Identification of A New Class of Potent Cdc7 Inhibitors Designed by Putative Pharmacophore Model: Synthesis and Biological Evaluation of 2,3-Dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-ones

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Abstract

Cell division cycle 7 (Cdc7) is a serine/threonine kinase that plays important roles in the regulation of DNA replication process. A genetic study indicates that Cdc7 inhibition can induce selective tumor-cell death in a p53-dependent manner, suggesting that Cdc7 is an attractive target for the treatment of cancers. In order to identify a new class of potent Cdc7 inhibitors, we generated a putative pharmacophore model based on in silico docking analysis of a known inhibitor with Cdc7 homology model. The pharmacophore model provided a minimum structural motif of Cdc7 inhibitor, by which

preliminary medicinal chemistry efforts identified a dihydrothieno[3,2-*d*]-pyrimidin-4(1*H*)-one scaffold having a heteroaromatic hinge-binding moiety. The structure-activity relationship (SAR) studies resulted in the discovery of new, potent, and selective Cdc7 inhibitors **14a,c,e**. Furthermore, the high selectivity of **14c,e** for Cdc7 over Rho-associated protein kinase 1 (ROCK1) is discussed by utilizing a docking study with Cdc7 and ROCK2 crystal structures.

Graphical abstract

Keywords

Cell division cycle 7 (Cdc7)

Pharmacophore model

Dihydrothieno[3,2-*d*]-pyrimidin-4(1*H*)-one

1. Introduction

Targeting DNA replication, a fundamental process for cell proliferation, is one of major strategies for the treatment of cancer. Various agents targeting DNA replication, platinum DNA cross linkers and topoisomerase inhibitors, have potent cancer cell growth inhibitory activities and are utilized in the clinic. However, in order to overcome the issue of cancer heterogeneity and emergence of resistant cancer cells, new agents having novel mechanism of action are required.

Cell division cycle 7 (Cdc7), an evolutionally conserved serine/threonine kinase, is a key regulator

for G1/S phase transition through phosphorylation of one or more residues of minichromosome maintenance 2 (MCM2) of the pre-replicative complex.^{1,2} The Cdc7-dependent phosphorylation causes loading of other accessory factors and subsequent generation of active replication forks, ultimately leading to the initiation of DNA replication. Overexpression of both Cdc7 and the regulatory partner, Dumbbell former 4 (Dbf4), is observed in various type of cancer cells, suggesting significant involvement of Cdc7 in tumor progression.³ The Cdc7 kinase is considered to be an attractive target based on the following findings: RNA interference silencing of Cdc7 in cancer cells induces a prolonged S phase, leading to subsequent p53-independent apoptotic cell death.⁴ On the other hand, the silencing in normal cells evokes G1 arrest in a p53-dependent manner without loss of viability.⁴

Several research groups have reported Cdc7 inhibitors and some of them have entered clinical trials; however, the proof of concept (POC) in humans has not been demonstrated so far.⁵⁻¹¹ We envisioned that it was mainly attributed to the difficulty of identifying the compound with sufficient kinase selectivity and/or cellular potency, particularly in the case of typical ATP-competitive Cdc7 kinase inhibitors. We sought, therefore, to discover and develop a new, potent and selective Cdc7 inhibitor.

In order to design new Cdc7 inhibitors, we firstly constructed a Cdc7 homology model on the basis of the reported crystal structure (PDB: 1DAW) of casein kinase 2 (CK2)¹² sharing high homology with Cdc7 in terms of the ATP binding pocket (74% identity). By utilizing the homology model, a docking study on an ATP-competitive Cdc7 inhibitor (**I**) reported by Nerviano⁶ was next carried out (Figure 1).

Figure 1. Docking study on Nerviano inhibitor (**I**) by utilizing Cdc7 homology model constructed on the basis of the crystal structure of CK2 (PDB: 1DAW).

The docking study provided the following clues: (i) The Pro135 and/or Leu137 residues can be exploited as a counterpart of hinge binding motif of Cdc7 inhibitors. (ii) The Lys90 and Asp196 residues making a salt bridge in the back pocket are considered to be important for the interaction with the amide group. (iii) It is speculated that the sulfur atom of the gate keeper Met134 residue can be used for an additional interaction with inhibitors. Based on the clues, we generated a putative pharmacophore model which suggests a heteroaromatic hinge-binding moiety and an amide group as two key binding motifs, and appropriate incorporation of a sulfur atom into the five-membered aromatic ring for an additional binding interaction. In the model, the aromatic core ring plays an important role to place the two key binding motifs in adequate positions. The pharmacophore model led us to design bicyclic thiophene-based Cdc7 inhibitors (Figure 2). A bicyclic scaffold, cyclic amide fused with the aromatic core ring, was adopted to fix the amide group to the appropriate direction toward the Lys90 and Asp196 residues. As a bicyclic scaffold, we chose thieno[3,2-*d*]pyrimidinone whose sulfur atom may effectively interact with the sulfur atom of the Met134 residue. Furthermore, the docking study suggested that the 2-fluoroethyl group of the Nerviano inhibitor extends toward solvent-exposed region which could be utilized to improve potency, kinase selectivity, and physicochemical properties by introducing desirable substituents into Rc and/or Rd.

Figure 2. (a) Pharmacophore model of Cdc7. (b) Design of bicyclic thiophene-based Cdc7 inhibitors.

Preliminarily, thienopyrimidinones **4a**, **5**, **8** and a ring-opening analogue **3a** were designed and prepared based on the pharmacophore model, and the Cdc7 kinase inhibitory activities were evaluated (Figure 3). As expected, inhibitory activities of compound **4a**, **5**, **8** were better than that of compound **3a**. With regard to the cyclic lactam ring, the pyrimidin-4-one **4a** was more potent inhibitor than the pyrimidin-2,4-dione **5**. Moreover, position of the sulfur atom in the bicyclic ring system significantly affected the potency. In fact, dihydrothieno[2,3-*d*]pyrimidin-4(1*H*)-one **8** was about 5-fold less potent than dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one **4a**, which may reflect the absence of the non-covalent S-S interaction. Among them, compound **4a** with an IC_{50} value of 26 nM was the best. In addition, we examined kinase selectivity of compound **4a** by using the in-house kinase panel (46 kinases),¹³ indicating that overall selectivity of **4a** was acceptable ($IC_{50} < 100$ nM for ROCK1 (1/46 kinases), IC_{50} 100–1000 nM for 4/47 kinases, IC_{50} 1000–10000 nM for 5/46 kinases, $IC_{50} > 10000$ nM for 36/46 kinases). ROCK acting downstream of the small GTPase Rho¹⁴ is known as one of the key players in cell cycle control; however, the kinase family is involved in a wide range of cellular functions.¹⁵ Thus, we considered improvement in selectivity for Cdc7 over ROCK1 would mitigate off-target effects.

Based on these results, dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one was selected as a scaffold and, starting from **4a** as a lead compound, we performed further optimization study to identify potent and selective Cdc7 inhibitors. The preliminary data described in Figure 3 suggested that the *gem*-dimethyl

aminal moiety extending toward the solvent-exposed region, which is significantly spacious in the Cdc7 homology model, can be potentially modified to improve kinase selectivity as well as Cdc7 inhibitory activity. Optimization of the hinge-binding moiety would also contribute to enhancing the in vitro potency.

Herein, we report the synthesis, structure-activity relationships (SARs), and biological effects of this chemical series. Furthermore, difference in binding modes of a selected compound with Cdc7 and ROCK in docking models is discussed to elucidate high selectivity.

Figure 3. Identification of dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one as a favorable scaffold of new Cdc7 inhibitors.

2. Chemistry

Dihydrothienopyrimidine derivatives possessing 4-pyridyl group as a hinge binder (**4a–k**, **5**, **8**) were synthesized according to the procedure in Scheme 1. After *N*-methylation of methyl 3-amino-5-(4-pyridyl)thiophene-2-carboxylate **1a**, the esters **1a,b** were then hydrolyzed under basic conditions to give the carboxylic acids **2a,b**. Compounds **2a,b** were condensed with appropriate amines to provide the corresponding amides **3a–d**. Similarly, ethyl 2-amino-5-(4-pyridyl)thiophene-3-carboxylate **6** was converted to amide **7** in 38% yield by saponification and subsequent amidation. The aminothiophenecarboxamides **3a–d**, **7** were reacted with various ketones in the presence of catalytic amount of *p*-toluenesulfonic acid to afford cyclized products **4a–k**, **8** in 11% to 74% yields. Moreover, the amide **3a** was subjected to ring-closure reaction using urea to yield thienopyrimidin-2,4-dione **5**.

2,3-Dihydro-6-(pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(*1H*)-ones possessing various substituents on pyrimidinone and pyrazole rings (**11a–c**, **12a–f**, **14a–e**) were synthesized as outlined in Scheme 2. Methylation of **9a** afforded *N*-methyl derivative **9b** and both of them were then subjected to ester saponification. However, attempts to isolate the corresponding carboxylic acids were unsuccessful because they are prone to decarboxylation. The result indicates that the electronic nature of the 5-substituent significantly influences the stability of the 2-carboxylic acid. After alkaline hydrolysis of **9a,b**, the reaction mixture was treated with 6 M hydrochloric acid to maintain pH > 7 and the solvent was removed by evaporation, yielding corresponding crude sodium salts. Without further purification, the sodium salts were next subjected to amidation reactions to furnish primary amides **10a,b**. Cyclization of **10a,b** with acetone by using *p*-toluenesulfonic acid as a catalyst afforded **11a,b**. However, reaction of **10b** with 2,2,2-trifluoroethyl methyl ketone didn't proceed under the same conditions presumably due to the poor reactivity. To overcome the problem, reaction in the presence of camphorsulfonic acid (CSA) and magnesium sulfate in dimethyl acetamide (DMA) was carried out at higher temperature (150 °C) to successfully produce cyclized product **11c**. Suzuki-Miyaura coupling of **11a–c** with pyrazole boronic acid esters (**A–D**), followed by deprotection under appropriate conditions gave the desired products **12a–f**. In the case of **12f**, removal of the dimethylsulfamoyl group by using trifluoroacetic acid (TFA) resulted in a mixture of **12f** and the monocyclic byproduct, which was again subjected to cyclization reaction to generate **12f** in a 31% yield. By utilizing the finding conversely, **12b** was treated with 1M hydrochloric acid in methanol to give ring-opening derivative **13**, which was re-cyclized with cyclohexanones to afford **14a–e**. Compounds **14b–e** have pseudo-chirality but were obtained as a single stereoisomer after purification by column chromatography. The absolute structures of **14b,c,e** were determined by X-ray crystallographic analysis as shown in Figure 4–6. As for **14d**, the absolute structure was unambiguously assigned as the antipode of **14e**.

Compounds having a substituent at the 7-position were prepared as described in Scheme 3. Trifluoroacetylation of aminothiophene **15**, followed by bromination at the 5-position yielded amide **16**. After *N*-methylation of **16**, removal of trifluoroacetyl group produced ester **17**. Saponification and

subsequent amidation gave amide **18** as in the case of **10a,b** (Scheme 2). Compound **18** was then subjected to acid-catalyzed cyclization and subsequent Suzuki coupling to afford 7-methyl derivative **20a**. Ionic bromination of **12a** worked well; however, a ring-opening side reaction partially proceeded presumably due to the strong acidic conditions. Thus, re-cyclization of the mixture was carried out to provide 7-bromo derivative **20b**.

Scheme 1

Reagents and conditions: (a) NaH, MeI, DMF, 0 °C to rt, 62%; (b) NaOH, MeOH–water, 70 °C, 92%; (c) LiOH, MeOH–water, 80 °C, quant.; (d) NH₄Cl or R₂NH₂, EDCI, HOBt, Et₃N, DMF, rt, 50–84%; (e) R₃COR₄, *p*-TsOH·H₂O, toluene or AcOH, 70–110 °C, 11–74%; (f) urea, 180 °C, 34%; (g) 1) LiOH, EtOH–water, 80 °C; 2) NH₄Cl, EDCI, HOBt, Et₃N, DMF, rt, 38%.

Scheme 2

Reagents and conditions: (a) K_2CO_3 , MeI, DMF, 60 °C, 90%; (b) NaOH, MeOH–water, 70 °C; (c) NH_4Cl , EDCI, HOBt, Et_3N , DMF, rt, 76–85%; (d) acetone, $p\text{-TsOH}\cdot H_2O$, AcOH, 70 °C, 60–91% for **11a, b** or methyl 2,2,2-trifluoroethyl ketone, CSA, $MgSO_4$, DMA, 150 °C, 31% for **11c**; (e) boronic acid ester A, $PdCl_2(dppf)$, DME–water, reflux, 38% for **12a**; (f) 1) boronic acid ester B, $PdCl_2(dppf)$, DME–water, reflux; 2) NaOH, 32–60% for **12b, c, d**; (g) boronic acid ester C, $PdCl_2(dppf)$, DME–water, reflux, 36% for **12e**; (h) 1) boronic acid ester D, $PdCl_2(dppf)$, DME–water, reflux; 2) TFA, rt; 3) acetone, $p\text{-TsOH}\cdot H_2O$, $MgSO_4$, DMF, 60 °C, 31% for **12f**; (i) 1 M HCl, MeOH, 50 °C, 41%; (j) $p\text{-TsOH}\cdot H_2O$, $MgSO_4$, DMF, 80 °C, 41% (2 steps) for **14a**; (k) CSA, $MgSO_4$, DMA, 80 °C, 19–34% for **14b-e**; The absolute structures were confirmed by X-ray crystallographic analysis (CCDC 1520567 for **14b**, CCDC 1520568 for **14c** and CCDC 1520566 for **14e**).

Figure 4. ORTEP of **14b**, thermal ellipsoids are drawn at 30% probability.

Figure 5. ORTEP of **14c**, thermal ellipsoids are drawn at 20% probability.

Figure 6. ORTEP of **14e**, thermal ellipsoids are drawn at 20% probability.

Scheme 3

Reagents: (a) 1) TFAA, pyridine, MeCN, 0 °C to rt; 2) NBS, acetic acid, 80 °C, 60%; (b) 1) MeI, K₂CO₃, DMF, 60 °C; 2) K₂CO₃, MeOH–water, rt, 62%; (c) 1) NaOH, MeOH–water, 70 °C; 2) NH₄Cl, Et₃N, DMF, WSC, HOBT, 56%; (d) acetone, *p*-TsOH·H₂O, MgSO₄, DMF, 60 °C, 24%; (e) *tert*-butyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-pyrazole-1-carboxylate pyrazole-4-ylboronic acid, PdCl₂(dppf), Cs₂CO₃, DME–water, 90 °C, 47%; (f) 1) bromine, acetic acid, rt; 2) acetone, *p*-TsOH·H₂O, AcOH, 60 °C, 25%.

3. Results and discussion

Biological evaluation of 2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-ones

All synthesized compounds were evaluated for the ability to inhibit Cdc7 kinase activity to investigate SARs. To assess kinase selectivity, the compounds were also evaluated for inhibitory activities against cyclin-dependent kinase 2 (Cdk2)/cyclin E (cycE) and ROCK1.

Firstly, starting from **4a** (Figure 3) as a lead compound, we evaluated effects of R₁ and R₂ groups of the pyrimidine ring on activity and selectivity, and the results are shown in Table 1. About 3-fold increase in Cdc7 inhibitory activity was observed by incorporation of a methyl group to the R₁-position (**4b** vs. **4a**) along with 2- to 4-fold improvement in kinase selectivity. By contrast, incorporation of a methyl or benzyl group to the R₂ -position (**4j,k** vs. **4a**) resulted in more than 17-fold decrease in Cdc7 inhibitory activity with decreased kinase selectivity. As expected from the pharmacophore model, incorporation of a substituent to the R₂ -position was detrimental to Cdc7 inhibition presumably due to disruption of the interaction with the Lys90 and Asp196 residues forming the salt bridge (see Figure 2). On the other hand, the substitution at the R₁-position was well tolerated or rather favorable for the potency as well as selectivity as speculated in the pharmacophore model.

Next, effects of R₃ and R₄ groups of the aminor moiety, were investigated on Cdc7 enzyme inhibition and kinase selectivity, and the results are presented in Table 2. Replacement of one of the geminal dimethyl substituents with a bulkier alkyl group (**4c–f**) enhanced Cdc7 enzyme inhibitory activity by 3- to 10-fold compared with that of the lead compound **4a**. Compounds **4g,h,i** having a spirocycloalkyl group showed a nanomolar inhibitory activity and moderate kinase selectivity which were similar to those of **4f**. The results indicated that the binding pocket accommodating the R₃ and R₄ groups is relatively spacious, which is consistent with our assumption. As shown in Table 2, the 2-methyl-2-(2',2',2'-trifluoro)ethyl group (**4f**) is the best among the alkyl series. On the other hand, there is not clear difference in potency and selectivity of the spiro-alkyl series. By considering synthetic feasibility of incorporation of additional substituents into the spiro-alkyl moiety, we selected the spiro-cyclohexyl (**4h**) as well as 2-methyl-2-trifluoroethyl (**4f**) groups as the 2-substituent.

To further improve Cdc7 inhibitory activity and, particularly kinase selectivity, modification of the hinge-binding moiety (R_5) and adjacent R_6 -substituent was carried out and the results are shown in Table 3. Replacement of the 4-pyridyl group with a 4-pyrazolyl group (**12a**) maintained Cdc7 activity but reduced kinase selectivity over Cdk2/cycE. We next incorporated an alkyl group onto the pyrazole ring to examine substituent effects on potency and selectivity. Incorporation of a methyl group to the 3-position of the pyrazole ring (**12c**) enhanced the Cdc7 activity by about 3-fold and partially recovered kinase selectivity over Cdk2/cycE relative to **12a**. Further introduction of a methyl group to the 5-position of **12c** (**12e**) or replacement of the 3-methyl group of **12c** with the 3-ethyl group (**12f**) significantly decreased the Cdc7 inhibitory activity with impaired kinase selectivity. Introduction of a bromo or methyl group to the R_6 -position of the scaffold didn't significantly affect the Cdc7 activity but led to marked reduction of kinase selectivity (**20a,b** vs. **12a**). The results strongly suggested that a spatial limitation exists in the hinge-binding pocket. We concluded that the 3-methylpyrazol-4-yl moiety was the best hinge-binding moiety based on the overall in vitro profiles.

Thus, incorporation of the preferable R_3 and R_4 groups (Table 1 and 2) to 2,3-dihydro-6-(3-methylpyrazol-4-yl)thienopyrimidin-4-ones was investigated and the results are summarized in Table 4. The 2-methyl-2-(2',2',2'-trifluoro)ethyl analogue **12d** showed the most potent Cdc7 inhibitory activity ($IC_{50} = 1.0$ nM) with moderate kinase selectivity over ROCK1. Spiro-cyclohexyl derivative **14a** was found to be almost equipotent to **12d** with slightly improved selectivity, which encouraged us to conduct further substitution on the cyclohexane ring. As described before, the SAR of the 2-substituent indicated that the binding pocket is relatively spacious. To further improve kinase selectivity, we attempted to incorporate both polar and lipophilic substituents to the cyclohexyl moiety, which can retain desirable lipophilicity ($clogP$: around 3).¹⁶ As a result, compound **14c** possessing 4-fluorophenyl and hydroxyl groups at the R_9 - and R_{10} -positions of the cyclohexane ring demonstrated significant improvement of selectivity over ROCK1 with maintaining a nanomolar Cdc7 inhibitory activity. On the other hand, the corresponding pseudo-enantiomer **14b** exhibited significantly reduced potency and selectivity. A similar tendency was observed in the case of spiro-cyclohexyl analogues **14d,e**

and the eutomer **14e** proved to show high kinase selectivity with retaining a nanomolar activity. Accordingly, compound **14c,e** were highly selective Cdc7 inhibitors with low nanomolar potency in this series.

Selected compounds **12d**, **14a,c,e** were evaluated for their cellular potency (Table 4). To assess Cdc7 kinase inhibition in cells, phosphorylation status of Ser40 in MCM2 was measured by using HeLa cells derived from human cervical cancer. COLO205 cells derived from human colon cancer were also used to evaluate inhibition of cancer cell growth in vitro.

Despite potent Cdc7 inhibitory activity in the cell-free assay, all the compounds tested showed moderate cellular potency at concentrations below 1 μ M (phosphorylation) and 10 μ M (cancer cell growth). Among them, compounds **12d**, **14a** exhibited submicromolar cellular activities. The inhibition of MCM2 phosphorylation and cell growth of **12d**, **14a** were observed in the same concentration range, suggesting that the cellular Cdc7 inhibition mainly contributed to the growth inhibition. However, the gap between cell-free and cellular Cdc7 inhibition still remains unclear because there are no significant defect and difference in solubility and membrane permeability of **12d**, **14a,c,e** (data not shown).

Consequently, we succeeded in the identification of new, potent, and selective Cdc7 kinase inhibitors represented by **12d**, **14a,c,e** designed based on the putative pharmacophore model.

Table 1. Effects of N^1 - and/or N^3 -substituents on Cdc7 enzyme inhibition and kinase selectivity.

Compound	R ₁	R ₂	Enzyme inhibition: IC ₅₀ (nM) ^a			Selectivity	
			Cdc7	Cdk2/cycE	ROCK1	Cdk2/Cdc7	ROCK1/Cdc7
4a	H	H	26 (17-41)	3200 (6900-9100)	90 (81-100)	x120	3.5
4b	Me	H	9.9 (5.2-19)	2100 (1800-2600)	140 (130-150)	x210	14
4j	H	Me	450 (280-700)	>10000	240 (210-280)	x22	0.53
4k	H	Bn	>10000	650 (570-740)	1.5 (1.3-1.7)	0.065	0.00015

^aNumbers in parentheses represent 95% confidence interval.

Table 2. Effects of 2-substituents on Cdc7 enzyme inhibition and kinase selectivity.

Compound	R ₃	R ₄	Enzyme inhibition: IC ₅₀ (nM) ^a			Selectivity	
			Cdc7	Cdk2/cycE	ROCK1	Cdk2/Cdc7	ROCK1/Cdc7
4a	Me	Me	26 (17-41)	3200 (2800-3700)	90 (81-100)	x120	3.5
4c	Et	Me	7.5 (4.4-13)	2000 (1800-2600)	36 (15-91)	x270	4.8
4d	<i>n</i> -Pr	Me	6.3 (3.3-12)	2000 (1800-2200)	33 (28-39)	x320	5.2
4e	<i>i</i> -Pr	Me	8.2 (5.9-11)	1400 (1200-1600)	35 (31-39)	x170	4.3
4f	CH ₂ CF ₃	Me	2.7 (1.8-4.1)	1200 (1000-1500)	31 (28-34)	x440	11
4g	—(CH ₂) ₄ —		8.2 (5.7-12)	4900 (4400-5500)	100 (93-110)	x600	12
4h	—(CH ₂) ₅ —		3.0 (2.2-4.0)	2000 (1700-2300)	53 (43-66)	x670	18
4i	—(CH ₂) ₆ —		3.6 (2.5-5.1)	2300 (2100-2500)	37 (32-41)	x640	10

^aNumbers in parentheses represent 95% confidence interval.

Table 3. Effects of 6- and/or 7-substituents on Cdc7 enzyme inhibition and kinase selectivity.

Compound	R ₅	R ₆	Enzyme inhibition: IC ₅₀ (nM) ^a			Selectivity	
			Cdc7	Cdk2/cycE	ROCK1	Cdk2/Cdc7	ROCK1/Cdc7
4b		H	9.9 (5.2-19)	2100 (1800-2600)	140 (130-150)	x210	x14
12a		H	8.4 (5.1-14)	160 (140-190)	130 (110-160)	x19	x15
12c		H	2.9 (1.5-5.5)	260 (220-310)	54 (49-59)	x90	x19
12e		H	290 (180-470)	1100 (930-1300)	2100 (1700-2500)	x3.8	x7.2
12f		H	13 (6.3-25)	360 (300-420)	350 (290-420)	x28	x27
20a		Me	14 (9.1-21)	26 (23-31)	44 (40-47)	x1.9	x3.1
20b		Br	9.5 (7.1-13)	26 (23-30)	22 (20-25)	x2.7	x2.3

^aNumbers in parentheses represent 95% confidence interval.

Table 4. Effects of 2-substituent including cyclohexane analogue on Cdc7 enzyme inhibition, kinase selectivity, MCM2 phosphorylation, and cancer cell growth.

Compound	R ₉	R ₁₀	Enzyme inhibition: IC ₅₀ (nM) ^a			Selectivity		clogP ^b	pMCM2 ^c IC ₅₀ (nM)	COLO205 ^d EC ₅₀ (nM)
			Cdc7	Cdk2/cycE	ROCK1	Cdk2/Cdc7	ROCK1/Cdc7			
12d	-	-	1.0 (0.67-1.6)	420 (370-490)	22 (20-25)	x420	x22	2.82	210	330
14a	H	H	1.5 (0.84-2.8)	1300 (1200-1400)	48 (42-54)	x870	x32	2.89	290	470
14b	OH	4-F-Ph	170 (110-260)	>10000	130 (110-160)	x59	x0.76	2.81	-	-
14c	4-F-Ph	OH	1.2 (0.39-3.8)	960 (860-1100)	820 (710-950)	x800	x680	2.81	>1000	4500
14d	OH	cyc-Hex	380 (150-950)	>10000	350 (300-420)	x26	x0.92	3.35	-	-
14e	cyc-Hex	OH	2.0 (0.80-5.1)	2200 (2000-2400)	2900 (2500-3400)	x1100	x1500	3.35	>1000	6200

^aNumbers in parentheses represent 95% confidence interval.

^bclog*P* values were calculated by Daylight Software.

^cInhibition of phosphorylation of Ser40 in HeLa cell line.

^dGrowth inhibition of COLO205 cell line.

Molecular modeling studies

In order to elucidate the kinase selectivity profile of compound **14c** showing favorable in vitro profiles, docking study with the reported Cdc7 co-crystal structure (PDB: 4F9C)¹⁷ and co-crystal structural analysis of **14c** with ROCK2, of which all the 34 amino acid residues in the ATP binding pocket are identical to those of ROCK1, were performed. As shown in Figure 7a, the docking model of **14c** with the Cdc7 crystal structure¹⁷ revealed the following findings: (i) Two hydrogen bonds between the two pyrazole nitrogen atoms and the Pro135/Leu137 residues in the hinge region are formed. (ii) The oxygen and nitrogen atoms of the lactam moiety effectively interact with the Lys90 and Asp196 residues, respectively. (iii) The two sulfur atoms of the thiophene ring and the Met134 residue face to each other, but a direct interaction between them isn't observed. (iv) The bulky 4,4-substituted-cyclohexyl moiety extends toward solvent-exposed region without steric clash with any amino acid residue in the pocket.

Co-crystal structure of **14c** with ROCK2 demonstrates that **14c** forms hydrogen bonding in a similar manner to that observed in the above-mentioned docking model with Cdc7 (Figure 7b). The results don't clearly explain the high selectivity for Cdc7 over ROCK1/2. Therefore, superposition of the X-ray co-crystal structure of **14c** with ROCK2 and crystal structure of ROCK2 apo-form was examined (Figure 7c). The study suggested that Asp176 and Asp218 residues located in the proximity of the 4-fluorophenyl group of **14c** slightly flipped out in comparison with the apo form. This may be attributed to unfavorable anion- π repulsion between the two Asp residues and the benzene ring. By considering the high amino-acid sequence homology between ROCK1 and ROCK2, the anion- π repulsion may similarly exist, resulting in attenuated ROCK1 inhibitory activity. As in the case of **14c**, compound **14e** bearing a cyclohexane ring on the spiro-cyclohexyl group displayed high selectivity for Cdc7 over ROCK1 (see Table 4), which can be ascribed to steric clash between the bulky cyclohexyl group and Asp residues. In

contrast, no unfavorable factor was observed in the putative binding mode of the bulky 4,4-disubstituted-cyclohexyl moiety of **14c** with any Cdc7 amino acid residue (Figure 7a), elucidating the high kinase selectivity.

On the basis of these finding, we concluded that the high selectivity of compounds **14c,e** for Cdc7 over ROCK1 mainly results from the electrostatic repulsion or steric clash between the bulky 4-substituent of the cyclohexyl moiety and the Asp residues of ROCK1.

(a)

(b)

(c)

Figure 7. (a) Docking model of compound **14c** with the Cdc7 crystal structure (PDB: 4F9C). (b) X-ray co-crystal structure of **14c** with ROCK2 (PDB: 5U7R). (c) Superposition of X-ray co-crystal structure of **14c** with ROCK2 (pink) and crystal structure of ROCK2 apo-form (green, PDB: 5U7Q).

4. Conclusion

To search for a new series of potent Cdc7 inhibitors, the putative pharmacophore model was generated on the basis of the Cdc7 homology model constructed by the crystal structure of CK2. Drug design based on the model suggested several candidate scaffolds for new Cdc7 inhibitors, leading to the identification of dihydrothieno[3,2-*d*]-pyrimidin-4(1*H*)-one based Cdc7 inhibitor **4a** as a lead compound. Medicinal chemistry program starting from **4a** revealed the following SARs: (i) introduction of substituents to 1- and/or 2-position was favorable as expected from the pharmacophore model; (ii) the 3-methylpyrazol-4-yl moiety was found as the best hinge-binding group in terms of both Cdc7 inhibition and kinase selectivity. Further SAR studies succeeded in the discovery of new, potent, and cell active Cdc7 selective inhibitors (**12d**, **14a**). Additionally, molecular modeling studies suggested that the high selectivity of **14c,e** for Cdc7 over ROCK1 is mainly due to the electrostatic repulsion or steric clash of the bulky 4-substituent of the cyclohexyl moiety with the Asp residues. These results could pave the way for developing highly potent and selective Cdc7 inhibitors for the treatment of cancer.

5. Experimental

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 μ M or NH 60 μ M, Fuji Silysia Chemical, Ltd.) or Combi-Flash. The proton nuclear magnetic resonance (^1H 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. $\times$ 50 mm, Shiseido, Japan) or L-column 2 ODS (2.0 mm i.d. $\times$ 30 mm, CERI, Japan) with a temperature of 50 $^{\circ}\text{C}$ 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.

Abbreviations

CSA: (+/-)-camphor-10-sulfonic acid

DCM: dichloromethane

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

NBS: *N*-bromosuccinimide

NH: amino silica gel

PdCl₂(dppf): 1,1'-bis(diphenylphosphino)ferrocenepalladium (II) dichloride dichloromethane adduct

PE: petroleum ether

PTSA: *p*-toluenesulfonic acid monohydrate

TFA: trifluoroacetic acid

TFAA: trifluoroacetic anhydride

THF: tetrahydrofuran

Methyl 3-(methylamino)-5-pyridin-4-ylthiophene-2-carboxylate (1b). To a solution of compound **1a**¹⁸ (300 mg, 1.29 mmol) in DMF (25 mL) was added NaH (60% in mineral oil, 63 mg, 1.56 mmol) at 0 °C. After being stirred for 10 min, to the solution was added dropwise MeI (200 mg, 1.42 mmol), and then the reaction mixture was stirred at room temperature for 3 h. The solvent was removed under reduced pressure, and the residue was purified by column chromatography on silica gel (PE/EtOAc, 1:1, v/v) to give crude **1b** (200 mg, 62%) as a yellow solid. This material was used in the next reaction without further purification.

3-Amino-5-(pyridin-4-yl)thiophene-2-carboxylic acid (2a). A mixture of **1a**¹⁸ (2.34 g, 10.0 mmol), sodium methoxide (1.62 g, 30.0 mmol), MeOH (40 mL) and water (10 mL) was refluxed for 4 h. The

mixture was allowed to cool to room temperature and stirring was continued overnight. The mixture was cooled under ice-bath, then conc. HCl (2.48 mL, 300 mmol) was added to adjust the pH approx. 4. The resultant yellow precipitate was collected by filtration, washed with water–MeOH, and dried under reduced pressure to afford **2a** (2.02 g, 92%) as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.20 (1H, s), 7.62 (2H, dd, *J* = 4.8, 1.5 Hz), 8.62 (2H, d, *J* = 6.3 Hz). The amino protons and carboxylic acid proton were not observed.

3-(Methylamino)-5-pyridin-4-ylthiophene-2-carboxylic acid (2b). Crude compound **2b** was prepared in quantitative yield from **1b** by a procedure similar to that described for **2a** as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 2.68 (3H, d, *J* = 3.0 Hz), 5.59 (1H, br s), 6.07 (1H, d, *J* = 1.8 Hz), 7.24 (1H, d, *J* = 1.8 Hz), 7.51–7.53 (2H, m), 8.52–8.54 (2H, m).

3-Amino-5-pyridin-4-ylthiophene-2-carboxamide (3a). Compound **3a** was prepared in 84% yield from **2a** by a procedure similar to that described for **3b** as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 6.54 (2H, br s), 7.06 (2H, br s), 7.18 (1H, s), 7.54–7.58 (2H, m), 8.61 (2H, d, *J* = 6.3 Hz).

3-(Methylamino)-5-pyridin-4-ylthiophene-2-carboxamide (3b). To a solution of compound **2b** (200 mg, 0.85 mmol), NH₄Cl (93 mg, 1.7 mmol), EDCI (195 mg, 1.02 mmol), HOBt (156 mg, 1.02 mmol) in DMF (20 mL) was added Et₃N (258 mg, 2.55 mmol) at room temperature. The reaction mixture was stirred overnight at room temperature. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (DCM/MeOH, 19:1, v/v) to give **3b** (110 mg, yield 55%) as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 2.94 (3H, d, *J* = 5.1 Hz), 7.00–7.09 (2H, m), 7.21–7.28 (1H, m), 7.49 (1H, s), 7.63–7.66 (2H, m), 8.60–8.63 (2H, m).

3-Amino-N-methyl-5-pyridin-4-ylthiophene-2-carboxamide (3c). Compound **3c** was prepared in 75% yield from **2a** by a procedure similar to that described for **3b** as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 2.69 (3H, d, *J* = 4.5 Hz), 6.50 (2H, br s), 7.19 (1H, s), 7.54 (2H, d, *J* = 6.0 Hz), 7.61–7.62 (1H, m), 8.61 (2H, d, *J* = 6.0 Hz).

3-Amino-N-benzyl-5-pyridin-4-ylthiophene-2-carboxamide (3d). Compound **3d** was prepared in 80% yield from **2a** by a procedure similar to that described for **3b** as a yellow solid. ¹H NMR (300 MHz,

DMSO-*d*₆) δ 4.38 (2H, d, J = 6.0 Hz), 6.56 (2H, br s), 7.21–7.35 (6H, m), 7.76 (2H, dd, J = 4.5, 1.5 Hz), 8.28 (1H, t, J = 5.7 Hz), 8.61 (2H, dd, J = 4.5, 1.5 Hz).

2,2-Dimethyl-6-pyridin-4-yl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (4a). A mixture of **3a** (150 mg, 0.68 mmol), PTSA (50 mg) and acetone (10 mL) in toluene (20 mL) was heated to reflux overnight. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (DCM/MeOH, 9:1, v/v) to afford **4a** (130 mg, yield 74%) as an orange solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.42 (6H, s), 7.62–7.65 (3H, m), 8.59–8.61 (2H, m). HRMS: Calcd for C₁₃H₁₄N₃OS [M+H]⁺: 260.0852. Found: 260.0834.

1,2,2-Trimethyl-6-pyridin-4-yl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (4b). A mixture of **3b** (69 mg, 0.30 mmol), PTSA (5.6 mg) and acetone (2 mL) in acetic acid (1 mL) was stirred at 80 °C overnight. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 95:5 to 0:100, v/v) and triturated with EtOAc. The precipitate was collected by filtration to afford **4b** (60 mg, yield 74%) as yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.42 (6H, s), 2.93 (3H, s), 7.56 (1H, s), 7.68 (2H, dd, J = 4.2, 1.8 Hz), 8.61 (2H, dd, J = 4.8, 1.8 Hz). MS: Calcd for C₁₄H₁₆N₃OS [M+H]⁺: 274. Found: 274. Anal. Calcd for C₁₄H₁₅N₃OS: C, 61.51; H, 5.53; N, 15.37. Found: C, 61.46; H, 5.60; N, 15.32.

2-Ethyl-2-methyl-6-pyridin-4-yl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (4c). Compound **4c** was prepared in 69% yield from **3a** by a procedure similar to that described for **4b** as yellow crystals. Mp 260–263 °C (from EtOAc–DCM). ¹H NMR (300 MHz, DMSO-*d*₆) δ 0.89 (3H, t, J = 7.5 Hz), 1.36 (3H, s), 1.63–1.77 (2H, m), 7.09 (1H, br s), 7.14 (1H, s), 7.59 (1H, br s), 7.62 (2H, dd, J = 4.5, 1.5 Hz), 8.60 (2H, dd, J = 4.5, 1.5 Hz). MS: Calcd for C₁₄H₁₆N₃OS [M+H]⁺: 274. Found: 274.

2-Methyl-2-*n*-propyl-6-pyridin-4-yl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (4d). Compound **4d** was prepared in 44% yield from **3a** by a procedure similar to that described for **4b** as yellow crystals. Mp 216–219 °C (from DCM). ¹H NMR (300 MHz, DMSO-*d*₆) δ 0.87 (3H, t, J = 7.2 Hz), 1.37–1.42 (5H, m), 1.63–1.67 (2H, m), 7.10 (1H, br s), 7.13 (1H, s), 7.60–7.63 (3H, m), 8.60 (2H, dd, J = 4.5, 1.5 Hz). MS: Calcd for C₁₅H₁₈N₃OS [M+H]⁺: 288. Found: 288.

2-Isopropyl-2-methyl-6-pyridin-4-yl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (4e).

Compound **4e** was prepared in 11% yield from **3a** by a procedure similar to that described for **4b** as yellow crystals. Mp 243–245 °C (from MeOH–EtOAc). ¹H NMR (300 MHz, DMSO-*d*₆) δ 0.90–0.94 (6H, m), 1.32 (3H, s), 2.01–2.08 (1H, m), 7.11 (1H, br s), 7.13 (1H, s), 7.60–7.62 (3H, m), 8.60 (2H, d, *J* = 6.3 Hz). MS: Calcd for C₁₅H₁₈N₃OS [M+H]⁺: 288. Found: 288. Anal. Calcd for C₁₅H₁₇N₃OS: C, 62.69; H, 5.96; N, 14.62. Found: C, 62.58; H, 5.94; N, 14.48.

2-Methyl-6-pyridin-4-yl-2-(2,2,2-trifluoroethyl)-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (4f).

Compound **4f** was prepared in 15% yield from **3a** by a procedure similar to that described for **4b** as yellow crystals. Mp 238–240 °C (from EtOAc–hexane). ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.56 (3H, s), 2.69–2.76 (2H, m), 7.20 (1H, s), 7.42 (1H, br s), 7.65 (2H, d, *J* = 5.1 Hz), 7.89 (1H, br s), 8.62 (2H, d, *J* = 4.8 Hz). MS: Calcd for C₁₄H₁₃F₃N₃OS [M+H]⁺: 328. Found: 328.

6'-Pyridin-4-yl-1'*H*-spiro[cyclopentane-1,2'-thieno[3,2-*d*]pyrimidin]-4'(3'*H*)-one (4g). Compound **4g** was prepared in 72% yield from **3a** by a procedure similar to that described for **4a** as an orange solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.66–1.69 (4H, m), 1.83–1.89 (4H, m), 7.16 (1H, s), 7.24 (1H, s), 7.62 (2H, d, *J* = 6.0 Hz), 7.80 (1H, s), 8.60–8.61 (2H, m). MS: Calcd for C₁₅H₁₆N₃OS [M+H]⁺: 286. Found: 286.

6'-(Pyridin-4-yl)-1'*H*-spiro[cyclohexane-1,2'-thieno[3,2-*d*]pyrimidin]-4'(3'*H*)-one (4h). Compound **4h** was prepared in 36% yield from **3a** by a procedure similar to that described for **4a** as yellow crystals. Mp 248–250 °C (from MeOH–DCM). ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.15–1.30 (1H, m), 1.40–1.66 (7H, m), 1.81–1.93 (2H, m), 7.15 (1H, br s), 7.18 (1H, s), 7.61 (2H, dd, *J* = 4.5, 1.5 Hz), 7.63 (1H, br s), 8.61 (2H, dd, *J* = 4.8, 1.8 Hz). MS: Calcd for C₁₆H₁₈N₃OS [M+H]⁺: 300. Found: 300.

6'-Pyridin-4-yl-1'*H*-spiro[cycloheptane-1,2'-thieno[3,2-*d*]pyrimidin]-4'(3'*H*)-one (4i). Compound **4i** was prepared in 14% yield from **3a** by a procedure similar to that described for **4b** as yellow crystals. Mp 233–235 °C (from MeOH–EtOAc). ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.52 (8H, m), 1.86–2.02 (4H, m), 7.13 (1H, s), 7.26 (1H, br s), 7.62 (2H, dd, *J* = 4.8, 1.8 Hz), 7.74 (1H, br s), 8.61 (2H, dd, *J* = 4.8, 1.5 Hz). MS: Calcd for C₁₇H₂₀N₃OS [M+H]⁺: 314. Found: 314.

2,2,3-Trimethyl-6-pyridin-4-yl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (4j). Compound **4j** was prepared in 32% yield from **3c** by a procedure similar to that described for **4b** as yellow crystals. Mp 231–234 °C (from MeOH–EtOAc). ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.47 (6H, s), 2.86 (3H, s), 7.16 (1H, s), 7.18 (1H, br s), 7.64 (2H, d, *J* = 6.0 Hz), 8.61 (2H, d, *J* = 6.0 Hz). MS: Calcd for C₁₄H₁₆N₃OS [M+H]⁺: 274. Found: 274.

3-Benzyl-2,2-dimethyl-6-pyridin-4-yl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (4k). Compound **4k** was prepared in 15% yield from **3d** by a procedure similar to that described for **4b** as yellow crystals. Mp 201–204 °C (from EtOAc–hexane). ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.42 (6H, s), 4.68 (2H, s), 7.19–7.34 (7H, m), 7.66–7.68 (2H, m), 8.61–8.63 (2H, m). MS: Calcd for C₂₀H₂₀N₃OS [M+H]⁺: 350. Found: 350. Anal. Calcd for C₂₀H₁₉N₃OS: C, 68.74; H, 5.48; N, 12.02. Found: C, 68.59; H, 5.52; N, 11.96.

6-Pyridin-4-ylthieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione (5). A mixture of **3a** (110 mg, 0.50 mmol) and urea (3.00 g, 50.0 mmol) was stirred at 180 °C for 2 h. After cooling to rt, 2 M NaOH (30 mL) was added. After stirred for 30 min, the precipitate was collected by filtration to give yellow solid. The obtained solid was suspended in water (10 mL), and AcOH was added to pH 7. The precipitate was collected by filtration. The obtained solid was triturated with MeOH (10 mL). The precipitate was collected by filtration to give product **5** (37 mg, 34%) as an orange solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.45 (1H, s), 7.74–7.77 (2H, m), 8.64–8.69 (2H, m, *J* = 6.2 Hz), 11.16–11.41 (1H, m), 11.58–11.83 (1H, m). MS: Calcd for C₁₁H₈N₃O₂S [M+H]⁺: 246. Found: 246. Anal. Calcd for C₁₁H₇N₃O₂S·0.4H₂O: C, 52.33; H, 3.11; N, 16.64. Found: C, 52.43; H, 3.09; N, 16.64.

2-Amino-5-pyridin-4-ylthiophene-3-carboxamide (7). To a solution of **6**¹⁹ (400 mg, 1.53 mmol) in EtOH (50 mL) was added a solution of LiOH monohydrate (256 mg, 6.1 mmol) in water (5 mL). The reaction mixture was heated to 80 °C for 2 h. After removal of the solvent under reduced pressure, the residue was dissolved in water and extracted with EtOAc (50 mL). The organic layer was discarded, and the aqueous layer was treated with 2 M HCl to pH 5–6. The precipitate was collected by filtration and dried over MgSO₄ to give 2-amino-5-pyridin-4-ylthiophene-3-carboxylic acid (277 mg, 77%) as an

orange solid. [^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 7.40 (2H, dd, $J = 4.5, 1.5$ Hz), 7.59 (1H, s), 7.65–7.72 (2H, m), 8.39–8.43 (2H, m), 12.19–12.27 (1H, m).] To a solution of the carboxylic acid (2.20 g, 10 mmol), NH_4Cl (1.09 g, 20 mmol), EDCI (2.30 g, 30 mmol) and HOBt (1.80 g, 12 mmol) in DMF (100 mL) was added Et_3N (3.0 g, 30 mmol) at room temperature. The reaction mixture was stirred overnight at room temperature. After removal of the solvent, the residue was purified by column chromatography on silica gel (DCM/MeOH, 19:1, v/v) to give **7** (1.10 g, 50 %) as a brown solid. ^1H NMR (400 MHz, CD_3OD) δ 7.44 (2H, d, $J = 6.0$ Hz), 7.79 (1H, s), 8.40 (2H, d, $J = 6.4$ Hz). MS: Calcd for $\text{C}_{10}\text{H}_{10}\text{N}_3\text{OS}$ $[\text{M}+\text{H}]^+$: 220. Found: 220.

2,2-Dimethyl-6-pyridin-4-yl-2,3-dihydrothieno[2,3-*d*]pyrimidin-4(1*H*)-one (8). Compound **8** was prepared in 10% yield from **7** by a procedure similar to that described for **4a** as an orange solid. ^1H NMR (400 MHz, CD_3OD) δ 1.58 (6H, s), 7.49 (2H, dd, $J = 4.8, 1.6$ Hz), 7.64 (1H, s), 8.43 (2H, d, $J = 6.0$ Hz). MS: Calcd for $\text{C}_{13}\text{H}_{14}\text{N}_3\text{OS}$ $[\text{M}+\text{H}]^+$: 260. Found: 260.

Methyl 5-bromo-3-(methyamino)thiophene-2-carboxylate (9b). A mixture of **9a**²⁰ (996 mg, 3.00 mmol), K_2CO_3 (829 mg, 6.00 mmol), DMF (6 mL) and MeI (0.225 mL, 3.60 mmol) was stirred at 60 °C for 4 h. Then, the mixture was poured into saturated NaHCO_3 aq. (100 mL), and extracted with EtOAc (100 mL). The organic layer was washed with brine, dried over MgSO_4 , filtered and concentrated in vacuo. The residue was mixed with K_2CO_3 (415 mg, 3.00 mmol), MeOH (20 mL) and water (10 mL). The mixture was stirred at room temperature overnight. Then, the reaction mixture was worked up as described above. The residue was purified by column chromatography on silica gel (hexane to 70:30 hexane/EtOAc) to afford **9b** (674 mg, 90%) as a white solid. ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 2.89 (3H, d, $J = 5.1$ Hz), 3.70 (3H, s), 6.82–6.88 (1H, m), 7.05 (1H, s).

3-Amino-5-bromothiophene-2-carboxamide (10a). A mixture of **9a**²⁰ (5.76 g, 24.4 mmol), NaOH (2.94 g, 73.5 mmol), water (25 mL) and MeOH (100 mL) was stirred at 70 °C overnight. After cooling under ice-water bath, 6 M HCl (8.17 mL, 49.0 mmol) was added and the mixture was concentrated in vacuo. NH_4Cl (26.3 g, 491 mmol), Et_3N (49.7 g, 491 mmol) and DMF (230 mL) were added to the residue. After being stirred for 5 min at room temperature, EDCI (28.2 g, 147 mmol) and HOBt (19.9 g,

147 mmol) were added and the stirring was continued for 5 days. The mixture was poured into saturated NaHCO₃ aq., and extracted with EtOAc. The organic layer was washed with brine, dried over MgSO₄, filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc) to afford **10a** (4.1 g, 76%) as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 6.56 (2H, br s), 6.70 (1H, s), 6.91 (2H, br s).

5-Bromo-3-(methylamino)thiophene-2-carboxamide (10b). Compound **10b** was prepared in 85% yield from **9b** by a procedure similar to that described for **10a** as a pale brown solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 2.83 (3H, d, *J* = 5.1 Hz), 6.92 (2H, br s), 6.98 (1H, s), 7.30–7.33 (1H, m).

6-Bromo-2,2-dimethyl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (11a). Compound **11a** was prepared in 90% yield from **10a** by a procedure similar to that described for **4b** as a brown solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.38 (6H, s), 6.69 (1H, s), 7.18 (1H, br s), 7.56 (1H, br s).

6-Bromo-1,2,2-trimethyl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (11b). Compound **11b** was prepared in 61% yield from **10b** by a procedure similar to that described for **4b** as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.39 (6H, s), 2.84 (3H, s), 7.07 (1H, s), 7.64 (1H, br s).

6-Bromo-1,2-dimethyl-2-(2,2,2-trifluoroethyl)-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (11c). A mixture of **10b** (940 mg, 4.0 mmol), 4,4,4-trifluorobutan-2-one (5.04 g, 40 mmol), CSA (93 mg, 0.40 mmol), MgSO₄ (576 mg, 6.0 mmol) and DMA (4 mL) was reacted under microwave irradiation at 150 °C for 1 h. The mixture was poured into saturated NaHCO₃ aq., and extracted with EtOAc. The combined extracts were washed with water and brine, dried over Na₂SO₄ and filtered. After removal of the solvent under reduced pressure, the residue was purified by column chromatography column chromatography on amino silica gel (*n*-hexane/EtOAc, 90:10 to 50:50, v/v) to give a yellow solid. The solid was purified by crystallization from EtOAc/heptane to give **11c** (431 mg, 31%) as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.58 (3H, s), 2.53–2.73 (1H, m), 2.84–3.05 (1H, m), 2.88 (3H, s), 7.10 (1H, s), 7.91 (1H, br s).

1,2,2-Trimethyl-6-(1*H*-pyrazol-4-yl)-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (12a). A mixture of **11b** (138 mg, 0.50 mmol), 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (291

mg, 1.50 mmol), Na₂CO₃ (265 mg, 2.50 mmol), DME (5 mL) and water (2.5 mL) was purged with argon gas. Then, PdCl₂(dppf) (40.8 mg, 0.050 mmol) was added, and the mixture was purged with argon gas again. This mixture was refluxed for 18 h. Then, the mixture was poured into saturated NaHCO₃ aq. (100 mL), and EtOAc (100 mL), and the mixture was stirred vigorously. An insoluble materials were filtered off. The organic layer was separated from the filtrate, washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. This residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 95:5 to 0:100, v/v, then EtOAc/MeOH, 100:0 to 90:10, v/v) to afford a white solid (60 mg). This solid was triturated with EtOAc/hexane and collected by filtration to afford **12a** (50.3 mg, 38%) as a white solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.40 (6H, s), 2.87 (3H, s), 6.97 (1H, s), 7.44 (1H, br s), 7.84 (1H, br s), 8.16 (1H, br s), 13.12 (1H, br s). HRMS: Calcd for C₁₂H₁₅N₄OS [M+H]⁺: 263.0961. Found: 263.0931.

2,2-Dimethyl-6-(3-methyl-1H-pyrazol-4-yl)-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1H)-one (12b).

Compound **12b** was prepared in 60% yield from **11a** and boronic acid ester B by a procedure similar to that described for **12a** as a pale yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.40 (6H, s), 2.37 (3H, br s), 6.53 (1H, s), 6.96 (1H, br s), 7.34 (1H, br s), 7.70 (0.6H, br s), 8.06 (0.4H, br s), 12.85 (1H, br s). HRMS: Calcd for C₁₂H₁₅N₄OS [M+H]⁺: 263.0961. Found: 263.0937.

1,2,2-Trimethyl-6-(3-methyl-1H-pyrazol-4-yl)-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1H)-one (12c).

Compound **12c** was prepared in 32% yield from **11b** by a procedure similar to that described for **12b** as a pale yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.40 (6H, s), 2.40 (3H, br s), 2.88 (3H, s), 6.86 (1H, s), 7.44 (1H, br s), 7.77 (0.6H, br s), 8.10 (0.4H, br s), 12.86 (1H, m). Anal. Calcd for C₁₃H₁₆N₄OS: C, 56.50; H, 5.84; N, 20.27. Found: C, 56.20; H, 5.87; N, 20.07.

1,2-Dimethyl-6-(3-methyl-1H-pyrazol-4-yl)-2-(2,2,2-trifluoroethyl)-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1H)-one (12d). Compound **12d** was prepared in 50% yield from **11c** by a procedure similar to that described for **12b** as a pale yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.59 (3H, s), 2.40 (3H, br s), 2.55–2.79 (1H, m), 2.84–3.06 (1H, m), 2.93 (3H, s), 6.88 (1H, s), 7.71 (1H, s), 7.79 (0.6H, br s), 8.12 (0.4H, br s), 12.87 (1H, br s). Anal. Calcd for C₁₄H₁₅F₃N₄OS·H₂O: C, 48.83; H, 4.39; N, 16.27. Found: C,

48.84; H, 4.47; N, 16.18.

6-(3,5-Dimethyl-1*H*-pyrazol-4-yl)-1,2,2-trimethyl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (12e). Compound **12e** was prepared in 36% yield from **11b** and boronic acid ester C by a procedure similar to that described for **12b** as a pale yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.41 (6H, s), 2.29–2.33 (6H, m), 2.88 (3H, s), 6.65 (1H, s), 7.44 (1H, br s), 12.53 (1H, br s). HRMS: Calcd for C₁₄H₁₉N₄OS [M+H]⁺: 291.1274. Found: 291.1249.

6-(3-Ethyl-1*H*-pyrazol-4-yl)-1,2,2-trimethyl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (12f). Compound **12f** was prepared in 31% yield from **11b** and boronic acid ester D²¹ by a procedure similar to that described for **12a** as a pale yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.23 (3H, t, *J* = 7.8 Hz), 1.40 (6H, s), 2.72–2.88 (5H, m), 6.84 (1H, s), 7.44 (1H, s), 7.76 (0.67H, br s), 8.08 (0.33H, br s), 12.79 (0.33H, br s), 12.88 (0.67H, br s). HRMS: Calcd for C₁₄H₁₉N₄OS [M+H]⁺: 291.1274. Found: 291.1253.

3-Amino-5-(3-methyl-1*H*-pyrazol-4-yl)thiophene-2-carboxamide (13). A mixture of **12b** (1.97 g, 7.51 mmol), 1 M HCl (38 mL) and MeOH (38 mL) was stirred at 50 °C for 4 h. After removal of the solvent under reduced pressure, the organic materials were extracted with EtOAc/THF. The combined extracts were dried over Na₂SO₄ and filtered. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (EtOAc/MeOH, 100:0 to 90:10, v/v) to give **13** (1.20 g, 72%) as a green solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 2.37 (3H, br s), 6.44 (2H, br s), 6.59 (1H, s), 6.75 (2H, br s), 7.63 (0.6H, br s), 7.95 (0.4H, br s), 12.83 (1H, br s).

6'-(3-Methyl-1*H*-pyrazol-4-yl)-1' *H*-spiro[cyclohexane-1,2'-thieno[3,2-*d*]pyrimidin]-4' (3' *H*)-one (14a). Compound **14a** was prepared in 41% yield from **12b** by a procedure similar to that described for **11c** as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.17–1.26 (1H, m), 1.43–1.62 (7H, m), 1.85–1.88 (2H, m), 2.38 (3H, m), 6.60 (1H, s), 6.98 (1H, br s), 7.30 (1H, br s), 7.68 (0.6H, br s), 8.03 (0.4H, br s), 12.77 (0.4H, br s), 12.87 (0.6H, br s). HRMS: Calcd for C₁₅H₁₉N₄OS [M+H]⁺: 303.1274. Found: 303.1271.

(1*r*,4*r*)-4-(4-Fluorophenyl)-4-hydroxy-6'-(3-methyl-1*H*-pyrazol-4-yl)-1' *H*-spiro[cyclohexane-1,2'-thieno[3,2-*d*]pyrimidin]-4' (3' *H*)-one (14b) and

(1*s*,4*s*)-4-(4-Fluorophenyl)-4-hydroxy-6'--(3-methyl-1*H*-pyrazol-4-yl)-1'*H*-spiro[cyclohexane-1,2'-thieno[3,2-*d*]pyrimidin]-4' (3' *H*)-one (**14c**). A mixture of **13** (100 mg, 0.450 mmol), 4-(4-fluorophenyl)-4-hydroxycyclohexanone²² (281 mg, 1.35 mmol), CSA (10.5 mg, 0.0450 mmol), MgSO₄ (108 mg, 0.900 mmol) in DMA (2 mL) was stirred at 80 °C for 45 min. The mixture was partitioned between EtOAc (20 mL) and saturated NaHCO₃ aq. (10 mL). The aqueous layer was extracted with EtOAc (5 mL × 2). The combined organic layers were washed with brine (5 mL), dried over Na₂SO₄ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc/MeOH, 100:0 to 95:5, v/v), followed by crystallization with MeOH–EtOAc to give **14b** (less polar isomer, 51.8 mg, 28%) as a yellow solid and **14c** (more polar isomer, 35.4 mg, 19%) as a pale yellow solid, respectively. **14b** (less polar isomer): ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.44–1.55 (2H, m), 1.85–2.06 (4H, m), 2.09–2.23 (2H, br s), 2.37 (3H, s), 4.94 (1H, s), 6.55 (1H, s), 7.04 (1H, s), 7.13 (2H, t, *J* = 8.9 Hz), 7.70 (1H, br s), 7.65 (2H, dd, *J* = 8.9, 5.7 Hz), 7.78 (1H, s), 12.82 (1H, br s). Anal. Calcd for C₂₁H₂₁FN₄O₂S·H₂O: C, 58.59; H, 5.39; N, 13.01. Found: C, 58.75; H, 5.44; N, 12.95. **14c** (more polar isomer): ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.48–1.58 (2H, m), 1.93–2.06 (6H, m), 2.38 (3H, s), 4.93 (1H, s), 6.65 (1H, s), 7.14 (2H, t, *J* = 8.9 Hz), 7.27 (1H, s), 7.40 (1H, s), 7.61 (2H, dd, *J* = 8.9, 5.7 Hz), 7.71 (1H, br s), 12.84 (1H, br s). Anal. Calcd for C₂₁H₂₁FN₄O₂S·0.2H₂O: C, 60.62; H, 5.18; N, 13.47. Found: C, 60.73; H, 5.04; N, 13.41. Absolute structures of **14b** and **14c** were determined by X-ray crystallographic analysis.

(1*r*,4*r*)-4-Cyclohexyl-4-hydroxy-6'--(3-methyl-1*H*-pyrazol-4-yl)-1'*H*-spiro[cyclohexane-1,2'-thieno[3,2-*d*]pyrimidin]-4' (3' *H*)-one (**14d**) and (1*s*,4*s*)-4-cyclohexyl-4-hydroxy-6'--(3-methyl-1*H*-pyrazol-4-yl)-1'*H*-spiro[cyclohexane-1,2'-thieno[3,2-*d*]pyrimidin]-4' (3' *H*)-one (**14e**). Compound **14d** and **14e** were prepared in 24% and 34% yield from **13** and 4-cyclohexyl-4-hydroxycyclohexanone²¹ by a procedure similar to that described for **14b** and **14c** as a yellow crystals, respectively. **14d** (less polar isomer): mp 200–202 °C (from MeOH–EtOAc). ¹H NMR (300 MHz, DMSO-*d*₆) δ 0.90–1.27 (6H, m), 1.29–1.41 (2H, m), 1.53–1.94 (11H, m), 2.36 (3H, s), 3.78 (1H, s), 6.53 (1H, s), 6.98 (1H, s), 7.39 (1H, s), 7.82 (1H, brs), 12.83 (1H, brs). Anal. Calcd for

$C_{21}H_{28}N_4O_2S \cdot 0.1H_2O$: C, 62.69; H, 7.06; N, 13.93. Found: C, 62.47; H, 7.04; N, 13.80. **14e** (more polar isomer): mp 178–180 °C (from EtOH–water). 1H NMR (300 MHz, DMSO- d_6) δ 0.90–1.25 (6H, m), 1.31–1.44 (2H, m), 1.45–1.92 (11H, m), 2.37 (3H, s), 3.74 (1H, s), 6.62 (1H, s), 6.93 (1H, s), 7.30 (1H, s), 7.80 (1H, brs), 12.83 (1H, brs). Anal. Calcd for $C_{21}H_{28}N_4O_2S \cdot H_2O$: C, 60.26; H, 7.22; N, 13.39. Found: C, 60.32; H, 7.25; N, 13.34. Absolute structure of **14e** was determined by X-ray crystallographic analysis.

Methyl 5-bromo-4-methyl-3-[(trifluoroacetyl)amino]thiophene-2-carboxylate (16). To a stirred solution of **15** (50 g, 292 mmol) in MeCN (600 mL) were added pyridine (28.6 mL, 350 mmol) and TFAA (58.6 mL, 421 mmol) at 0 °C. After 5 min, the mixture was allowed to room temperature and the stirring was continued for 2 h. The mixture was poured into ice-water (2.5 L) and the mixture was stirred for 20 min. Then, extraction with EtOAc (1 L), washing with brine, drying over $MgSO_4$, filtration and concentration under reduced pressure gave a brown oil. This residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 100:0 to 50:50, v/v) to afford methyl 4-methyl-3-[(trifluoroacetyl)amino]thiophene-2-carboxylate (74.8 g, 96%) as an orange oil. 1H NMR (300 MHz, $CDCl_3$) δ 2.24 (3H, s), 3.90 (3H, s), 7.72 (1H, s), 9.68 (1H, br s). To a stirred this intermediate (11.7 g, 43.8 mmol) and AcOH (150 mL) was added NBS (15.6 g, 88.0 mmol) at room temperature. Then, this mixture was stirred at 80 °C for 6 h. After cooling to room temperature, the mixture was poured into water (600 mL) and brine (300 mL). The precipitate was collected by filtration, washed with water, dried under vacuum to afford **16** (9.40 g, 62%) as a white solid. 1H NMR (300 MHz, $CDCl_3$) δ 2.15 (3H, s), 3.89 (3H, s), 9.62 (1H, br s).

Methyl 5-bromo-4-methyl-3-(methyldamino)thiophene-2-carboxylate (17). A mixture of **16** (9.40 g, 27.2 mmol), K_2CO_3 (7.51 g, 54.3 mmol), MeI (2.04 mL, 32.6 mmol) and DMF (60 mL) was stirred at 60 °C for 6 h. The mixture was poured into water (200 mL) and extracted with EtOAc (200 mL), and the extract was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was mixed with K_2CO_3 (4.13 g, 29.9 mmol), MeOH (200 mL) and water (100 mL). The mixture was stirred at room temperature overnight. Then, the mixture was concentrated under reduced pressure to a half volume, and worked up as described above. This residue was purified by column chromatography

on silica gel (*n*-hexane/EtOAc, 100:0 to 50:50, v/v) to afford **17** (4.46 g, 62%) as a pale yellow solid. ¹H NMR (300 MHz, CDCl₃) δ 2.08 (3H, s), 3.24 (3H, s), 3.86 (3H, s).

5-Bromo-4-methyl-3-(methylamino)thiophene-2-carboxamide (18). Compound **18** was prepared in 56% yield from **17** by a procedure similar to that described for **10a** as a brown oil. ¹H NMR (300 MHz, DMSO-*d*₆) δ 2.26 (3H, s), 2.98 (3H, d, *J* = 5.4 Hz), 5.35 (2H, br s), 6.82 (1H, br s).

6-Bromo-1,2,2,7-tetramethyl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (19). Compound **19** was prepared in 43% yield from **18** by a procedure similar to that described for **4a** as a pale orange solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.39 (6H, s), 2.08 (3H, s), 2.56 (3H, s), 7.87 (1H, br s).

1,2,2,7-Tetramethyl-6-(1*H*-pyrazol-4-yl)-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (20a). Compound **20a** was prepared in 47% yield from **19** by a procedure similar to that described for **12a** as a white solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.39 (6H, s), 2.15 (3H, s), 2.57 (3H, s), 7.70 (1H, s), 7.76 (1H, br s), 8.09 (1H, br s), 13.21 (1H, br s). HRMS: Calcd for C₁₃H₁₇N₄OS [M+H]⁺: 277.1118. Found: 277.1097.

7-Bromo-1,2,2-trimethyl-6-(1*H*-pyrazol-4-yl)-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (20b). To a stirred solution of **12a** (0.111 g, 0.423 mmol) in AcOH (5 mL) was added bromine (0.030 mL, 0.582 mmol) at room temperature. After 1 h, the precipitate was collected by filtration and washed with EtOAc to give a yellow solid (111 mg). The obtained solid was mixed with acetone (4 mL), AcOH (1 mL) and PTSA (4.0 mg, 0.021 mmol), and this mixture was stirred at 60 °C for 2 h. The mixture was poured into saturated NaHCO₃ aq., and extracted with EtOAc, and the extract was dried over MgSO₄ and concentrated under reduced pressure to give a yellow solid. This residue was mixed with acetone (4 mL), AcOH (1 mL) and PTSA (4.0 mg, 0.021 mmol). The mixture was stirred at 60° C overnight. Then, the mixture was worked up as described above. The residue was purified by column chromatography on silica gel (95:5 hexane/EtOAc to EtOAc) to give a white solid. This solid was triturated with EtOAc/hexane and collected by filtration to afford **20b** (36.2 mg, 25%) as a white solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.41 (6H, s), 2.68 (3H, s), 7.97–8.33 (3H, m), 13.36 (1H, br s). HRMS: Calcd for C₁₂H₁₄BrN₄OS [M+H]⁺: 341.0066. Found: 341.0031.

X-ray structure analysis

All measurements were made on a Rigaku R-Axis RAPID diffractometer using graphite monochromated Cu-K α radiation. The structure was solved by direct methods with SHELXS-97²³ or SIR-92²⁴ and was refined using full-matrix least-squares on F^2 with SHELXL-97.²³ All non-H atoms were refined with anisotropic displacement parameters.

Single crystal of **14b** was prepared from DMSO, and analyzed as follows.

Crystal data for 14b: C₂₁H₂₁FN₄O₂S·0.5C₂H₆OS·H₂O, $MW = 469.56$; crystal size, $0.20 \times 0.07 \times 0.01$ mm; colorless, platelet; monoclinic, space group $P2_1/c$, $a = 21.4974(4)$ Å, $b = 8.9821(2)$ Å, $c = 12.1415(2)$ Å, $\alpha = \gamma = 90^\circ$, $\beta = 110.3665(12)^\circ$, $V = 2197.86(7)$ Å³, $Z = 4$, $D_x = 1.419$ g/cm³, $T = 100$ K, $\mu = 2.130$ mm⁻¹, $\lambda = 1.54187$ Å, $R_1 = 0.101$, $wR_2 = 0.215$.

Figure 8. ORTEP of **14b**, thermal ellipsoids are drawn at 50% probability.

Single crystal of **14c** was prepared from MeOH/EtOAc, and analyzed as follows.

Crystal data for 14c: C₂₁H₂₁FN₄O₂S, $MW = 412.48$; crystal size, $0.26 \times 0.10 \times 0.08$ mm; yellow, chip; monoclinic, space group $P2_1/c$, $a = 6.97071(17)$ Å, $b = 20.6197(5)$ Å, $c = 13.0525(4)$ Å, $\alpha = \gamma = 90^\circ$, $\beta =$

107.0450(14)°, $V = 1793.67(8) \text{ \AA}^3$, $Z = 4$, $D_x = 1.527 \text{ g/cm}^3$, $T = 100 \text{ K}$, $\mu = 1.934 \text{ mm}^{-1}$, $\lambda = 1.54187 \text{ \AA}$, $R_1 = 0.101$, $wR_2 = 0.274$.

Figure 9. ORTEP of **14c**, thermal ellipsoids are drawn at 30% probability.

Single crystal of **14e** was prepared from EtOAc/THF, and analyzed as follows.

Crystal data for 14e: $\text{C}_{21}\text{H}_{28}\text{N}_4\text{O}_2\text{S} \cdot 0.5 \text{ C}_4\text{H}_8\text{O}$, $MW = 436.59$; crystal size, $0.20 \times 0.10 \times 0.09 \text{ mm}$; yellow, prism; monoclinic, space group $C2/c$, $a = 29.7681(5) \text{ \AA}$, $b = 12.6156(2) \text{ \AA}$, $c = 23.3913(4) \text{ \AA}$, $\alpha = \gamma = 90^\circ$, $\beta = 90.6729(8)^\circ$, $V = 8783.8(3) \text{ \AA}^3$, $Z = 16$, $D_x = 1.320 \text{ g/cm}^3$, $T = 100 \text{ K}$, $\mu = 1.551 \text{ mm}^{-1}$, $\lambda = 1.54187 \text{ \AA}$, $R_1 = 0.099$, $wR_2 = 0.334$.

CCDC 1520567 for compound **14b**, CCDC 1520568 for compound **14c** and CCDC 1520566 for compound **14e** contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via <http://www.ccdc.cam.ac.uk/Community/Requestastructure/Pages/DataRequest.aspx?>

Figure 10. ORTEP of **14e**, thermal ellipsoids are drawn at 20% probability.

Biology

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'-TATGCATCATCATCATCACGGATCCCATCATCATCATCACTGAGC-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 $0.22\text{ }\mu\text{m}$ filter to give an *Escherichia coli* cell-free 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 1.0 μM (K_m) or 50 μM ($K_m \times 50$) 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 Eu³⁺-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.

$$\text{Inhibitory rate (\%)} = (1 - (\text{count of test compound} - \text{blank}) \div (\text{control} - \text{blank})) \times 100$$

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-GloTM (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.

$$\text{Inhibitory rate (\%)} = (1 - (\text{count of test compound} - \text{blank}) \div (\text{control} - \text{blank})) \times 100$$

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 MgCl₂ and 1 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.

$$\text{Inhibitory rate (\%)} = (1 - (\text{count of test compound} - \text{blank}) \div (\text{control} - \text{blank})) \times 100$$

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% CO₂ 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 IC₅₀ 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 EC₅₀ value of test compound was calculated by using Prism software.

Homology model and docking study

Cdc7 homology models were constructed with MOE (Chemical Computing Group, Inc.) by using the Boltzmann-weighted randomized modeling procedure. The X-ray crystal structure of CK2 (PDB ID: 1DAW) was utilized as a template. Docking studies were performed using Glide (Schrödinger, Inc.) and the correct poses of each ligand were identified by scoring with the MM/PBSA (Molecular Mechanics/Poisson Boltzmann Surface Area) approach.

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Graphical abstract