aqueous layer was acidified to pH 3 by addition of 2 M HCl, and extracted with EtOAc (150 mL) three times. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo to give **19** (2.45 g, 27%) as a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.42–1.55 (2H, m), 1.62–1.82 (4H, m), 1.90–2.05 (2H, m), 4.26 (1H, t, J = 4.6 Hz), 5.05 (2H, s), 7.28–7.41 (5H, m), 12.58 (1H, br s).

Benzyl 1-(chlorocarbonyl)-7-azabiciclo[2.2.1]heptane-7-carboxylate (20). To a mixture of **19** (550 mg, 2.00 mmol), DMF (0.02 mL), and THF (10 mL) was added dropwise oxalyl chloride (0.800 mL, 9.32 mmol). The mixture was stirred at room temperature for 30 min, and concentrated in vacuo. To the residue was added THF, and concentrated in vacuo to give crude **20** as a yellow oil. This material was used in the next reaction without further purification.

***tert*-Butyl 4-(2-hydroxyethyl)piperidine-1-carboxylate (22).** To a mixture of 2-(piperidin-4-yl)ethanol (100 g, 774 mmol), NaOH (34.1 g, 851 mmol), *t*-BuOH (300 mL) and water (400 mL) was added Boc_2O

(180 mL, 774 mmol) dropwise over 30 min, maintaining the inner temperature within 10 to 23 °C by ice-cooling. The mixture was stirred at room temperature overnight. The mixture was poured into water (1 L), and extracted with EtOAc (1 L). The organic layer was washed with saturated $NaHCO_3$ aq. and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to afford **22** (180 g, quant.) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.04–1.20 (2H, m), 1.36 (1H, t, *J* = 5.1 Hz), 1.43–1.47 (9H, m), 1.47–1.72 (5H, m), 2.69 (2H, t, *J* = 12.4 Hz), 3.65–3.76 (2H, m), 4.00–4.16 (2H, m).

***tert*-Butyl 4-(2-oxoethyl)piperidine-1-carboxylate (23).** To a solution of **22** (180 g, 785 mmol) in DMSO (440 mL) was added Et_3N (328 mL, 2.35 mol) at 10 °C. After 5 min, pyridine sulfur trioxide (250 g, 1.57 mol) was added portionwise over 1 h. The inner temperature was maintained below 20 °C in an ice-water bath. The mixture was stirred at room temperature for a further 30 min. The mixture was poured into ice-water (2 L), and extracted with EtOAc (2L × 1, 1L × 1). The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc 100:0 to 80:20, v/v) to give **23** (144 g, 81 %) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.08–1.30 (2H, m), 1.45 (9H, s), 1.69 (2H, d, *J* = 13.9 Hz), 1.97–2.14 (1H, m), 2.39 (2H, dd, *J* = 6.7, 1.4 Hz), 2.74 (2H, t, *J* = 12.8 Hz), 4.00–4.17 (2H, m), 9.78 (1H, s).

***tert*-Butyl 4-(2-cyano-2-hydroxyethyl)piperidine-1-carboxylate (24).** To a mixture of **23** (144 g, 634 mmol) and NaCN (37.3 g, 760 mmol) in Et_2O (440 mL) and water (300 mL) was added 6 M HCl (106

mL, 634 mmol) dropwise over 30 min at 0 °C, maintaining the inner temperature below 10 °C. After being stirred at 0 °C for 1 h, to the mixture was added saturated $NaHCO_3$ aq. (400 mL). After 10 min, EtOAc (550 mL) was added and the organic layer was collected, washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to afford crude **24** (161 g, quant.) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.07–1.29 (2H, m), 1.45 (9H, s), 1.63–1.90 (5H, m), 2.65–2.78 (2H, m), 3.45 (1H, br s), 4.01–4.16 (2H, m), 4.56 (1H, t, J = 6.8 Hz).

***tert*-Butyl 4-(2-cyano-2-((methylsulfonyl)oxy)ethyl)piperidine-1-carboxylate (25).** To a solution of **24** (161 g, 633 mmol) in THF (700 mL) was added Et_3N (115 mL, 823 mmol) at 0 °C. After 10 min, MsCl (58.8 mL, 760 mmol) was added dropwise over 1 h, maintaining the inner temperature below 10 °C. The mixture was stirred at 0 °C for a further 1 h. The mixture was poured into saturated $NaHCO_3$ aq. (1300 mL), and extracted with EtOAc (1000 mL + 300 mL). The organic layer was washed with saturated $NaHCO_3$ aq. and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to afford crude **25** (211 g, quant.) as yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.05–1.31 (2H, m), 1.39–1.53 (9H, m), 1.63–2.12 (5H, m), 2.72 (2H, t, J = 12.6 Hz), 3.21 (3H, s), 4.12 (2H, q, J = 7.1 Hz), 5.25 (1H, dd, J = 8.2, 5.9 Hz).

Quinuclidine-2-carbonitrile (26). To a solution of **25** (80.0 g, 633 mmol) in CH_2Cl_2 (200 mL) was added dropwise a solution of TFA (137 g, 1.20 mol) in CH_2Cl_2 (200 mL) cooled under ice-water bath. The mixture was allowed to room temperature for 30 min. The resulting mixture was concentrated, and

the residue was dissolved in MeCN (200 mL), and then Et_3N (98.0 g, 0.97 mol) was added dropwise cooled under ice-water bath. The mixture was then heated under reflux, and stirred overnight. The mixture was concentrated, and the residue was diluted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated, and the residue was purified by column chromatography on silica gel (petroleum ether/EtOAc, 2:1, v/v) to give **26** (13.0 g, 40%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.58–1.62 (3H, m), 1.80–1.84 (3H, m), 2.00–2.02 (1H, m), 2.88–2.92 (3H, m), 3.23–3.27 (1H, m), 3.86–3.90 (1H, m).

Quinuclidine-2-carboxylic acid hydrochloride (27). A mixture of **26** (28.4 g, 209 mmol) and concentrated HCl (280 mL) was stirred at 110 °C for 5 h. The mixture was concentrated in vacuo. To the residue was added water (100 mL), and the mixture was concentrated in vacuo to afford a wet solid (68.0 g). This solid was collected by filtration, and washed with water (15 mL) to give a white solid (31.8 g). Analysis by ^{1}H-NMR indicated that this material included 1.3 eq of NH_4Cl (7.0–7.4 ppm). The material (31.8 g) was dissolved in 2 M NaOH (166 mL, 332 mmol), and the solution was concentrated in vacuo to remove generated ammonia. To the residue was added water (50 mL), and the mixture was concentrated in vacuo to give a wet solid (67 g). To the residue was added water (50 mL), then 6 M HCl (90 mL, 540 mmol) was added. The mixture was concentrated in vacuo to give crude **27** (45.3 g, ca.135 mmol, 65%) as a white solid. Content rate of **27** was 57.2%, calculated by the estimated amount of NaCl present in the crude product derived from the used NaOH (332 mmol). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.66–1.97 (5H, m), 2.05–2.24 (2H, m), 3.13–3.48 (4H, m), 4.40 (1H, t, J = 9.5 Hz), 9.91 (1H, br s), 14.03 (1H, br s).

Biology

General

All animal experiments performed in the manuscript were conducted in compliance with institutional guidelines.

Preparation of human-derived MCM2 protein

The genetic engineering methods described below followed the method described in a book (Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, 1989) or a method described in the protocol attached to the reagent. N terminal Histagged recombinant human MCM2 protein corresponding to the 10–294th amino acids from the N terminal was cloned to Escherichia coli expression vector pET-21. The vector pET21-HH was prepared by inserting the following 6 × Histag synthetic DNA

5’-TATGCATCATCATCATCATCACGGATCCCATCATCATCATCATCACTGAGC-3’ (SEQ ID NO: 1); and

5’-GGCCGCTCAGTGATGATGATGATGATGGGATCCGTGATGATGATGATGATGCA-3’ (SEQ ID NO: 2)

into the Nde I-Not I site of pET-21a(+) (Novagen).

The Mcm2(10–294 a.a.) gene encoding the 10–294th amino acids from the N terminal side of human MCM2 protein was cloned by PCR using synthetic DNA

5’-CGCGGATCCATGGCATCCAGCCCGGCCCA-3’ (SEQ ID NO: 3); and

5’-ATTCTTATGCGGCCGCTCACAGCTCCTCCACCAGAGGCA-3’ (SEQ ID NO: 4)

prepared by reference to the base sequence described in GenBank accession No.: NM_004526, as a primer set and human testis cDNA library (TAKARA bio inc.) as a template. PCR reaction was performed according to the protocol attached to Pyrobest (TAKARA bio inc.).

The obtained 883 bp fragment was digested with restriction enzymes BamHI and NotI, inserted into the BamHI-NotI site of pET21-HH, and the inserted base sequence was confirmed to give pET21-HHhMcm2(10–294) plasmid. The pET21-HHhMcm2(10–294) plasmid was introduced into Escherichia coli BL21(DE3) cell line (American Type Culture Collection).

Escherichia coli cells introduced with the above-mentioned plasmid were cultured in LB medium (1% tripton, 0.5% yeast extract, 0.5% NaCl) containing 50 mg/L ampicillin, and MCM2 expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 h. Escherichia coli cells expressing MCM2 were recovered by centrifugation (6000 rpm, 10 min), washed with phosphate-buffered saline, and cryopreserved at –80 °C. The above-mentioned cryopreserved Escherichia coli cells were thawed on ice, and suspended in complete ethylenediaminetetraacetic acid (EDTA) (Roche Diagnostics GmbH, Mannheim, Germany)-added buffer A (25 mM tris-hydrochloride (pH 7.4), 2.7 mM KCl, 137 mM NaCl). The above-mentioned suspended Escherichia coli cells were lysed with 1 mg/mL lysozyme, and sonicated 4 times in Insonator 201M (Kubota) at 170W for 30 sec while cooling with ice water. This extract was ultracentrifuged at 15000 rpm, at 4 °C for 20 min, and the obtained supernatant was passed through a 0.22 μm filter to give an Escherichia coli cell-fee cell extract. The Escherichia coli cell-free cell extract was passed through nickel-NTA Superflow resin, and the resulting resin was washed

with buffer A, and eluted with buffer B (25 mM tris-hydrochloride (pH 7.4), 2.7 mM KCl, 137 mM NaCl, 10% glycerol, 200 mM imidazole). The eluate was concentrated using Amicon Ultra 4 (5K MWCO, Millipore, MA, USA), and purified by gel filtration using HiLoad 16/60 Superdex 200 pg (GE healthcare, Chalfont St. Giles, UK) equilibrated with buffer C (25 mM tris-hydrochloride (pH 7.4), 2.7 mM KCl, 137 mM NaCl, 10% glycerol, 200 mM imidazole). The fraction containing MCM2 protein was concentrated as a purified sample, and cryopreserved at –80 °C.

Cdc7 kinase assay

Full-length Cdc7 co-expressed with full-length Dbf4 was purchased from Carna Biosciences (Kobe). The enzyme activity of Cdc7/Dbf4 complex was detected by homogeneous time-resolved fluorescence method Transcreener ADP assay (Cisbio Inc., MA, USA). The enzyme reaction was performed in a kinase buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 10 mM $Mg(OAc)_2$, 1 mM dithiothreitol (DTT)) supplemented with 1.0 μM ATP, 10 μg/mL MCM2, and 0.1 μg/mL Cdc7/Dbf4. Prior to the addition of ATP, test compounds and enzyme were pre-incubated for 10 min. For time dependent inhibition assay, the enzyme reactions were performed in the kinase buffer containing 50 μM (K_m × 50) ATP. Prior to the addition of ATP, test compounds and enzyme were pre-incubated for 0 or 60 min. Free ADP produced by ATP hydrolysis was detected by Eu^{3+}-Cryptate-labeled anti-ADP monoclonal antibody competitively with d2-labeled ADP, and the production amount thereof was measured. The obtained time-resolved fluorescence resonance energy transfer signal was measured with EnVision (Perkin Elmer Inc., MA, USA) by excitation at 320 nm and

emission donor at 615 nm or emission acceptor 665 nm, respectively. The inhibitory rate (%) of the test compound to Cdc7 was calculated by the following formula.

Inhibitory rate (%) = (1– (count of test compound–blank) ÷ (control–blank)) × 100

The count of the Cdc7/Dbf4 reaction mixture under compound-free conditions was taken as the control, and that under compound-free and Cdc7/Dbf4-free conditions was taken as the blank.

Cdk2/CyclinE kinase assay

The Kinase-Glo™ (Promega, USA) assay was performed in 384-well plate format. The enzyme reaction was run in a reaction buffer consisting of 25 mM HEPES (pH 7.5), 10 mM $Mg(OAc)_2$, 0.01% bovine serum albumin (BSA), 0.01% Tween 20, and 1 mM DTT. The final concentrations of substrate Histone H1 and ATP were 100 μg/ml and 500 nM, respectively. The final concentration of Cdk2/CyclinE (Carnabiosciences, Japan) was 750 ng/ml. After incubation at room temperature for 90 min, the reaction was terminated by the addition of the reagent supplied with the Kinase-Glo reagent. The luminescence correlated with the amount of ATP remaining in solution was measured on EnVision (PerkinElmer, MA, USA) after incubation at room temperature for 10 min.

The inhibitory rate (%) of the test compound to Cdc7 was calculated by the following formula.

Inhibitory rate (%) = (1– (count of test compound–blank) ÷ (control–blank)) × 100

ROCK1 kinase assay

TR-FRET assay was used to assess ROCK1 (Carnabiosciences, Japan) enzyme activity (CisBio,

France, KinEASE HTRF kit (Cat# 62ST3PEB)). The enzyme reaction was run in a reaction buffer consisting of 50 mM HEPES (pH 7.5), 0.1 mM orthovanadate, 0.01% BSA, 10 mM $MgCl_2$ and l mM DTT. The assay was done in a 384-well plate assay format. Before initiation of the enzymatic reaction, ROCK1, test compounds, and the substrate peptide (Biotin-STK substrate-2 (Cat# 61ST2BLC)) were incubated in the reaction buffer at room temperature for 5 min. The final concentration of ROCK1 was 300 ng/mL. The enzymatic reaction was started with the addition of ATP at a final concentration of 2 μM. After incubation at room temperature for 2 h, the reaction was terminated by adding 10 mM EDTA in a detection buffer containing 15 nM streptavidin-linked XL665. Time-resolved fluorescence was monitored with an EnVision Multilabel Plate Reader (PerkinElmer Life Sciences, Fremont, CA, USA) with an excitation wavelength of 320 nm and emission donor and acceptor wavelengths of 615 and 665 nm, respectively. The total reaction without enzyme as 0% activity and the total reaction as 100% activity were set.

The inhibitory rate (%) of the test compound to Cdc7 was calculated by the following formula.

Inhibitory rate (%) = (1– (count of test compound–blank) ÷ (control–blank)) × 100

Cell lines

HeLa cells from ATCC were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS). COLO205 cells from ATCC were cultured in Roswell Park Memorial Institute (RPMI) medium with 10 % FBS. Cell lines were incubated at 37 °C with 5% CO_2 gas.

Cell-based MCM2 phosphorylation

HeLa cells were seeded at 3.0×10^4 cells/well in a 24-well plate. After 1-day incubation, the plate was treated with the test compound for 7 h. At end of the incubation, HeLa cells were lysated by 200 μL of sodium dodecylsulphate (SDS) buffer. Phosphorylation level of MCM2 in each sample was determined by Western blotting. Western blotting was carried out by using the following antibodies; pSer40 MCM2 (EPITOMICS, Inc., #3378-1), horseradish peroxidase (HRP)-labeled rabbit IgG polyclonal antibody (Amersham Biosciences, NA9340). Band intensity of each sample was detected by LAS1000 and the corresponding IC_{50} value was calculated by using Prism software.

Growth inhibition assay

COLO205 cells were seeded at 3000 cells/well in a 96-well plate. After 1-day incubation, the plate was treated with test compound and incubated for a further 3 days. At end of the incubation, cell viability of each well was measured by using CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega). An EC_{50} value of test compound was calculated by using Prism software.

In vivo PD study

COLO205 cells were suspended in 50% Matrigel solution, and transplanted into female BALB/c mice (CLEA Japan, Inc.) by subcutaneous injection at 5.0×10^6 cells. After approximately 7 days from inoculation, diameter of the tumor was measured and tumor volume was calculated by the following formula.

Tumor volume = long diameter × short diameter × short diameter × (1/2)

When tumors grew enough volume (approximately 300~500 mm^3), *in vivo* PD study was carried out with test compound that were suspended in 0.5% methylcellulose solution. At 1 h, 2 h, 4 h, 8 h or 16 h after oral administration, tumor was removed from mice and homogenized in Cell Lysis Buffer (Cell Signaling). After protein amount of the cell extract from each tumor was adjusted, phosphorylation level of MCM2 in each sample was detected by Western blotting using following antibodies: pSer40/41 MCM2 (Bethyl laboratories, A300-788A), MCM2 (Santa Cruz, sc-9839), anti-PARP (Cell Signaling Technology, #9542), anti-CyclinB1 (Santa Cruz, sc-752), anti-GAPDH (Chemicon, MAB374). The band intensity of phosphorylated MCM2 (pMCM2) was normalized by that of MCM2. Percent (%) inhibition of pMCM2 was calculated by following formula.

% inhibition = 100 – 100 × (relative pMCM2 band intensity of test compound treated tumor) / (relative pMCM2 band intensity of vehicle treated tumor)

In vivo efficacy study

Mice having a COLO205 tumor which size was approximately 200 mm^3 were selected, and 5 mice per group were used for the experiment. Compound **3d** was suspended in 0.5% methylcellulose solution and orally administrated twice daily for 14 days. Tumor volume and body weight of mice were measured every 2~3 days. T/C was calculated by following formula.

T/C(%) = (tumor volume change of test compound treated group) / (tumor volume change of vehicle treated group) × 100

Formaldehyde adduct formation test

LC/MS (liquid chromatography mass spectrometry) system, consisted of ultra high performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) and SYNAPT quadrupole time-of-flight (QTOF) mass spectrometer (Waters) equipped with an electrospray ionization source, was used for the test.

Compounds **3d'**, **11b**, and **3o** (5 nmol each) in MeCN were treated with an excess of formaldehyde (12.65 equiv) and the mixture was incubated at 37 ºC for 30 min. After the mixture was diluted with purified water by 8-fold, an aliquot was analyzed with a QTOF mass spectrometer equipped with an UPLC. Aliquots were separated on a BEH C_{18} column (particle size 1.7 μm, 2.1 mm i.d. × 100 mm, Waters) using solvent A (0.2% formic acid in 10 mM aqueous ammonium formate) and solvent B (0.2% formic acid in MeOH). At a flow rate of 0.4 mL/min, the initial elution gradient was 5% solvent B with a linear gradient to 98% solvent B over 6 min and held for 4.1 min. The initial concentration was then reinstated and held for 1.9 min for re-equilibration. The column temperature was 40 °C and the eluates were monitored with a photodiode array (PDA) detector. The mass spectrometry was run in positive ion mode. The source settings were as follows: 1.20 kV capillary voltage, 40 V sampling cone voltage, 120 °C source temperature, and 350 °C desolvation temperature.

Docking study

Docking model of **11b** with Cdc7 was constructed utilizing the Cdc7 crystal structure (PDB code:

4F9C). Docking was performed with Glide (Schrödinger, Inc.) in standard precision mode with further minimization with an extra precision mode. The correct binding mode of **11b** was determined by scoring with the MM/PBSA (Molecular Mechanics/Poisson Boltzmann Surface Area) approach.

ASSOCIATED CONTENT

Supporting Information: The supporting information is available free of charge on the ACS publication website at DOI://xxxxxxx

・The HPLC traces for compound **3d** and **11b**

・Molecular formula strings including screening data (CSV)

Accession Codes: Atom coordinates and structure factors for complexes of ROCK2/compound **I**, and ROCK2/compound **11b** have been deposited in the Protein Data Bank with accession codes 6P5M, and 6P5P, respectively.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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ABBREVIATIONS

Cdc7, cell division cycle 7; MCM2, minichromosome maintenance 2; POC, proof of concept; PD, pharmacodynamic; SAR, structure-activity relationship; DIEA, *N,N*-diisopropylethylamine; DMA, *N,N*-dimethylacetamide; DME, 1,2-dimethoxyethane; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; HATU, 2-(3*H*-[1,2,3]triazolo[4,5-*b*]pyridin-3-yl)-1,1,3,3-tetramethylisouronium hexafluorophosphate (V); IPA, isopropyl alcohol; IPE, isopropyl ether; $PdCl_2$(dppf), 1,1'-bis(diphenylphosphino)ferrocenepalladium (II) dichloride dichloromethane adduct; TBAF, tetra-*n*-butylammonium fluoride; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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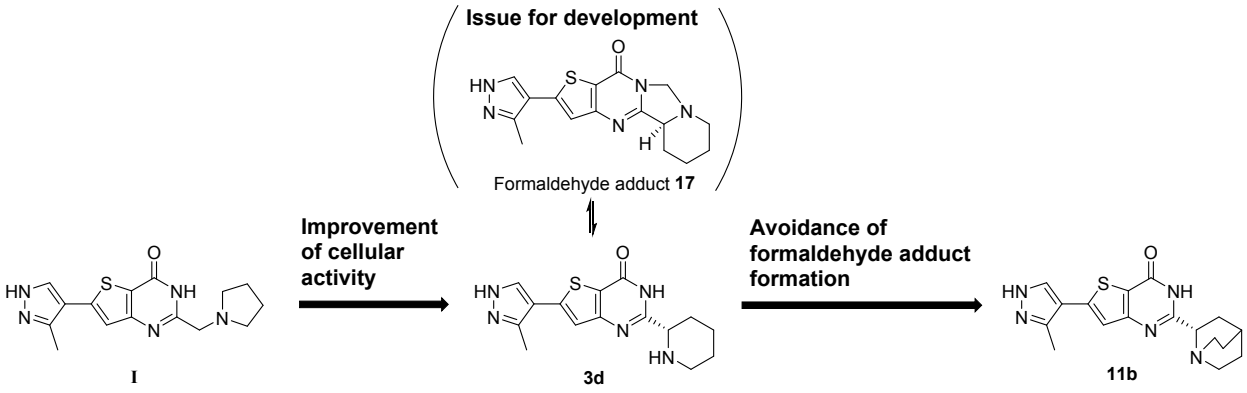

Cdc7: IC_{50} = 0.70 nM
Koff: 4.94 x 10^{-4} (sec^{-1})
Residence time: 34 min
pMCM2: IC_{50} = 250 nM
COLO205: EC_{50} = 1100 nM

Cdc7: IC_{50} = 0.44 nM
Koff: 6.30 x 10^{-4} (sec^{-1})
Residence time: 26 min
pMCM2: IC_{50} = 36 nM
COLO205: EC_{50} = 130 nM

Cdc7: IC_{50} = 0.26 nM
Koff: 6.30 x 10^{-4} (sec^{-1})
Residence time: 26 min
pMCM2: IC_{50} = 17 nM
COLO205: EC_{50} = 81 nM