博士論文

ATP 濃度依存性低減を指向した
分子設計に基づく
新規経口 Cdc7 阻害薬の合成研究

倉澤　修

目次

理論の部

実験の部

略語表

AUC	area under the blood concentration time curve
ATP	adenosine triphosphate
B-ALL	B-cell precursor lymphoblastic leukaemia
Bcr-Abl	breakpoint cluster region-Abelson
Btk	Bruton's tyrosine kinase
CAR-T	chimeric antigen receptor T-cell
Cdc2	cell division cycle 2
Cdc7	cell division cycle 7
Cdk2	cyclin-dependent kinase 2
Cdk4/6	cyclin-dependent kinase 4/6
Cdk8	cyclin-dependent kinase 8
CK2	casein kinase 2
CLK1	Cdc2-like kinase 1
CLK2	Cdc2-like kinase 2
CLK4	Cdc2-like kinase 4
Cmax	maximum drug concentration
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DAPK1	death-associated protein kinase 1
DAPK3	death-associated protein kinase 3
DMPK	dystrophia myotonica protein kinase
DNA	deoxyribonucleic acid
DYRK1A	dual specificity tyrosine-phosphorylation-regulated kinase 1A
DYRK1B	dual specificity tyrosine-phosphorylation-regulated kinase 1B
EGFR	epidermal growth factor receptor
EML4-ALK	echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase
ET1	endothelin 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin releasing hormone
GSK3	glycogen synthase kinase 3
HER2	human epidermal growth factor receptor-related 2
HIPK4	homeodomain-interacting protein kinase 4
HMBC	heteronuclear multiple bond coherence
MCM	minichromosome maintenance
MCM2	minichromosome maintenance 2
Orc1	origin recognition complex subunit 1
PARP	poly (adenosine diphosphate-ribose) polymerase
PD-1	programmed cell death 1
ROCK1	rho-associated, coiled-coil-containing protein kinase 1
ROCK2	rho-associated, coiled-coil-containing protein kinase 2

STK17A	serine/threonine kinase 17a
VEGFR2	vascular endothelial growth factor receptor 2

第一章　緒言

第一節　抗悪性腫瘍薬開発の歴史

抗悪性腫瘍薬（抗がん薬）の研究開発の歴史について紹介する（Figure 1）。古代ギリシャの医学者ヒポクラテスが、ギリシャ語で「カニ」を意味する「カルキノス」という言葉を使って、がんについて記載した例が示す通り、古来よりがんは病として存在してきた。しかし、その治療薬の開発の歴史の始まりは比較的遅く、20 世紀半ばのナイトロジェンマスタードの血液がんへの適用に始まり、シスプラチン、5-フルオロウラシルなど DNA 複製を標的とした細胞障害性抗がん剤の開発が続いた。その後、微小管重合阻害を作用機序としたパクリタキセルが上市された後、20 世紀後半のゲノム解析技術の進展に伴い、がんの分子を標的とした分子標的薬の研究開発が中心となっていった。がんで高発現している EGFR や HER2 等の上皮成長因子受容体、栄養供給に必要な血管新生に関連する VEGF、融合遺伝子による発がんをもたらす Bcr-Abl や EML4-ALK、がんで遺伝子変異が見られる B-Raf や Btk、細胞周期関連酵素である Cdk4/6 など、主にキナーゼを標的とする分子標的薬が上市されてきた。また、多発性骨髄腫における蛋白品質調整機構の脆弱性をついたプロテアソーム阻害薬、molecular glue として転写因子に対してセレブロンによる分解誘導を引き起こすサリドマイド誘導体など、臨床開発中あるいは後にその標的や作用機序が明らかとされた例も存在する。また、2011 年の抗 CTLA4 抗体であるイプリムマブの上市に始まり、抗 PD-1 抗体であるニボルマブ/ペムロリズマブに代表される、免疫関連分子を標的とし、患者の細胞性免疫反応を利用した薬剤へと開発対象が広がってきた。それらの研究開発過程で、低分子薬のみならず、細胞表面の受容体や血中蛋白を標的とした抗体薬物、また患者から取り出した T 細胞を遺伝子加工するキムリアに代表される CD19 陽性 B-ALL の CAR-T 細胞療法など、多様なモダリティの薬剤が実用化されてきていることも、他の疾患領域の治療薬では多くは見られない特徴である。一口に悪性腫瘍（がん）といっても、その分類は複雑で、それぞれの特徴に差異があり、多様な切り口での治療薬の研究開発が進展した結果、このような歴史が形成されたと考えられる。また、難治性がんに対する新規薬剤へのアンメットニーズが依然として高いことから、研究開発活動が活発に継続されている。

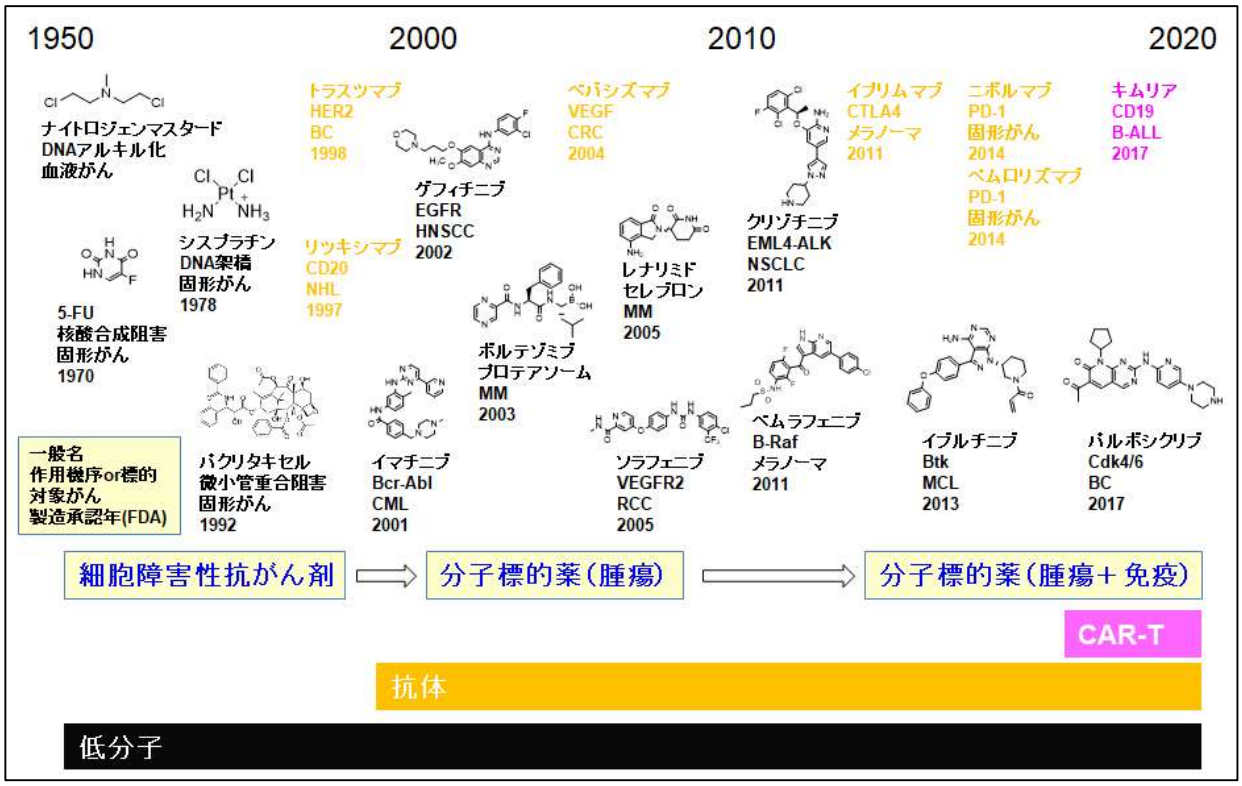


Figure 1. History of the development of the part of the anti-tumor agents.

第二節　　標的分子としての Cdc7 の重要性

我々が着目した Cell division cycle 7(Cdc7)は、真核細胞の DNA 複製期(S 期）において、DNA 複製開始ポイントを形成する Orc1-6 に結合する MCM complex の一部となる MCM2 をリン酸化し、そのヘリカーゼ活性を活性化することで、DNA 複製を制御する Ser/Thr キナーゼである (Figure 2)[1-3]。また、種々のがんにおいて高発現していることが知られており、siRNA を用いた Cdc7 の発現阻害による表現型の解析の報告からも、創薬ターゲットとしての有望性が示唆される。すなわち Cdc7 を阻害することにより、p53 野生型の繊維芽細胞においては MCM2 リン酸化阻害により MCM 複合体の機能異常が生じ、p53 によるチェックポイント機構が活性化され、細胞の G1 期停止を誘導する。一方で p53 変異がん細胞においては、チェックポイント機構また DNA 損傷修復機構が破綻しており、それに由来する S 期における DNA ダメージが蓄積されることで、アポトーシスを誘導することが示されている。したがって、Cdc7 阻害薬には従来の細胞障害性抗がん剤と比較して副作用の軽減、また幅広い固形がんへの適用が期待される（Figure 3）[4]。

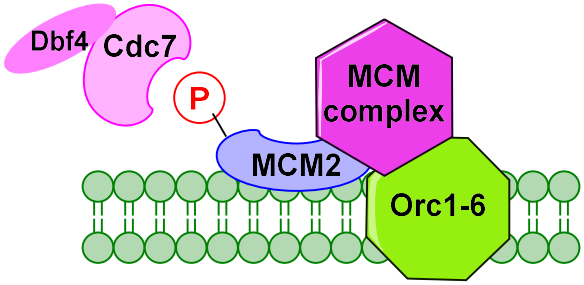


Figure 2. Initiation point of DNA replication at S phase.

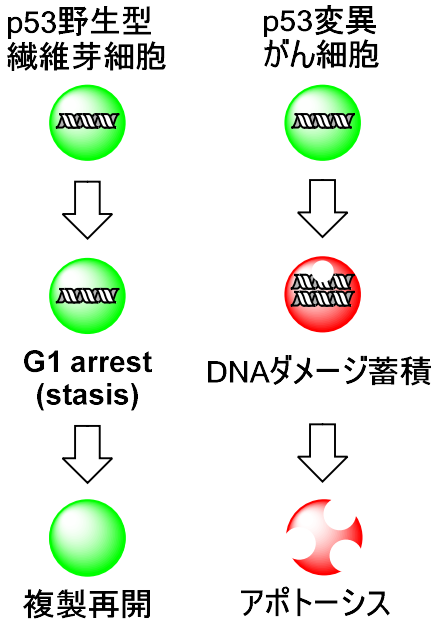
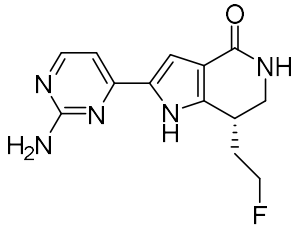


Figure 3. Cancer cell selective apoptosis by siRNA of Cdc7.

第二章　新規縮合チオフェン系 Cdc7 阻害薬のデザイン

第一節　Cdc7 阻害薬リード創出戦略

Cdc7 は細胞内に局在する蛋白であることから、抗体や細胞治療など標的細胞内への移行性に乏しいモダリティの阻害剤の開発は難しいと考えられる。一方で、基質である MCM2 をリン酸化する源となる ATP 結合ポケットを有するキナーゼであり、ATP が形成する相互作用をミミックする ATP 拮抗型低分子性阻害剤の開発は可能と考えられる。そこで、我々は化合物ライブラリーを用いて Cdc7 阻害薬のハイスループットスクリーニングを実施した。しかしながら、良好なヒット化合物は得られなかった。そこで、筆者は、研究開始時点において唯一 Nerviano 社から報告されていた Cdc7 inhibitor (**1**) [5-6] に着目した（Figure 4）。Nerviano Cdc7 inhibitor (**1**) は、Cdc7 酵素阻害活性評価系で nM オーダーの活性(Cdc7 酵素阻害活性 IC_{50} = 2 nM)を示し、担癌マウスモデルにおいて経口投与で薬効を示すことが報告されている。しかしながら複数のキナーゼ活性を阻害すること、 また比較的細胞系活性が低く、高い薬剤暴露量を必要とすることから潜在的にオフターゲット効果を惹起する懸念点を有している。後に臨床試験段階に移行したが、明確な薬効の報告はなされていない。以上の情報を基に、Cdc7 阻害薬のリード創出と臨床応用に適用可能な薬剤の創出を指向し、研究を開始した。



Nerviano Cdc7 inhibitor (1)
Cdc7: IC_{50} = 0.002 +/- 0.001 uM
Cdk9: IC_{50} = 0.028 +/- 0.004 uM
A2780: IC_{50} = 0.5 uM

Figure 4. The Cdc7 inhibitor (**1**) reported by Nerviano.

リード創出にあたり、Cdc7 とアミノ酸相同性が高い Casein kinase 2(CK2)の結晶構造(PDB: 1DAW)を利用して、Cdc7 ホモロジーモデルを作成し、docking study を行い、Nerviano 化合物(1)の ATP ポケットへの結合様式を推定した(Figure 5)。キナーゼのヒンジ部位に位置する Leu137-アミノピリミジン環、また Lys90-Asp196 の塩橋-ラクタムと、二カ所での水素結合を形成し、アデニンポケットへ結合することで、ATP 分子と拮抗する結合様式と推定した。

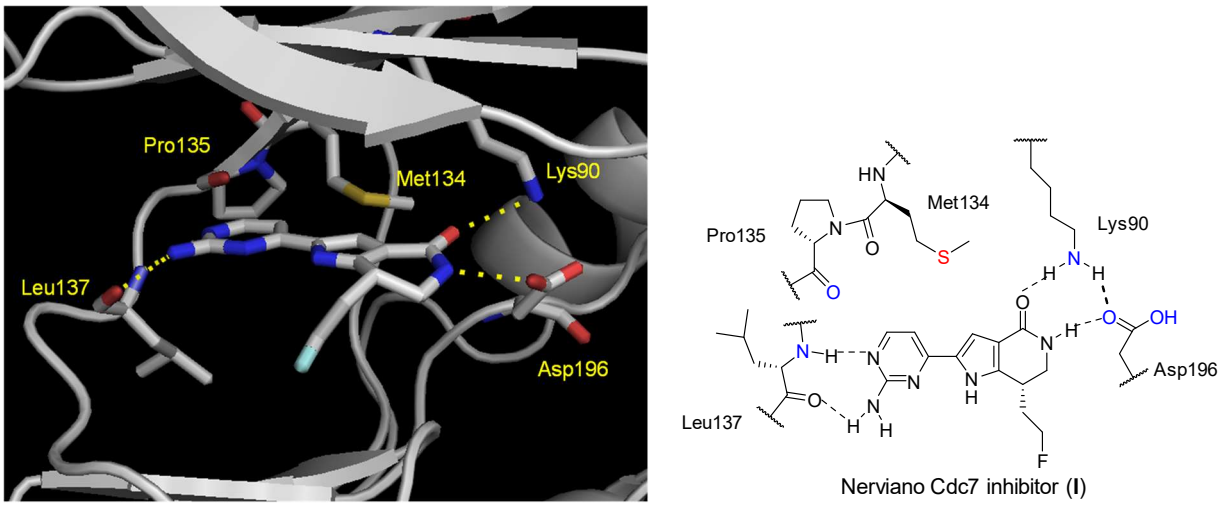


Figure 5. Docking study on Nerviano inhibitor (**1**) by utilizing Cdc7 homology model constructed on the basis of the crystal structure of CK2 (PDB: 1DAW).

第二節　縮合チオフェン誘導体のデザイン

薬剤を開発するにあたり、化学構造の変換により多様な受容体や酵素に対する有益なリガンドを生成しうる基本骨格として、privileged structure という定義が提唱されている[7]。Privileged structure は既知の医薬品や良好な薬理活性が報告されている化合物の分子構造中、統計的に再現性のある分子骨格として抽出された構造である。すなわち薬剤の基本骨格として有用性が証明された骨格は、他の標的の薬剤の基本骨格となりうると考えられる。そこで筆者は、5-6 縮合環に 6 員環が結合した privileged structure であり、過去の GPCR 拮抗薬 (ET antagonist, GnRH antagonist)の研究[8-10]で用いられていた縮合チオフェン骨格に着目した。また、前節で述べた Nerviano inhibitor (1)の推定結合様式から得られた情報を基に pharmacophore model を作成し、特徴的な水素結合形成に加え、ゲートキーパーアミノ酸の Met134 との S-S 相互作用[11] (n(S)→σ*(S) 軌道間相互作用)の付与を期待し、かつ溶媒側方向へさまざまな置換基導入が可能な、硫黄原子の位置の異なる縮合チオフェン骨格[A]をデザインした（Figure 6)。

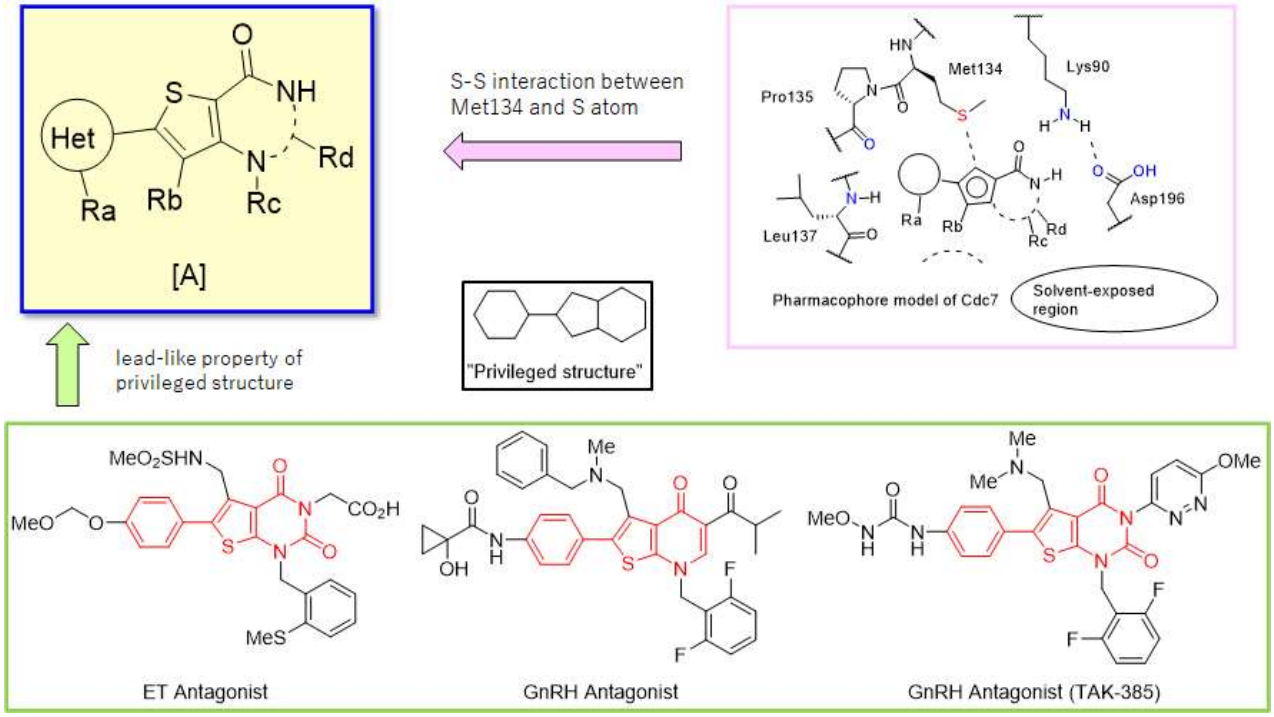
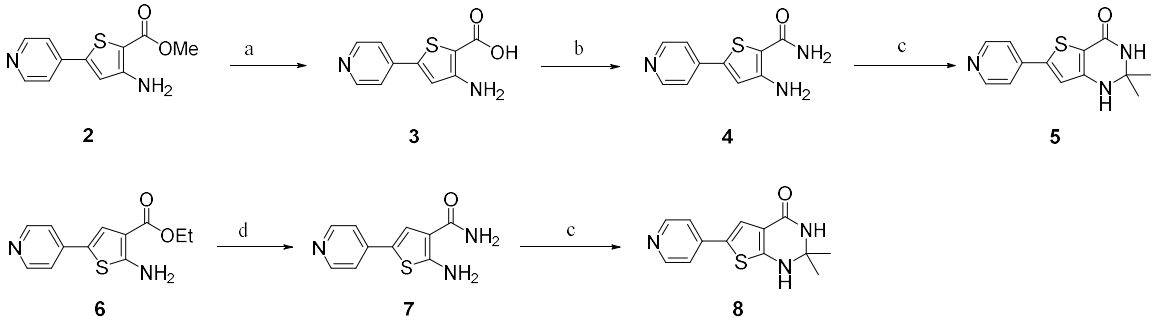


Figure 6. Design of bicyclic thiophene-based Cdc7 inhibitors A based on the pharmacophore model and the concept of the privileged structure.

第三節　予備検討結果

予備検討として、硫黄原子の位置のみが異なる化合物 **5**, **8** を Scheme 1 に示す手法で合成した。すなわち化合物 **2**[12] を加水分解し、得られたカルボン酸 **3** をアンモニアとの縮合反応に付した。得られた化合物 **4** をアセトンと反応させ、環形成させることにより、化合物 **5** を合成した。また同様の手法を用いて、化合物 **6**[13] から硫黄原子の位置の異なる化合物 **8** を合成した。

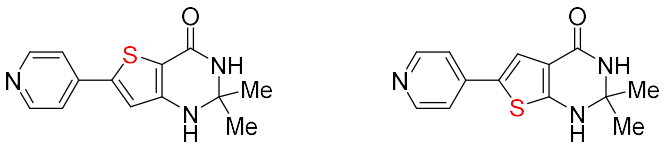
Scheme 1



Reagents and conditions: (a) NaOH, MeOH–water, 70 °C, 92%; (b) NH_4Cl, EDCI, HOBt, Et_3N, DMF, rt, 84%; (c) acetone, *p*-TsOH·H_2O, toluene, 110 °C, 74%; (d) 1) LiOH, EtOH–water, 80 °C; 2) NH_4Cl, EDCI, HOBt, Et_3N, DMF, rt, 38%.

化合物 **5** と **8** の Cdc7 酵素阻害活性を評価した結果、それぞれ IC_{50} = 26 nM および IC_{50} = 140 nM の Cdc7 酵素阻害活性を確認した。また、他のキナーゼ阻害活性に対する選択性を調べるためにキナーゼパネル（46 キナーゼ）試験に付したところ、化合物 **5** と **8** は Rho-associated, coiled-

coil-containing protein kinase 1 (ROCK1)に対しても同等あるいは 1/3 程度の阻害活性を示した。化合物間の比較では、化合物 **5** により高い活性と選択性が認められた。以上のことから、予備検討結果として、縮合チオフェン骨格[A]が Cdc7 阻害薬の骨格として有用であること、また期待通り、硫黄原子が Met134 側を向く配置が、活性および選択性の面で好ましいという構造活性相関情報を得ることができた。



Cdc7: IC_{50} = 26 nM
ROCK1: IC_{50} = 90 nM

Cdc7: IC_{50} = 140 nM
ROCK1: IC_{50} = 100 nM

Figure 7. Identification of dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one as a favorable scaffold of new Cdc7 inhibitors.

第三章　ATP 濃度依存性低減を指向したリード化合物の探索

第一節　推定結合様式に基づく分子設計

前述の予備検討の結果、縮合チオフェン骨格[A]を有する化合物 **5** が、期待通り Cdc7 阻害活性(IC_{50} = 26 nM)を示し、キナーゼパネル（46 キナーゼ）試験で、ROCK1(IC_{50} = 90 nM)を除く 45 キナーゼに対して選択性を示すという結果を得た。しかしながら、細胞系における高濃度 ATP の存在を考慮すると、さらに強力な Cdc7 酵素阻害活性が必要と考えられる。Cdc7 酵素阻害アッセイは一般的な条件として、*Km* 付近の ATP 濃度（1 uM）で実施されている。推定結合様式より、化合物 **5** は Nerviano Cdc7 inhibiotr (1)と同様に ATP 拮抗型の阻害剤であり、細胞系では数 mM の ATP と拮抗することから、Cdc7 酵素阻害活性が大きく減弱すると予想される。また、選択性の面においても、ROCK1 阻害活性が認められていることから、好ましくない作用発現の懸念となりうるため、改善の余地がある。そこで、細胞内高濃度 ATP に打ち勝つべく、かつ選択性を改善する目的で、次に示す観点に着目した。

(1)水素結合の最適化：水素結合の強度は約 1-40 kcal/mol[14] であり、ファンデルワールス力(0.24 kcal/mol)と比較して、大幅に高い。水分子との競合を排除した環境においては、特に高い結合エネルギーが期待できる。また、水素結合数を増やすことで、標的への結合強度の向上が期待できる。

(2)空間充填：水素結合に対し、外からの水分子が競合しないように蓋をすることで、水素結合能の最大化を図る。また Cdc7 のアデニンポケットに対して最適な空間充填をすることで、Cdc7 に対する最大の結合活性と、アミノ酸配列の違いから微細な差がある他のキナーゼのアデニンポケットに対しての選択性を獲得する。

上記観点に基づき、1)ピラゾール環をキナーゼのヒンジ結合部位に用いることで二本の水素結合を形成、2)水素結合の保護効果を期待し、ピラゾール環へ水分子の影響を低減しうる置換基の導入、3)Lys90-Asp196 塩橋部位へ水素結合を追加するための塩基性部位の導入、4)Cdc7 の ATP ポケットに最適な空間充填、また塩橋部位との水素結合保護効果を期待したかさ高い構造の導入、という最適化戦略 1 を立案した（Figure 8）。

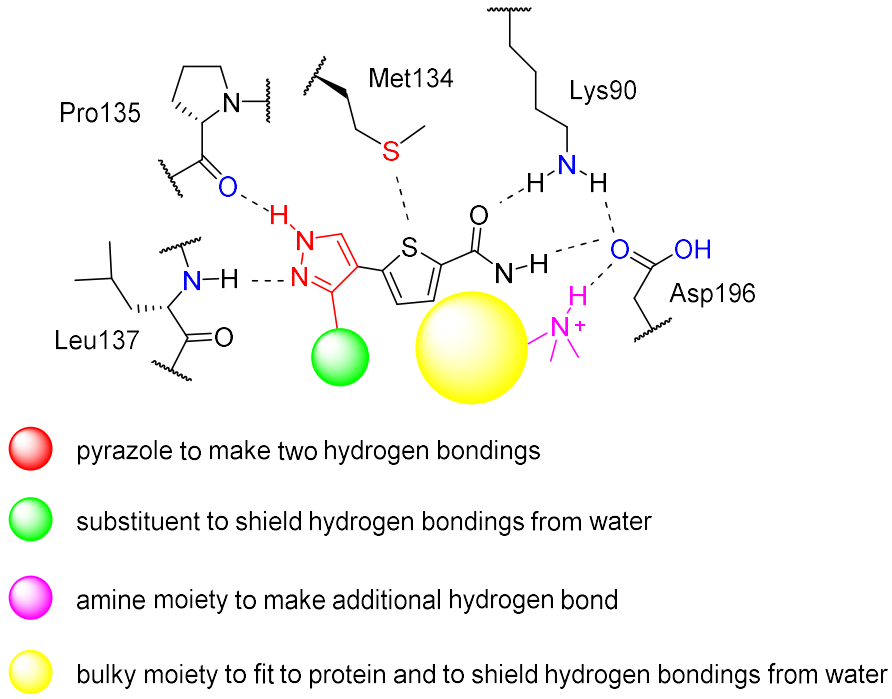


Figure 8. Strategy of optimization (1).

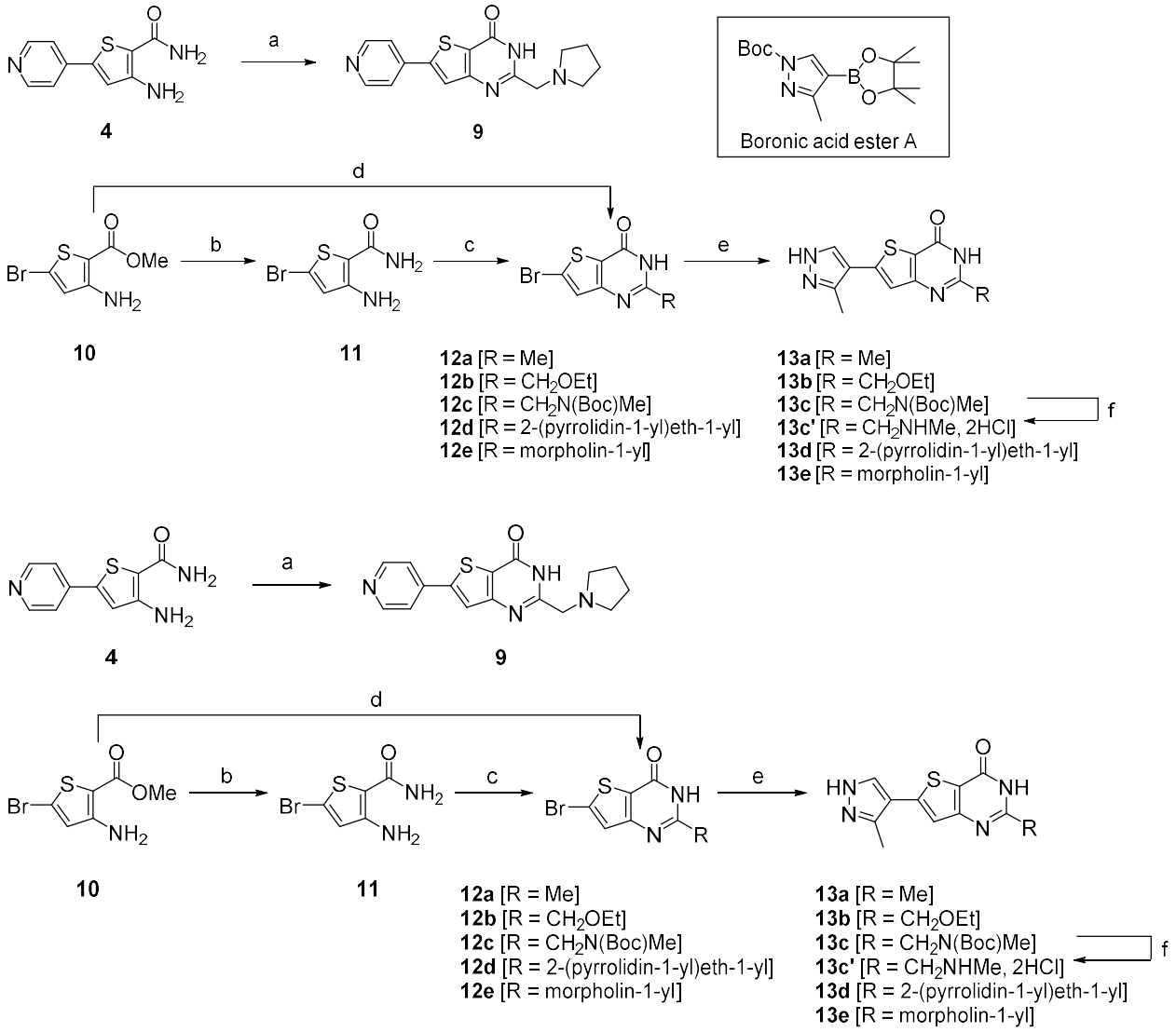
第二節　合成

Scheme 2 および Scheme 3 に示す手法で、チエノピリミジン誘導体の合成を実施した。まず Scheme 2 について述べる。化合物 **4** のアミノ基に対し、クロロアセチルクロリドを用いてクロロアセチル化を行い、導入された塩素原子を *Sn2* 反応で *N*-ピロリジニル基へと置換し、さらに塩基性条件に付すことで、ピリミジン環への閉環反応を引き起こし、4-ピリジル基を有する化合物 **9** を合成した。3-メチルピラゾール-4 イル基を有する化合物については、ブロモチオフェン **10**[15] を原料とし、側鎖を含むピリミジン環を構築した後に、鈴木カップリング反応で 3-メチルピラゾール-4 イル基を導入することで合成した。すなわち、化合物 **10** を塩基性加水分解反応に付し、反応系を中和することなく（反応を中和すると、カルボン酸の脱炭酸反応が生じる）、濃縮することで得られたカルボン酸塩を縮合反応に用いることで、一級アミド **11** を得た。得られた **11** を、化合物 **9** の合成で述べたのと同様に、酸クロリド原料を用いて、側鎖が導入されたピリミジン誘導体 **12a,b,d** へと誘導した。また、原料のカルボン酸を混合酸無水物とし、化合物 **11** のアミノ基と反応させ、塩基性条件に付すことで化合物 **12c** を合成した。化合物 **12e** については、化合物 **11** とモルホリン - 4-カルボニトリルを酸性条件に付すことで、1 工程で得ることに成功した。得られた **12a-e** はピラゾールボロン酸エステル **A** との鈴木カップリング反応に付した。弱塩基性の反応系中でピラゾールボロン酸の *N*-Boc 基の脱保護も進行し、**13a,c** を得た。**13b,d,e** の合成の際には、鈴木カップリング反応後のピラゾールボロン酸の *N*-Boc 基の脱保護反応の進行が不十分であったため、反応系中に水酸化ナトリウム水溶液を加えることで脱保護反応を完遂し、化合物 **13b,d,e** を得た。**13c** の側鎖の Boc 基を、酸性条件で脱保護し、目的とする **13c'**へと導いた。

次に Scheme 3 について述べる。Scheme 2 の化合物 **12e** の合成と同様の手法で、化合物 **10** とクロロアセトニトリルを酸性条件に付し、化合物 **14** を得た。化合物 **14** に導入された塩素原子を *Sn2* 反応で種々のアミンで置換し、**15a-d** を得た。続いて、化合物 **15b-d** をピラゾールボ

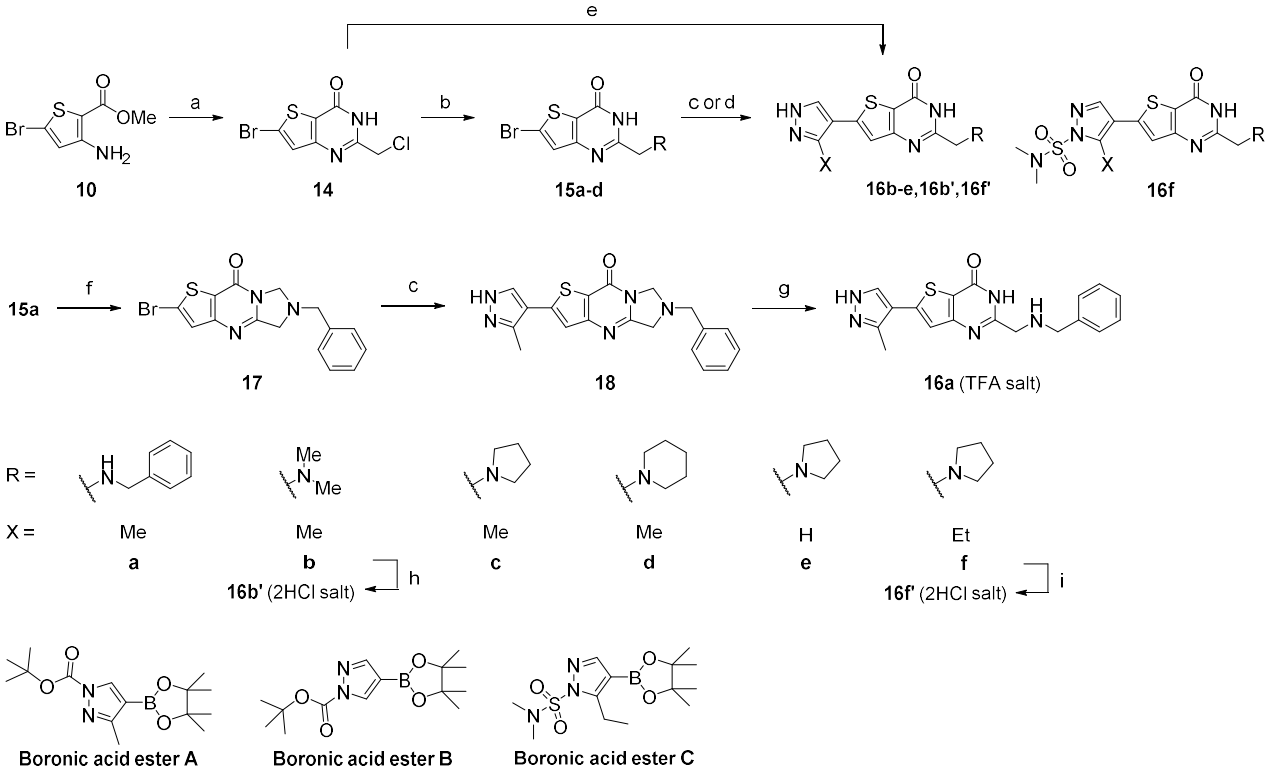
ロン酸エステル **A-C** との鈴木カップリング反応に付して、**16b-f** へと導いた。しかしながら、化合物 **15a** を同様の反応に付したところ、鈴木カップリング反応が進行せず、分子内における二級アミンが付加することによりパラジウムの触媒活性を妨げているのではないかと推定した。そこで、化合物 **15a** をホルムアルデヒドと縮合させ、二級アミンとラクタム部位を保護した **17** を合成した。得られた **17** に対する鈴木カップリング反応は、期待通り問題なく進行し、化合物 **18** を得た。化合物 **18** を酸性条件下の開環反応に付し、化合物 **16a** を得た。また、化合物 **16b** は酸性条件に付し、二塩酸塩 **16b'**とした。化合物 **16f** についても同様に酸性条件に付すことでスルファモイル基を脱保護し、二塩酸塩 **16f'**を得た。

Scheme 2



Reagents and conditions: (a) $ClCH_2COCl$, TEA, THF, rt, then pyrrolidine, 80 °C, then 2 M NaOH, 100 °C, 28%; (b) NaOH, MeOH–water, 70 °C, then NH_4Cl, TEA, HOBt, EDCI, DMF, rt, 76%; (c) RCOCl, TEA, THF, rt, then 2 M NaOH, 70 °C, 59–89% for **12a-b**; RCOOH, *i*-BuOCOCl, TEA, 60 °C, then 2 M NaOH, EtOH, 70 °C, 97% for **12c**; $ClCH_2CH_2COCl$, TEA, THF, rt, then pyrrolidine, rt, then 2 M NaOH, 100 °C, 74% for **12d**; (d) morpholine-4-carbonitrile, 4 M HCl in CPME, 110 °C for **12e**; (e) *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate, $PdCl_2$(dppf), Na_2CO_3 or Cs_2CO_3, DME–water, 80–100 °C, 57% for **13a,c**; *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate, $PdCl_2$(dppf), Na_2CO_3, DME–water, 100 °C, then 8 M NaOH, 100 °C, 42–58% for **13b,d-e**; (f) 4 M HCl in EtOAc, MeOH, rt, 13% (2 steps).

Scheme 3



Reagents and conditions: (a) chloroacetonitrile, 4 M HCl in CPME, 70 °C, 100%; (b) RH (amine), K_2CO_3, NaI, DMF, 70 °C, 37–84% for **15a,c,d**; (c) pyrazolylboronic acid derivative[a], $PdCl_2$(dppf), Na_2CO_3, DME–water, 100 °C, 32–75% for **16d,e,f** and **18**; (d) pyrazolylboronic acid derivative[b], $PdCl_2$(dppf), Na_2CO_3, DME–water, 100 °C then 8 M NaOH, 100 °C, 44% for **16c**; (e) RH, DMF, 100 °C then pyrazolylboronic acid derivative[a], $PdCl_2$(dppf), Na_2CO_3, DME–water, reflux for **16b**; (f) 37% formaldehyde aq., THF, rt, 54%; (g) TFA, MeOH, 70 °C, 44% (2 steps); (h) 10% HCl in MeOH, MeOH, 13% (3 steps); (i) 1 M HCl in Et_2O, MeOH, 60 °C, 75% (2 steps).

[a]**Boronic acid ester A** was used for the preparation of **16b-d, 18**. **Boronic acid ester B** and **C** were used for the preparation of **16e,f**.

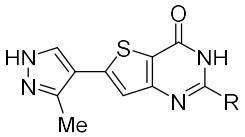
第三節　構造活性相関

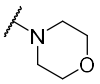
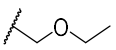
3-メチルピラゾール-4-イル構造を有するチエノピリミジン骨格を固定し、ピリミジン環 2 位側鎖に Lys90-Asp196 塩橋との新たな水素結合の形成を指向した官能基の導入を検討した結果について述べる(Table 1)。生物活性として、Cdc7 酵素阻害活性、また Cdc7 と同じく細胞周期関連キナーゼである Cdk2/cyclin E に対する酵素阻害活性も測定した。その理由を説明する。Cdc7 は MCM2 の Ser40 をリン酸化するキナーゼであるが、Cdk2/cyclin E は MCM2 の Ser41 をリン酸化する酵素である。Cdk2/cyclin E を阻害すると、がん細胞においても細胞周期が G1 期で停止してしまい、Cdc7 阻害の作用機序であるがん細胞選択的な S 期での DNA ダメージ作用が発現しなくなると考えられる。そのため、Cdk2/cyclin E 阻害活性に対する選択性を確認する必要があると考えた。また、第二章で述べたように予備検討の際、化合物 **5** に Cdc7 酵素阻害活性の 1/3 程度の ROCK1 阻害活性が見られたことから、ROCK1 に対する酵素阻害活性も選択

性の指標として測定を実施した。

その結果、単純なメチル基を有する **13a** と比較して、4-モルホリノ基、エトキシメチル基、*N*-メチルアミノメチル基を導入した化合物 **13e**, **13b**, **13c'**はいずれも高い Cdc7 酵素阻害活性を示した。また、化合物 **13e**, **13b**, **13c'**は化合物 **13a** と比較して、Cdk2/cyclin E および ROCK1 阻害活性に対する、より高い選択性を示した。中でも *N*-メチルアミノメチル基を有する **13c'**に最も高い Cdc7 酵素阻害活性と Cdk2/cyclin E 阻害活性を確認した。また ROCK1 阻害活性に対する選択性について、化合物 **13a** と比較して化合物 **13c'**では約 10 倍の改善（x260 vs. x20）が見られた。

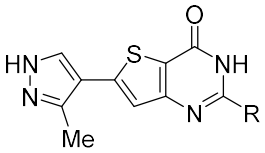
Table 1. Effects of 2-substituent on Cdc7 kinase inhibition and kinase selectivity (1)

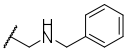
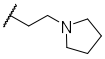


Compound	R	Enzyme inhibition: IC_{50} (nM)			Selectivity	
		Cdc7	Cdk2/cycE	ROCK1	Cdk2 /Cdc7	ROCK1 /Cdc7
13a		75	990	1500	x13	x20
13e		4.1	1100	1700	x270	x410
13b		25	3600	2100	x140	x84
13c'		1.4	5300	360	x3800	x260

次に、良好なプロファイルを示した化合物 **13c'**の構造を基に、種々のアミン誘導体を検討した結果について述べる(Table 2)。*N*-メチルアミノメチル基末端にフェニル基を導入した化合物 **16a** は、化合物 **13c'**の 1/3 程度の Cdc7 酵素阻害活性を示すにとどまり、また Cdk2/cyclin E と ROCK1 阻害活性に対する選択性も 1/2～1/6 に低下した。一方で *N,N*-ジメチルアミノメチル誘導体 **16b'**における Cdc7 酵素阻害活性と ROCK1 阻害活性に対する選択性は、化合物 **13c'**と比較して、1/2 程度の低下にとどまっていることから、三級アミン構造は許容されることが示唆された。そこで、側鎖への環状アミン構造の導入を検討した。その結果、ピペリジニル基を有する化合物 **16d** の Cdc7 酵素阻害活性と Cdk2/cyclin E と ROCK1 阻害活性に対する選択性は、化合物 **16b'**と比較して同等であり、ピロリジニル基を有する化合物 **16c** には約 3 倍強力な Cdc7 酵素阻害活性と、より高い Cdk2/cyclin E と ROCK1 阻害活性に対する選択性が確認された。塩基性窒素原子とピリミジン環の距離の変換が生物活性に及ぼす影響を調べるため、化合物 **13d** を検討したが、化合物 **16c** と比較して、約 200 倍の Cdc7 酵素阻害活性の減弱が認められたことから、メチレン鎖の伸長は望ましくないことが示唆された。

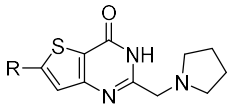
Table 2. Effects of 2-substituent on Cdc7 kinase inhibition and kinase selectivity (2)

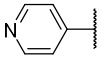
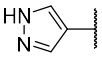
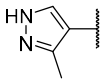
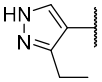


Compound	R	Enzyme inhibition: IC_{50} (nM)			Selectivity	
		Cdc7	Cdk2/cycE	ROCK1	Cdk2 /Cdc7	ROCK1 /Cdc7
13c'		1.4	5300	360	x3800	x260
16a		5.1	3400	610	x670	x120
16b'		2.2	6800	260	x3100	x120
16c		0.7	>10000	140	x14000	x200
16d		2.4	>10000	200	x4200	x83
13d		140	>10000	1600	x70	x11

さらに、活性および選択性の両面で良好なプロファイルを示した **16c** を基に、ヒンジ結合部位の構造活性相関を検討した(Table 3)。4-ピリジル構造へと変換した化合物 **9** は **16c** と比較して Cdc7 酵素阻害活性が約 1/17 に減弱した。またピラゾール環上のメチル基を除去した化合物 **16e** およびエチル基へ伸長した化合物 **16f'**においても、Cdc7 酵素阻害活性の減弱が観測されたが、活性減弱の程度は限定的であった。ピリジン環はピラゾール環と比較して、ヒンジ結合部位における水素結合の形成可能な複素原子の数が少ないため、ピラゾール環を有する化合物群が比較的高い活性を示したと推察される。またピラゾール環上のメチル基の除去による活性減弱は、水素結合部位の保護効果の消失に由来すると推定される。これらの結果は第一節にて述べた推定結合様式に基づく研究戦略より予想された結果と合致している。一方でメチル基と同様の水素保護効果が期待されるエチル基への伸長においても、活性が減弱した。ヒンジ結合部位の空間が限定されているため、メチル基と比較して嵩高いエチル基では最適な親和性が得られなかったためと考えられる。また、Cdk2/cyclin E と ROCK1 阻害活性に対する選択性の面においても、3-メチルピラゾール構造を有する化合物 **16c** が最良のプロファイルを示した。

Table 3. Effects of hinge binding moiety on Cdc7 kinase inhibition and kinase selectivity

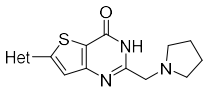


Compound	R	Enzyme inhibition: IC_{50} (nM)			Selectivity	
		Cdc7	Cdk2/cycE	ROCK1	Cdk2/Cdc7	ROCK1/Cdc7
9		12	>10000	180	x830	x15
16e		4.5	>10000	820	x2200	x180
16c		0.7	>10000	140	x14000	x200
16f'		2.5	>10000	830	x4000	x330

化合物 **16c** に、Nerviano Cdc7 inhibitor (1)の報告値(IC_{50} = 2 nM)より強力な活性(IC_{50} = 0.7 nM)と、Cdk2/cycE および ROCK1 に対する高い選択性(Cdk2/Cdc7:x14000, ROCK1/Cdc7: x200)を確認することができたことから、より詳細に化合物 **16c** の Cdc7 酵素阻害活性をプロファイリングすることとした。その結果を、後述する細胞系における生物活性評価結果と共に示す(Table 4)。化合物 **16c** の Cdc7 酵素阻害活性を、通常の酵素阻害活性の条件(ATP = 1 μM、*Km*)と比較して、50 倍高い ATP 濃度で評価した。その際、ATP を加える前に酵素と化合物をプレインキュベーションする時間を 0 分、60 分と異なる複数の条件を適用した。その結果、化合物 **16c** は ATP を加える前に酵素と化合物をプレインキュベーションする時間を 0 分とした場合、すなわちプレインキュベーション時間なしの場合は、IC_{50} = 190 nM と、通常の条件での活性値(IC_{50} = 0.70 nM)と比較して、大きな活性減弱が見られた。しかしながら、プレインキュベーション時間を 60 分とした場合、IC_{50} = 1.7 nM と、通常の条件での活性値(IC_{50} = 0.70 nM)と比較して約 1/3 の活性値への低減にとどまった。すなわち、化合物 **16c** は slow binder であり、また、プレインキュベーション時間を十分とった場合、ATP 高濃度による活性減弱への影響は限定的であることが示された。次に、化合物 **16c** のヒンジ結合部位と ATP 拮抗性の相関を検討した。メチル基を除去した化合物 **16e** および 4-ピリジル基へと変換した **9** においては、通常のアッセイ条件において、**16c** と比較して 1/7～1/17 の Cdc7 酵素阻害活性を示した。一方で、ATP 濃度 50 uM の条件においては、プレインキュベーションなしの場合、化合物 **16c** と同等の Cdc7 酵素阻害活性を示すが、プレインキュベーション時間を 60 分とした場合、IC_{50} = 77-150 nM と、化合物 **16c** (IC_{50} = 1.7 nM)と比較して、1/45～1/88 に減弱した。また、Proteros reporter displacement assay[16-17] を用いて、化合物 **16c**, **16e**, **9** の Cdc7/Dbf4 への結合プロファイルを調べたところ、*Kd*, *Koff*, residence time の指標から、化合物 **16c** が Cdc7/Dbfe4 へ、最も高い結合親和性を有し、また標的から最も乖離しづらいプロファイルを有するという結果を得た。次に、化合物 **16c**, **16e**, **9** を細胞系における生物活性評価へと進めた。HeLa 細胞株における Cdc7 の基質である MCM2

の Ser40 のリン酸化阻害活性(pMCM2)、また結腸癌由来細胞株 colo205 の増殖阻害活性を評価した。その結果、細胞内リン酸化阻害活性値は IC_{50} = 250 nM であり、また colo205 細胞増殖阻害活性値は EC_{50} = 1100 nM であったことから、中程度の細胞系における生物活性を確認することができた。一方で、化合物 **16e**, **9** の明確な細胞内リン酸化阻害活性(pMCM2)は確認できず、colo205 の増殖阻害活性も弱いものであった。以上の結果は、化合物 **16c** が化合物 **16e**, **9** と比較して、標的への結合速度および乖離速度が遅い slow binding/slow dissociation の性質を持ち、また十分プレインキュベーション時間を取った場合は、高濃度 ATP との拮抗による活性減弱の影響を受けにくい性質を有することを示すものである。また、その性質は細胞系活性発現にも好ましいことが示唆された。すなわち、ピラゾール-4-イル基が 4-ピリジル基よりも活性面で好ましく、またピラゾール環 3 位でのメチル基の導入が活性面でさらに好ましく、ヒンジ結合部位の水素結合をより強固なものとし、ATP 濃度の影響を受けにくい性質をもたらし、細胞系活性発現に好ましいという構造活性相関情報を得たことで、本最適化戦略の有用性を確認することに成功した。

Table 4. Effect of 2-substituent on Cdc7 inhibition, time-dependency, phosphorylation of Ser40 in MCM2, and cancer cell growth



Compound	Het	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)
16c		0.70	190	1.7	5.41×10^{-10}	4.94×10^{-4}	34	250	1100
16e		4.5	190	77	1.26×10^{-9}	2.67×10^{-3}	6	>1000	7500
9		12	350	150	2.80×10^{-9}	2.15×10^{-3}	8	>1000	9500

[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.

第四章　細胞系活性と選択性の向上を指向した構造最適化

第一節　　推定結合様式に基づく最適化戦略

第三章で述べた研究により、Cdc7 へ強固に結合し、ATP 高濃度の影響を受けにくい性質を有するリード化合物 **16c** を発見した。一方で化合物 **16c** の細胞系活性(細胞内リン酸化阻害活性値:IC_{50} = 250 nM、colo205 細胞増殖阻害活性値:EC_{50} = 1100 nM)の絶対値は、開発段階に進めるにあたって不十分であり、さらなる活性の向上が必要である。そこで、さらに細胞系活性の向上を図るため、推定結合様式を見直すこととした。Table 2 で示した結果から、塩基性窒素原子の位置の変更、また単純な置換基の導入による活性向上は見込めないことが示唆された。そこで、環状アミン直結構造として、塩基性部位のコンホメーションを固定化し、エントロピーロスを低減することで、より強固な結合を図る戦略を立案した（Figure 9)。

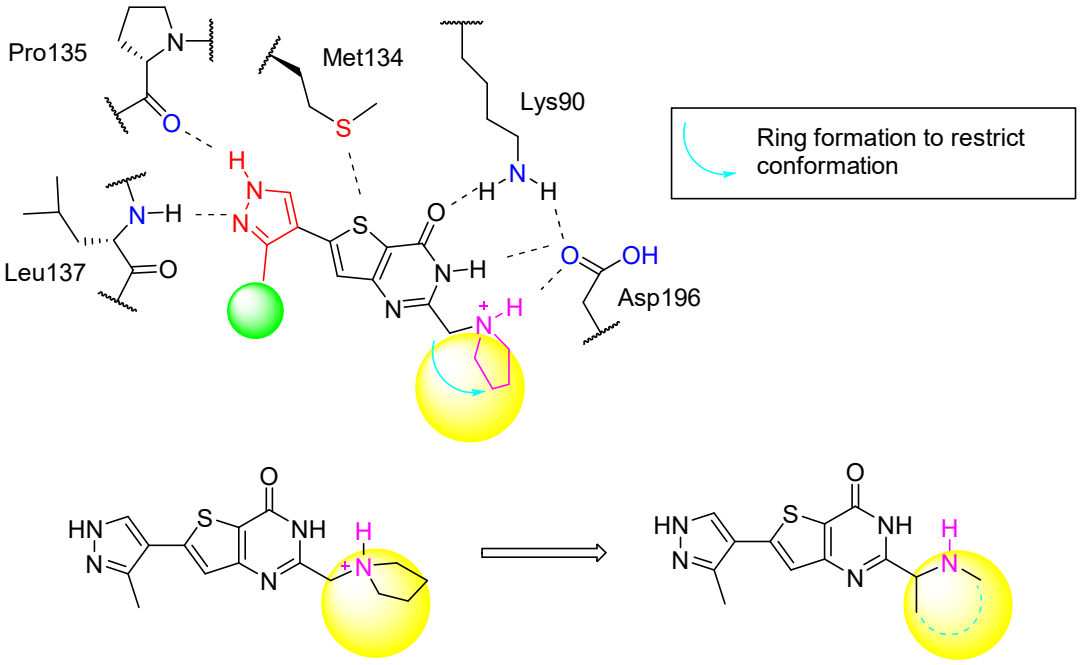


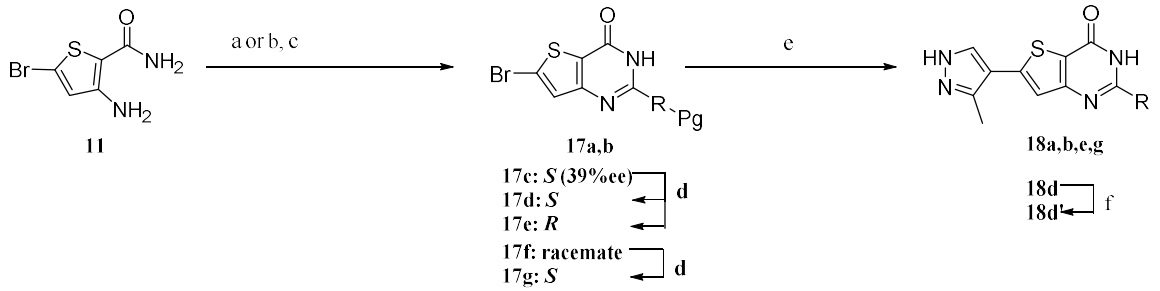
Figure 9. Strategy of optimization (2).

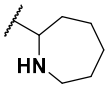
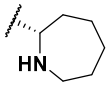
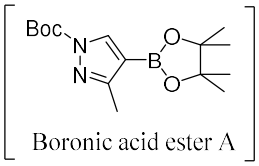
第二節　合成

デザインした化合物は Scheme 4 に示す手法で合成した。化合物 **11** のアミノ基に対し、光学活性アミノ酸原料を用いて、縮合剤 HATU あるいは混合酸無水物法を適用することにより、アシル化反応を行い、さらに塩基性条件に付すことで、化合物 **17a**,**b**,**c** を合成した。得られた化合物 **17a**,**b**,**c** をキラル HPLC で分析したところ、化合物 **17a**,**b** においては、複数のピークは認められず、光学純度の低下は生じていないと推測された。一方で、化合物 **17c** においては 2 本のピークが見られ、光学純度は 39%ee と、低下が認められた。そこで、化合物 **17c** をキラル HPLC で光学分割し、光学純度 99.9%ee に高めた化合物 **17d,e** を得た。得られた化合物 **17a**,**b**,**d**,**e** を鈴木カップリング法に付し、メチルピラゾール部位を導入し、化合物 **18a**,**b**,**d**,**e** を得た。化合物 **18d**,**e** をキラル HPLC で分析したところ、いずれも単一ピークであり、 鈴木カップリングの工程では光学純度の低下が生じていないことがわかった。また、7 員環のアゼパン誘導体については、ラセミ体のアミノ酸を用いて、前述と同様の手法で **17f** を合成したのち、キラル HPLC

を用いた光学分割により **17g** を得た。**17g** の絶対構造は X 線結晶構造解析により *S* 体であることを確認した。**17g** から同様の手法により、**18g** を得た。

Scheme 4



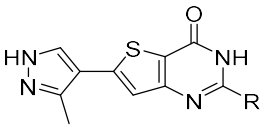
	R	*N*-Protective group (2)	Salt (3)		R	*N*-Protective group (2)	Salt (3)		R	*N*-Protective group (2)	Salt (3)
a		Boc	free	d		Boc	2HCl	f		Boc (racemate)	-
b		Boc	2HCl	d'		—	free	g		Boc	free
c		Boc (39%ee)	-	e		Boc	2HCl		 Boronic acid ester A		

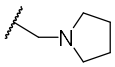
Reagents and conditions: (a) *N*-Boc-amino acid, *i*-BuOCOCl, Et_3N, THF for **17a,c,f,h**; (b) *N*-Boc-*L*-proline, HATU, DIEA, DMF for **17b**; (c) NaOH, EtOH, water, 43%–quant. (2 steps); (d) chiral HPLC separation for **17d,e,g** (e) 1) **boronic acid ester A**, $PdCl_2$(dppf), Cs_2CO_3, DME–water for **18a,b,d,e,g**. 2) 4 M HCl in EtOAc for **18a,b,d,e,g**, 17–66% (2 steps); (f) Et_3N, MeOH, 88%.

第三節　構造活性相関

ピリミジン環 2 位に環状アミン直結構造の導入を検討した結果について述べる(Table 5)。前章と同様に、合成した化合物について、主活性である Cdc7 酵素阻害活性、また Cdk2/cyclin E および ROCK1 酵素阻害活性を測定した。リード化合物 **16c** と比較して、4 員環、7 員環アミン構造を有する化合物 **18a**,**g** は Cdc7 酵素阻害活性が約 1/2 – 1/3 に低下した。5 員環アミンである *(2S)*-ピロリジニル構造を導入した化合物 **18b** はリード化合物 **16c** と同等の活性を示し、ROCK1 阻害活性に対する選択性が約 2-3 倍向上した(x510 vs. x200)。一方で Cdk2/cyclin E 阻害活性に対する選択性が約 1/2 – 1/3 に低下した(x5500 vs. x14000)。しかしながら、6 員環アミンである *(2S)*-ピペリジニル構造を導入した化合物 **18d** は、リード化合物 **16c** と比較して、約 2 倍強力な Cdc7 酵素阻害活性(IC_{50}: 0.44 nM vs. 0.7 nM)と約 5 倍高い ROCK1 阻害活性に対する選択性を示した(x950 vs. x200)。また、Cdk2/cyclin E 阻害活性に対する選択性も同等であった。また、*(2R)*-ピペリジニル構造を導入した **18e** は *S* 体の **18d** と比較して、約 1/2 に活性が低下しており、ユートマーは *S* 体であることが示唆された。

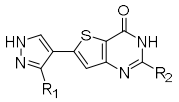
Table 5. Effects of ring size and chirality of the 2-cyclic amine group on Cdc7 inhibitory activity and kinase selectivity



Compound	R	Enzyme inhibition: IC_{50} (nM)			Selectivity	
		Cdc7	Cdk2/cycE	ROCK1	Cdk2 /Cdc7	ROCK1 /Cdc7
16c		0.7	>10000	140	x14000	x200
18a		2.1	6900	760	x3300	x360
18b		0.71	3900	360	x5500	x510
18d		0.44	6500	420	x15000	x950
18e		0.91	4800	370	x5300	x410
18g		1.2	2800	450	x2300	x380

リード化合物 **16c** と比較して、2 倍強力な Cdc7 酵素阻害活性と 5 倍優れた ROCK1 酵素阻害活性に対する選択性を示した*(2S)*-ピペリジニル構造を有する化合物 **18d** について、前章と同様、細胞系における生物活性を評価した(Table 6)。その結果、化合物 **18d** は、リード化合物 **16c** と比較して、約 7 倍強力な細胞内リン酸化阻害活性値(IC_{50}:36 nM vs. 250 nM)と、約 8-9 倍強力な colo205 細胞増殖阻害活性値(EC_{50}:130 nM vs. 1100 nM)を示した。Cdc7 酵素阻害活性のプロファイリングを実施したところ、**18d** は高濃度 ATP(50 μM)存在下においても低濃度 ATP(1 μM, *Km*)存在下と同等の Cdc7 阻害活性(IC_{50}:0.41 nM vs. 0.44 nM)を示し、**16c** と比較して、高濃度 ATP と拮抗することによる活性減弱の影響をさらに受けにくい性質を有していることが明らかとなった。一方で、Proteros reporter displacement assay[16-17] を用いて、化合物 **16c**, **18d** の Cdc7/Dbf4 への binding property を測定したところ、*Kd*, *Koff*, residence time の指標からは、化合物 **18d** の標的への親和性は化合物 **16c** より低く、乖離しにくさの点は同等であることが判明した。これらの結果は、細胞系活性の発現にあたり、標的への結合能ではなく、実際の酵素阻害活性を発現する環境下での、高濃度 ATP への拮抗性の低下(ATP 濃度依存性の低減)が特に重要であることを示唆している。以上、本最適化戦略により、細胞系活性の向上が達成された。

Table 6. Effect of 2-substituent of the thienopyrimidinone scaffold 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]		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]					
16c			0.70	190	1.7	5.41×10^{-10}	4.94×10^{-4}	34	250	1100
18d			0.44	120	0.41	1.96×10^{-9}	6.30×10^{-4}	26	36	130

[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.

化合物 **18d** の広範なキナーゼ選択性を調べる目的で、キナーゼパネル（>300 キナーゼ）試験を実施したところ、300 を超えるキナーゼに対しては、化合物濃度 1000 nM で<80%の阻害率であり、非常に高い選択性を示した（Table 7）。また、キナーゼパネルにおける阻害率上位 3,4,5 番目のキナーゼである DYRK1A. DYRK1B, DMPK に対する IC_{50} 値を測定したところ、IC_{50} = 59-79 nM であり、Cdc7 酵素阻害活性(IC_{50} = 0.44 nM)と比較して、100 倍以上の選択性が担保されていることを確認した。

Table 7. Kinase selectivity data of **18d**.

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 **18d** against 317 kinases are reported by Invitrogen Corp.

本研究で示した化合物群は脂溶性が低いためか、一般的な ADME-Tox 評価項目において、特に問題となる結果を示さないことが多く、化合物 **18d** においても、特に問題となる結果は見られなかった。また、マウスカセットドージングで経口投与における薬物暴露(Cmax = 0.978 μg/mL, $AUC_{0\text{-}8h}$ = 1.35 μg/mL·h at 10 mg/kg, po) を確認できたことから、colo205 担がんマウスモデルを用いた in vivo PD 試験を実施した。結果を Figure 10 に示す。化合物 **18d** (100 mg/kg)を経口投与し、投与後 1,2,4,8,16 時間後の Cdc7 の基質である pSer40 of MCM2 を anti-pSer40/41 抗体、Cdk2 の基質である pSer41 of MCM2 を anti-pSer41 抗体で染色することで、計測した。Anti-pSer40/41 抗体による染色は、投与後、2, 4, 8 時間後に強く抑制され、16 時間後には抑制は回復した。一方で、anti-pSer41 抗体による Cdk2 の基質である pSer41 of MCM2 の染色の変動は認められなかった。すなわち、酵素阻害評価系における Cdk2/cyclin E 阻害活性に対する高い選択性が反映された結果と考えられる。また、pSer40 of MCM2 の変動後である 8, 16 時間後に、S 期後期または G2/M 期で発現上昇が見られる cyclin B1 の増大が見られたことも、化合物 **18d** の Cdc7 阻害薬としての作用機序と齟齬がない結果である。一方で、アポトーシスの指標である cleaved PARP の変動は特にみられず、短時間での Cdc7 阻害ではアポトーシス誘導を起こさないことが示唆された。また、基質である MCM2、コントロールの GAPDH の発現量は特に変動なく、異常は認められなかった。以上の結果から、化合物 **18d** の 100 mg/kg の投与量における単回経口投与は、Cdc7 による基質である MCM2 のリン酸化を強く抑制し、二次マーカーの cyclin B1 の変動を引き起こすことがわかった。一方でアポトーシス誘導には、より長期間、複数回の投与が必要であることが示唆された。

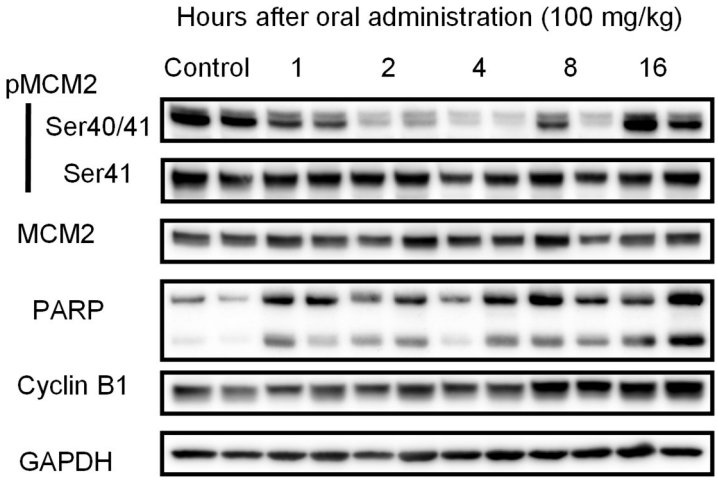


Figure 10. In vivo pharmacodynamic (PD) effects in a COLO205 xenograft mouse model. Compound **18d** (100 mg/kg) was orally administrated to mice bearing COLO205 xenografted tumor. At each time point, xenografted tumor was removed from the mice and homogenized. Protein level or phosphorylation level 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.

In vivo PD 試験結果に基づき、1 日 2 回、50 or 100 mg/kg の投与量で、2 週間経口投与を連投という条件を設定し、colo205 担がんマウスモデルを用いた抗腫瘍試験を実施した(Figure 11)。その結果、50 mg/kg では T/C = 36%、100 mg/kg では T/C = 6%と、投与量依存的な抗腫瘍活性が確認された。また、投与期間におけるマウスの体重減少は軽微であった。以上の結果から、**18d** を精査候補化合物として選定した。

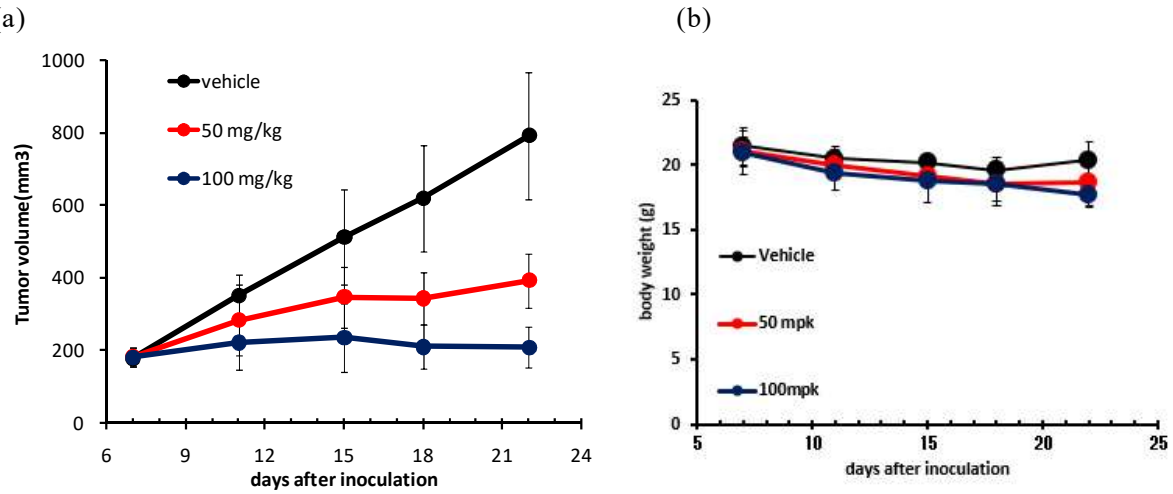


Figure 11. (a) Anti-tumor effects of **3d** in a COLO205 xenograft mouse model. Compound **18d** (50 or 100 mg/kg) was orally administrated twice daily to mice bearing COLO205 xenografted tumor for 14 days (n = 5). Tumor size and body weight mass were measured twice weekly. (b) Body weight measured during the anti-tumor efficacy study.

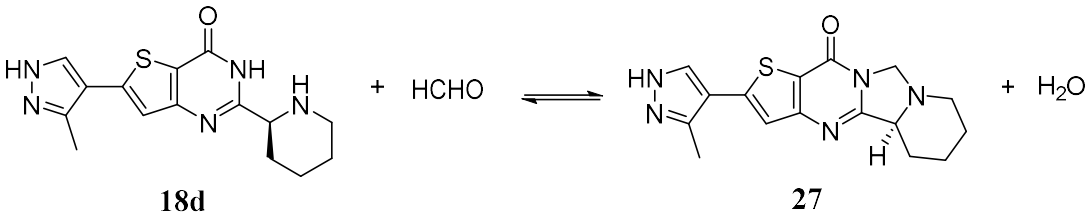
第五章　新規経口 Cdc7 阻害薬 TAK-931 の創製

第一節　ホルムアルデヒドアダクト形成の課題と推定結合様式に基づく回避戦略

精査試験を進めるにつれ、**18d** のホルムアルデヒドアダクト体 **27** が in vivo 試験を含む各種生物試験下や製剤化検討中に検出されることが判明した（Figure 12）。賦形剤中に微量のホルムアルデヒドが存在するため、製剤化の際に **27** が検出されたと考えられる。一方で、各種生物試験下における原材料中に、特にホルムアルデヒドが混入しているという情報はない。また、ホルムアルデヒドは生物に有害な物質であることから、生物試験の原材料に多量に含まれることはあり得ないと考えられることから、各種生物試験下でのホルムアルデヒド体形成に関与するホルムアルデヒドの由来は不明である。

ホルムアルデヒドアダクト体 **27** の非細胞系における Cdc7 酵素阻害活性を測定したところ、親化合物 **18d** と同等の結果が得られた。推定結合様式からは、ホルムアルデヒドアダクト体 **27** は Lys90-Asp196 間の塩橋との水素結合を形成しえない。また、ホルムアルデヒドアダクト体 **27** を水に溶解し、HPLC で分析すると、**18d** と **27** の混合物として計測されたことから、化合物 **27** そのものではなく、原体である化合物 **18d** に分解したものが、Cdc7 酵素阻害活性に寄与したと考えられる。また、化合物 **27** は水中で不安定であることから、検量線の設定が不可能であり、化合物 **18d** については、これ以上の開発には支障があると判断した。

そこで、化合物 **18d** の優れた薬理活性を維持しつつ、アダクト形成を回避する方策として、塩基性部位のビシクロ化戦略を立案した（Figure 13）。すなわち、環状アミン部位のサイズを大きく増大させることなく、コンホメーションを固定化した二級アミンまたは三級アミン構造とすることで、ホルムアルデヒドアダクト形成を回避するという戦略である。ビシクロ化は、活性および選択性に寄与する塩基部位に対し、よりコンホメーションを固定化する方策でもあることから、さらなる活性および選択性の向上の可能性も期待し、検討を進めた。



18d　　**27**

Figure 12. Interconversion of **18d** and its formaldehyde adduct **27** in aqueous media.

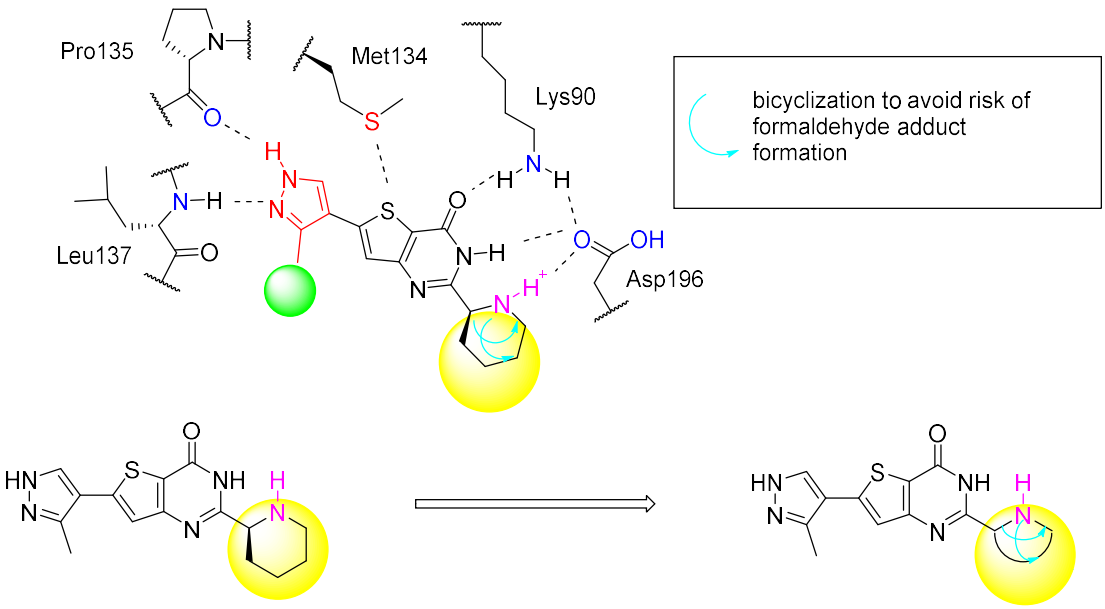
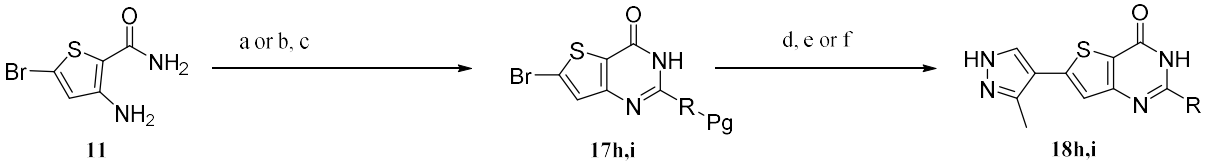


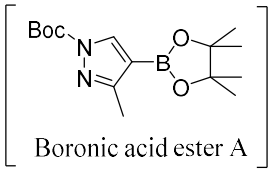
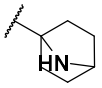
Figure 13. Strategy of optimization (3).

第二節　合成

第二章で述べた合成法と同様の手法で、ビシクロ構造を有する二級アミン誘導体 **18h,i** を合成した(Scheme 5)。キヌクリジン構造を有する **26b,c** の合成については、Scheme 6 に示す手法で実施した。ジケトン **18** を *N,N*-ジメチルホルムアミドジメチルアセタールと作用させ、増炭した後に、PMB 基で保護したヒドラジンを反応させ、ピラゾール誘導体 **20** を合成した。得られたピラゾール誘導体 **20** を Vilsmeier 試薬と反応させた後、ヒドロキシルアミンを作用させる二段階の反応により、アクリロニトリル **21** へと導いた。アクリロニトリル **21** をメルカプト酢酸メチルと反応させ、チオフェン誘導体 **22** を合成した。キヌクリジンカルボン酸 **37** に対し塩化チオニルを用いて酸クロリドとし、チオフェン誘導体 **22** へアシル化してキヌクリジン部位を導入し、化合物 **23** を合成した。化合物 **23** のエステル部位を加水分解し、塩化アンモニウムを用いて縮合反応を行い、一級アミド **24** へと導いた。一級アミド **24** を塩基性条件に付すことでピリミジン **25** を合成した。ピリミジン **25** の PMB 基を、アニソール共存下、トリフルオロ酢酸中で加熱して脱保護し、化合物 **26a** を合成した。化合物 **26a**（ラセミ体）をキラル HPLC による光学分割に付し、化合物 **26b**,**26c** をそれぞれ得た。化合物 **26b** を *D*-DTTA 塩 **26b'**とし、さらに再結晶することで得られた 0.5 D-DTTA 塩 0.5 MeOH-0.5 水付加物 **26b"**の単結晶を X 線結晶構造解析することにより、不斉炭素の立体を含め、その絶対構造を決定した(Figure 14)。したがって、化合物 **26b** を *S* 体と決定し,その光学異性体 **26c** を *R* 体と決定することに成功した。

Scheme 5

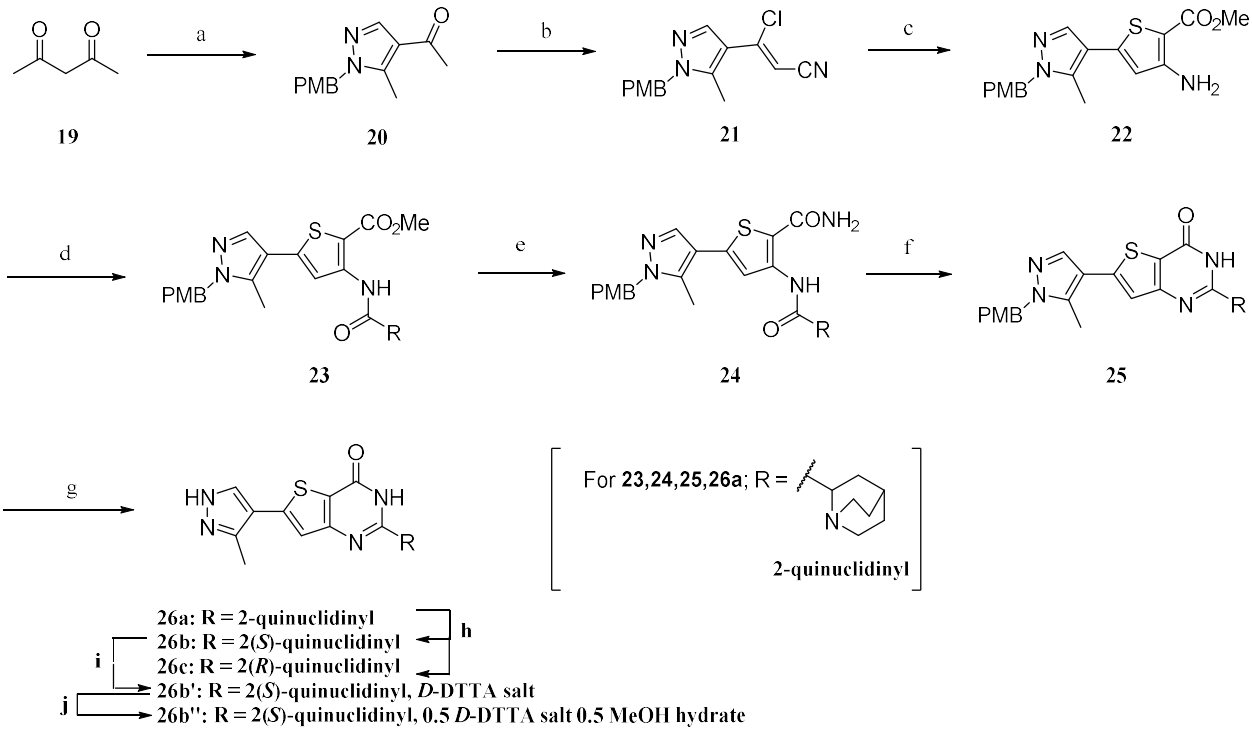


	R	*N*-Protective group (2)	Salt (3)	
h		Boc	2HCl	
i		Cbz	HCl	

Boronic acid ester A

Reagents and conditions: (a) *N*-Boc-amino acid, *i*-BuOCOCl, Et_3N, THF for **17h**; (b) benzyl 1-(chlorocarbonyl)-7-azabiciclo[2.2.1]heptane-7-carboxylate (**30**), DIEA, THF for **17i**; (c) NaOH, EtOH, water, 29%–40% (2 steps); (d) **boronic acid ester A**, $PdCl_2$(dppf), Cs_2CO_3, DME–water for **18h,i**; (e) 4 M HCl in EtOAc for **18h**, 70% (2 steps); (f) 1) Pd/C, HCO_2H. 2) HCl in MeOH for **18i**, 24% (2 steps).

Scheme 6



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 (**37**), $SOCl_2$, 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 **26b** and **26c**; (i) *D*-DTTA, MeOH, 72%; (j) single crystal preparation from MeOH–methyl ethyl ketone.

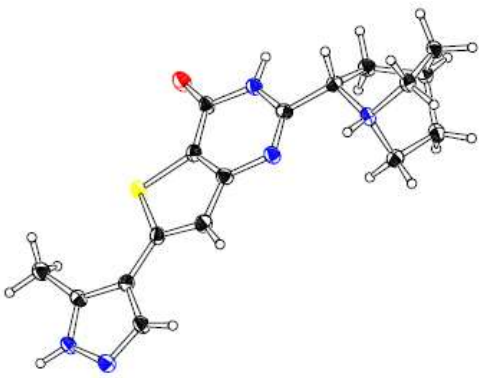
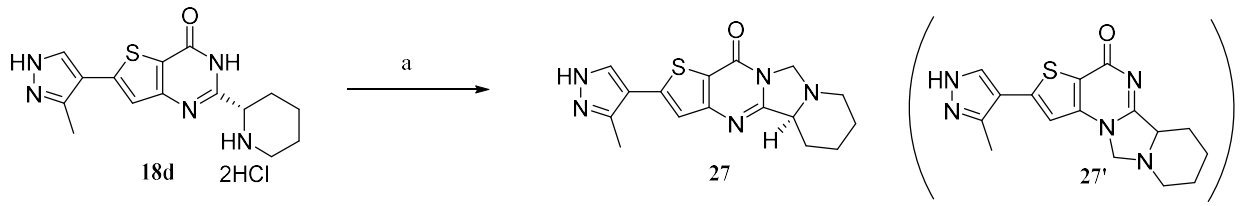


Figure 14. ORTEP of **26b''** (CCDC 1918344, only host ion is displayed). Thermal ellipsoids are drawn at 30% probability.

ホルムアルデヒド付加体 **27** は Scheme 7 に示す手法で、化合物 **18d** をホルムアルデヒドと反応させ、合成した。化合物 **18d** の互変異性体より環化様式の異なる化合物 **27'**が得られる可能性も考えられるが、反応系中で特に生成はみられなかった。また実験の部で述べるように HMBC の手法により化合物 **27** の構造を決定した。

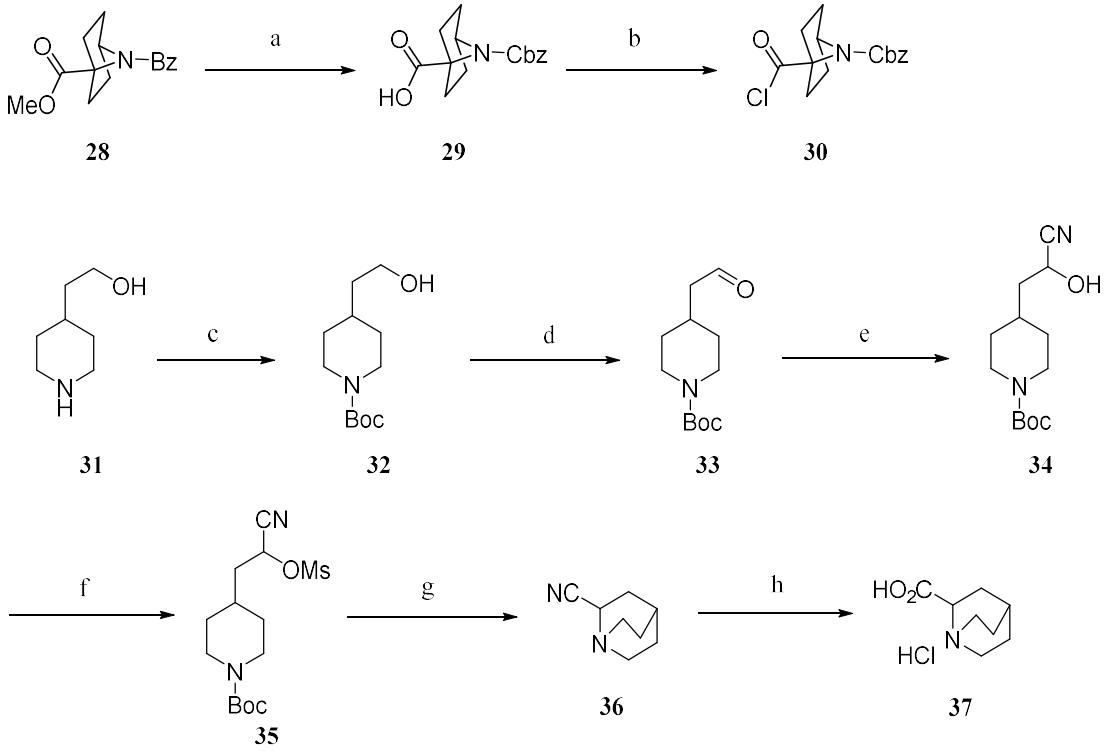
Scheme 7



Reagents and conditions: (a) HCHO, Et_3N, MeOH, 81%.

Scheme 5 で用いた酸クロリド **30** および Scheme 6 で用いたカルボン酸 **37** は Scheme 8 に示す手法で合成した。化合物 **30** については、報告されている合成法 [18] と異なる合成ルートを用いた。すなわち、化合物 **28**[19] を濃塩酸中加熱し、エステル部位とベンゾイル基を加水分解した後に、Schotten-Baumann 条件で窒素原子を Cbz 基で保護し、化合物 **29** を合成した。化合物 **29** を塩化オキサリルと反応させ、酸クロリド **30** を合成した。キヌクリジンカルボン酸 **37** の合成については、報告されている合成法 [20] では最終工程でニトリル基由来のアンモニア分子が塩化アンモニウムとして混入するため、アシル化反応での使用の際に一級アミド形成のリスクがあると考えた。そこで、一部後処理の操作法を変更することで、塩化ナトリウムとの混合物として合成した。

Scheme 8



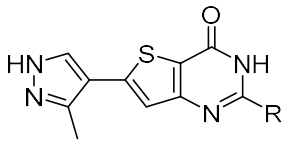
Reagents and conditions: (a) 1) conc. HCl. 2) Cbz-Cl, Na_2CO_3, 1,4-dioxane, water, 27% (2 steps); (b) oxalyl chloride, DMF (cat.), THF, quant; (c) $(Boc)_2O$, *t*-BuOH, water; (d) pyridine sulfur trioxide, Et_3N, DMSO, 81% (2steps); (e) NaCN, HCl, Et_2O, water; (f) MsCl, Et_3N, THF; (g) 1) TFA, CH_2Cl_2. 2) Et_3N, MeCN, 40% (from **33**, 4 steps); (h) 1) conc. HCl, 2) 2 M NaOH, 3) 6 M HCl, 65% (3 steps).

第三節　構造活性相関

ピリミジン環 2 位にビシクロアミン構造の導入を検討した結果について述べる(Table 8)。前章と同様に、合成した化合物について、主活性である Cdc7 酵素阻害活性、また Cdk2/cyclin E および ROCK1 酵素阻害活性を測定した。その結果、ディストマーである *R* 体の **26c** を除いて、いずれのビシクロアミン誘導体(**18i, 26b, 18h**)も 2-ピペリジン誘導体 **18d** より優れた Cdc7 酵素阻害活性を示した。選択性においては、**26b**, **18h** の 2 化合物が **18d** よりも優れた結果を示した。そこで、化合物 **26b**, **18h** について、より詳細な非細胞系における Cdc7 酵素阻害活性と細胞系における生物活性を評価した (Table 9)。その結果、化合物 **26b** は高濃度 ATP(50 μM)存在下において、プレインキュベーション時間 60 min の条件で、低濃度 ATP(1 μM, *Km*)存在下と比較して約 1/2 の Cdc7 阻害活性(IC_{50}: 0.54 nM vs. 0.26 nM)を示し、その阻害活性値は化合物 **18d** と同等であった。また、プレインキュベーションなし(0 min)の条件における高濃度 ATP(50 uM)存在下での化合物 **26b** の Cdc7 酵素阻害活性(IC_{50} = 43 nM)は、化合物 **18d** (IC_{50} = 120 nM)と比較して約 3 倍高かった。一方で、化合物 **18h** の高濃度 ATP(50 μM)存在下、プレインキュベーション時間 60 min における Cdc7 酵素阻害活性(IC_{50} = 21 nM)は、低濃度 ATP(1 uM, *Km*)存在下における Cdc7 酵素阻害活性(IC_{50} = 0.16 nM)と比較して、100 倍以上に減弱しており、化合物 **18h** は ATP 濃度の影響を受けやすいことが明らかとなった。また、Proteros reporter displacement assay[16-17] を用いて、化合物 **26b** の Cdc7/Dbf4 への binding property を測定し、化合物 **18d** と比較したと

ころ、*Kd*, *Koff*, residence time の指標からは、化合物 **26c** の標的への親和性が化合物 **18d** より高く、乖離しにくさの点は同等であることが判明した。細胞系における生物活性を評価したところ、**18h** は **18d** より低い細胞内リン酸化阻害活性(pMCM2:IC_{50} = 130 nM)および低い細胞増殖阻害活性(colo205: EC_{50} = 750 nM)を示したが、**26b** においては、**18d** と比較して細胞系活性の向上[細胞内リン酸化阻害活性(pMCM2:IC_{50} = 17 nM)、細胞増殖阻害活性(colo205: EC_{50} = 81 nM)]が認められた。化合物 **26b** が、高濃度 ATP(50 μM)存在下において、プレインキュベーション時間 60 min の条件で、化合物 **18d** と同等の活性(IC_{50}: 0.54 nM vs. 0.41 nM)であったにもかかわらず、細胞系活性向上が見られたのは、プレインキュベーションなし(0 min)の条件における高濃度 ATP(50 μM)存在下での活性値が化合物 **26b** の方が高く(IC_{50}: 43 nM vs. 120 nM)、高濃度 ATP 存在下において、より素早く標的に作用する性質も、細胞系活性の発現に重要である可能性を示唆していると考えられる。

Table 8. Effect of introduction of azabicycloalkane into the 2-position on Cdc7 inhibitory activity and kinase selectivity



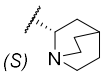
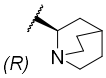
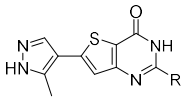
Compound	R	Enzyme inhibition: IC_{50} (nM)			Selectivity	
		Cdc7	Cdk2/cycE	ROCK1	Cdk2 /Cdc7	ROCK1 /Cdc7
18d		0.44	6500	420	x15000	x950
18i		0.16	1500	96	x9400	x600
26b		0.26	6300	430	x24000	x1700
26c		1	>10000	460	x10000	x460
18h		0.16	2700	220	x17000	x1400

Table 9. 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]					
18d		0.44	120	0.41	1.96×10^{-9}	6.30×10^{-4}	26	36	130
18h		0.16	77	2.1	NT	NT	NT	130	750
26b		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.[20]

塩基部位のビシクロ化による化合物 **18d** のホルムアルデヒドアダクト形成能の回避を、水溶液中に 12 当量のホルムアルデヒドを添加し、30 分間後に LCMS で解析することにより、確認する実験を実施した(Figure 15)。その結果、化合物 **18d'**(**18d** のフリー体)においては、LCMS で解析すると、ホルムアルデヒドアダクト **27** の生成が確認されたが、化合物 **18i** と化合物 **26b** においては、同条件でホルムアルデヒドアダクトが検出されなかった。したがって、ビシクロ化によるコンホメーションの固定、あるいは 3 級アミン構造の導入により、ホルムアルデヒド形成の課題が回避可能であることが示唆された。また、詳細は割愛するが、後の薬理精査試験や製剤化試験において、化合物 **18i** および化合物 **26b** のホルムアルデヒドアダクト体は検出されなかった。したがって、塩基性部位をビシクロ化することにより、期待通り、ホルムアルデヒドアダクト形成の課題を解決することができた。

最良の活性と選択性のプロファイルを示し、ホルムアルデヒド形成能の課題の回避を達成した化合物 **26b** を、精査候補化合物として選定した。化合物 **26b** は後の精査試験[21]を経て、開発化合物 TAK-931 として臨床試験で評価中である。

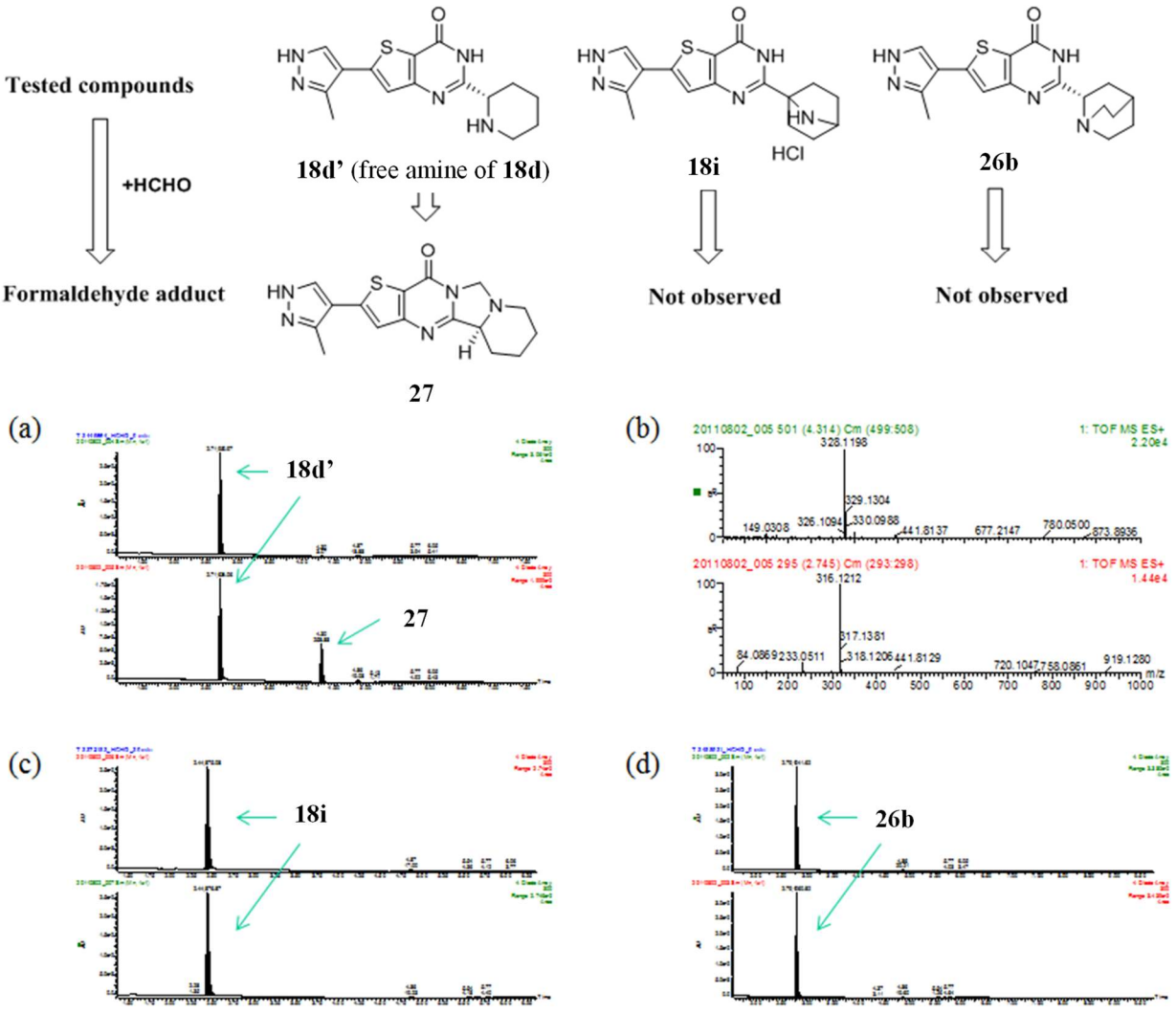


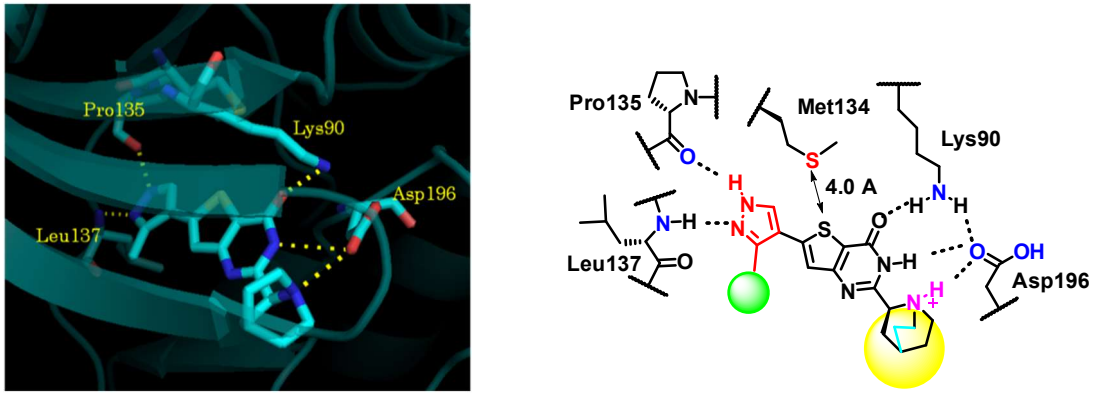
Figure 15. Results of formaldehyde adduct formation study. (a) UV chromatogram of **18d'** at initial (upper) and after 30 min incubation (lower). (b) MS spectra for the peaks of **27** (RT = 4.31 min, upper) and **18d'** (RT = 2.75 min, lower). (c) UV chromatogram of **18i** at initial (upper) and after 30 min incubation (lower). (d) UV chromatogram of **26b** at initial (upper) and after 30 min incubation (lower).

第四節　三次元構造情報からの考察

既知の Cdc7 結晶構造(4F9C)[22] と化合物 **26b** のドッキングモデルを作成し、結合様式を解析した（Figure 16a)。その結果、化合物 **26b** は Cdc7 に対して、合理的に複数の水素結合を形成している様子が観測され、ヒンジ結合部位のピラゾール環上のメチル基およびキヌクリジン環の炭素鎖は溶媒側方向に位置し、重要な水素結合の保護に寄与していると推定された。一方で、チオフェン環の硫黄原子と Met134 の硫黄原子の原子間距離は 4.0 Å であり、ファンデルワールス半径の和(3.6 Å)と同等であり、これらの硫黄原子は近傍に位置するが、明確な S-S 相互作用を示していない。水素結合を含む他の部位での相互作用に影響されたと考えられる。選択性に関する考察を行うべく、ROCK1 と ATP ポケット内の 34 アミノ酸配列相同性が一致している ROCK2 と化合物 **26b** の共結晶を取得し、X 線結晶構造解析を実施した。その結果、キヌクリジン部位の電子密度は不明確であり、すなわちキヌクリジン窒素原子は水素結合を未形成であ

った(Figure 16b)。また、ヒンジ結合部位のピラゾール環上のメチル基は蛋白奥側に位置し、水素結合の保護に寄与しえない結合様式であった。また、チオフェン環の硫黄原子と Met169 の硫黄原子の原子間距離は 5.9Å と、S-S 相互作用は全く期待しえない距離であった。以上の三次元構造情報は、化合物 **26b** の強力な Cdc7 酵素阻害活性および高濃度 ATP 条件における活性低減効果の構造的な要因、また ROCK1 を含めた他のキナーゼに対する高い選択性を説明しうるものである。

(a)



(b)

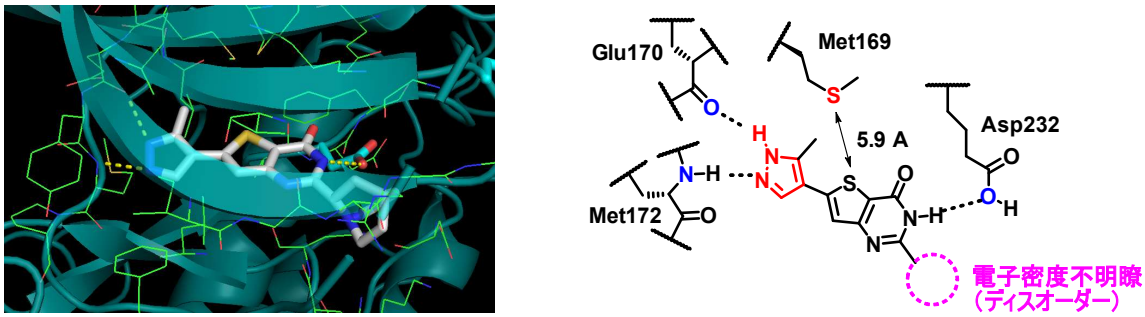


Figure 16. (a) Docking study on compound **26b** with the Cdc7 crystal structure (PDB: 4F9C). (b) Superposition of X-ray co-crystallographic data of **26b** (beige, PDB: 6P5P) bound to the ROCK2 protein.

結語

筆者は、新規経口 Cdc7 阻害薬の創出を指向した戦略を立案した。Cdc7 ホモロジーモデルに対する Nerviano 化合物(1)の結合様式を推定し、ファーマコフォアモデルを作成した。privileged structure であるチエノピリミジン骨格を基に、ファーマコフォアモデルの情報を加えて、縮合チオフェン誘導体を着想した。さらに細胞内高濃度 ATP に打ち勝つ強固な水素結合能と Cdc7 に対する高選択性の獲得を指向した最適化戦略を立案した。検討を進め、ヒンジ部位の構造と、高濃度 ATP による活性低下の低減(ATP 濃度依存性の低減)という ATP 拮抗型阻害剤として希少な性質についての構造活性相関情報を明らかにした。さらに、塩基性部位のコンホメーションを固定化する戦略を進め、大幅に向上した細胞系活性を示す*(2S)*-ピペリジン誘導体 **18d** を見出した。**18d** の高いキナーゼ選択性と、経口投与における担がんマウスモデル抗腫瘍性活性の確認に成功した。しかしながら、新たにホルムアルデヒドアダクト形成の課題を有することが判明し、アダクト形成を回避する方策として、塩基性部位のビシクロ化戦略を立案し、検討を進めた。その結果、さらに細胞系活性が向上し、ホルムアルデヒドアダクト形成を回避した **26b** を見出すことに成功した。**26b** は後の精査試験を経て、開発化合物 TAK-931 として臨床試験で評価中である。フェーズ 1 試験での用量依存的な薬剤暴露と良好な PK/PD 相関を伴う PD 抑制効果、また忍容性と初期の臨床徴候についての速報が ASCO national meeting 2018 (*J. Clin. Oncol.* **36**, no. 15_suppl (May 20, 2018) 2506-2506)で報告されている。

以上の結果から、本研究は、privileged structure とファーマコフォアモデルからデザインした骨格を基に、細胞内高濃度 ATP に打ち勝つ強固な水素結合能の形成と Cdc7 に対する適切な空間充填を指向して立案した最適化戦略が、臨床応用に適用可能な新規経口 Cdc7 阻害薬の創出に有用な方法論であることを示した。本戦略は他の ATP 拮抗型キナーゼ阻害薬にも応用可能と考えられる。

謝辞

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実験の部

ABBREVIATIONS

CSA, (+/-)-camphor-10-sulfonic acid; DCM, dichloromethane; 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; PE, petroleum ether; PTSA, *p*-toluenesulfonic acid monohydrate; TBAF, tetra-*n*-butylammonium fluoride; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

化学に関する実験

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 ºC 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.

3-Amino-5-(pyridin-4-yl)thiophene-2-carboxylic acid (3). A mixture of **2**[12] (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 **3** (2.02 g, 92%) as a yellow solid. ^{1}H 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-Amino-5-pyridin-4-ylthiophene-2-carboxamide (4). A mixture of **3** (0.220 g, 1.00 mmol), NH_4Cl (0.267 g, 5.00 mmol), Et_3N (0.70 mL, 5.0 mmol) and DMF (3.0 mL) was stirred for 5 min. HOBt (0.204 g, 1.50 mmol) and EDCI (0.286 g, 1.50 mmol) were added and the stirring was continued overnight. After this time, DMF (2 mL), NH_4Cl (0.267 g, 5.00 mmol), Et_3N (0.70 mL, 5.0 mmol), HOBt (0.204 g, 1.50 mmol) and EDCI (0.286 g, 1.50 mmol) were added again. After 6 h, the mixture was poured into sat.$NaHCO_3$ (50 mL). Extraction with EtOAc/THF (2:1, 2 x 30 mL), drying over $MgSO_4$, filtration and concentration under reduced pressure gave **4** (0.185 g, 84%) as a yellow solid. ^{1}H 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).

2,2-Dimethyl-6-pyridin-4-yl-2,3-dihydrothieno[3,2-*d*]pyrimidin-4(1*H*)-one (5). A mixture of **4** (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 **5** (130 mg, yield 74%) as an orange solid. ^{1}H 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 $C_{13}H_{14}N_3OS$ $[M+H]^+$: 260.0852. Found: 260.0834.

2-Amino-5-pyridin-4-ylthiophene-3-carboxamide (7). To a solution of **6**[13] (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 $MgSO_4$ to give 2-amino-5-pyridin-4-ylthiophene-3-carboxylic acid (277 mg, 77%) as an orange solid. [^{1}H NMR (300 MHz, 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. ^{1}H 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 $C_{10}H_{10}N_3OS$ $[M+H]^+$: 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 **5** as an orange solid. ^{1}H 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 $C_{13}H_{14}N_3OS$ $[M+H]^+$: 260. Found: 260.

6-(Pyridin-4-yl)-2-(pyrrolidin-1-ylmethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (9). To a stirred

suspension of **4** (219 mg, 1.0 mmol), Et_3N (0.152 mL, 1.1 mmol) and THF (20 mL) was added chloroacetyl chloride (0.087 mL, 1.1 mmol). After 10 min, pyrrolidine (1.65 mL, 20 mmol) was added and the mixture was stirred at 80 °C for 1 h. The mixture was concentrated in vacuo, and the residual oil was mixed with 2 M NaOH (10 mL). The mixture was stirred at 100 °C for 1 h. The mixture was concentrated in vacuo and the residue was poured in to saturated $NaHCO_3$ aq., and extracted with EtOAc/THF. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on amino silica gel (EtOAc/MeOH, 100:0 to 80:20, v/v) to afford light yellow solid. The solid was triturated with EtOAc and the precipitate was collected by filtration to afford **3** (87 mg, yield 28%) as white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.70–1.74 (4H, m), 2.55–2.59 (4H, m), 3.60 (2H, s), 7.82–7.85 (2H, m), 8.11 (1H, s), 8.68–8.70 (2H, m), 12.32 (1H, br s). HRMS: Calcd for $C_{16}H_{17}N_4OS$ $[M+H]^+$: 313.1118. Found: 313.1090.

3-Amino-5-bromothiophene-2-carboxyamide (11). A mixture of **10**[15] (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 $NaHCO_3$ aq., and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc) to afford **11** (4.1 g, 76%) as a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.56 (2H, br s), 6.70 (1H, s), 6.91 (2H, br s).

6-Bromo-2-methylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (12a). To a solution of **11** (0.200 g, 0.90 mmol) and Et_3N (0.190 mL, 1.36 mmol) in THF (4 mL) was added AcCl (0.096 mL, 1.36 mmol) at 0 °C. The mixture was stirred at room temperature for 0.5 h. The mixture was quenched with water and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. To the residue were added 2 M NaOH (0.450 mL, 0.90 mmol) and EtOH (2.0 mL), and the mixture was stirred at 70 °C for 2 h. The mixture was neutralized with 1 M HCl (1.3 mL) at 0 °C. After addition of water (2.0 mL), the precipitate was collected by filtration to give **12a** (131 mg, yield 59%) as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.36 (3H, s), 7.54 (1H, s), 12.55 (1H, br s).

6-Bromo-2-(ethoxymethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (12b). Compound **12b** was prepared in 89% yield from **11** by a procedure similar to that described for **12a** as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.14 (3H, t, J = 7.0 Hz), 3.53 (2H, q, J = 7.0 Hz), 4.30 (2H, s), 7.48 (1H, s), 12.60 (1H, br s).

***tert*-Butyl (6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)methyl(methyl)carbamate (12c).** Isobutyl chloroformate (614 mg, 4.49 mmol) was added to a solution of Et_3N (0.741 mL, 5.35 mmol) and 2-(*tert*-butoxycarbonyl(methyl)amino)acetic acid (850 mg, 4.49 mmol) in THF (5.0 mL) at 0 °C. The mixture was stirred at room temperature for 1 h. To the mixture was added **11** (473 mg, 2.14

mmol). The resulting mixture was stirred at 60 °C for 15 h. The mixture was extracted with EtOAc, washed with water, brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was dissolved in 2 M NaOH (5.35 mL, 10.70 mmol) and EtOH (10 mL), and the mixture was stirred at 70 °C for 15 h. The mixture was neutralized with 1 M HCl at 0 °C and extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residual solid was collected and washed with EtOAc–hexane to give **6f** (780 mg, yield 97%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.31–1.49 (9H, m), 2.77–2.87 (3H, m), 3.79–3.90 (2H, m), 7.60 (1H, s), 12.63 (1H, br s).

6-Bromo-2-(2-pyrrolidin-1-ylethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (12d). To a suspension of **11** (120 mg, 0.543 mmol) and Et_3N (0.083 mL, 0.60 mmol) in THF (3.0 mL) was added 3-chloropropanoyl chloride (0.057 mL, 0.60 mmol) at room temperature. After stirring at that temperature for 10 min, pyrrolidine (0.227 mL, 2.72 mmol) was added. The mixture was stirred at room temperature for 10 min. After removal of the solvent, 2 M NaOH (1.0 mL) was added. The mixture was stirred at 100 °C for 1 h. The organic materials were extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give **12d** (131 mg, yield 74%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.63–1.70 (4H, m), 2.43–2.49 (4H, m), 2.78–2.82 (4H, m), 7.57 (1H, s), 12.61 (1H, br s).

6-Bromo-2-morpholin-4-ylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (12e). A mixture of **10**[15] (200 mg, 0.847 mmol), morpholine-4-carbonitrile (0.17 mL, 1.69 mmol) and 4 M HCl in cyclopentylmethylether (3.0 mL) was stirred at 110 °C for 4 h. The reaction mixture was concentrated under reduced pressure, and saturated $NaHCO_3$ aq. was added. The precipitate was collected by filtration and washed with water and EtOAc/hexane (1:1) to give **12e** (144 mg, yield 54%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.51–3.60 (4H, m), 3.61–3.71 (4H, m), 7.29 (1H, s), 11.50 (1H, br s).

2-Methyl-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (13a). A mixture of **12a** (130 mg, 0.53 mmol), *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate (327 mg, 1.06 mmol), Na_2CO_3 (169 mg, 1.59 mmol), $PdCl_2$(dppf) (43.3 mg, 0.053 mmol), DME (3.0 mL) and water (1.5 mL) was stirred at 100 °C for 3 h under Ar. The organic materials were extracted with EtOAc, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography on amino silica gel (EtOAc/MeOH, 100:0 to 90:10, v/v) to give pale yellow solid. The solid was triturated with MeOH, and the precipitate was collected by filtration to afford **13a** (74.0 mg, yield 57%) as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.36 (3H, s), 2.44 (3H, s), 7.31 (1H, s), 7.98 (1H, br s), 12.30 (1H, br s), 12.96 (1H, br s). HRMS: Calcd for $C_{11}H_{11}N_4OS$ $[M+H]^+$: 247.0648. Found: 247.0625.

2-(Ethoxymethyl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (13b). A mixture of **12b** (140 mg, 0.48 mmol), *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole-1-carboxylate (448 mg, 1.45 mmol), Na_2CO_3 (116 mg, 1.94 mmol), $PdCl_2$(dppf) (40 mg, 0.0484 mmol), DME (3.0 mL) and water (1.5 mL) was stirred at 100 °C for 0.5 h under Ar. Then 8 M NaOH

(0.5 mL) was added, and the mixture was stirred at 100 °C for further 1 h. The organic materials were extracted with EtOAc/THF, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on amino silica gel (hexane/EtOAc, 85:15 to 0:100, EtOAc/MeOH, 100:0 to 90:10, v/v), then triturated with 2-PrOH/heptane, and the precipitate was collected by filtration to afford **13b** (59 mg, 42%) as a pale brown solid: 1H NMR (300 MHz, DMSO-d6) δ 1.17 (3H, t, J = 7.0 Hz), 2.38–2.50 (3H, m), 3.56 (2H, q, J = 7.0 Hz), 4.38 (2H, s), 7.39 (1H, s), 7.89 (0.6H, br s), 8.26 (0.4H, br s), 12.34 (1H, br s), 12.77–13.18 (1H, m); Anal. Calcd for $C_{13}H_{14}N_4O_2S$: C, 53.78; H, 4.86; N, 19.30. Found: C, 53.78; H, 4.91; N, 19.09.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-((methylamino)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (13c'). A mixture of **12c** (400 mg, 1.07 mmol), *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate (659 mg, 2.14 mmol), Cs_2CO_3 (696 mg, 2.14 mmol), $PdCl_2$(dppf) (44.0 mg, 0.054 mmol), DME (5.0 mL) and water (0.5 mL) was stirred at 80 °C overnight. The mixture was diluted with water and extracted with EtOAc. The combined extracts were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 9:1 to 1:1, v/v). The residue was dissolved in MeOH (4.0 mL) and 4 M HCl in EtOAc (1.0 mL, 4.00 mmol). The mixture was stirred at room temperature for 3 h. The resulting solid was collected and washed with EtOAc to give **13c'** (50.0 mg, yield 13%) as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.47 (3H, s), 2.69 (3H, br s), 4.23 (2H, br s), 7.31–8.30 (5H, m), 9.54 (2H, br s). Anal. Calcd for $C_{12}H_{15}N_5OSCl_2 \cdot 0.15H_2O$: C, 41.07; H, 4.39; N, 19.96. Found: C, 41.29; H, 4.58; N, 19.67.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-(2-pyrrolidin-1-ylethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (13d). Compound **13d** was prepared in 53% yield from **12d** by a procedure similar to that described for **13b** as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.51–1.78 (4H, m), 2.40–2.49 (7H, m), 2.75–2.89 (4H, m), 7.34 (1H, s), 8.02 (1H, br s), 11.93–13.36 (2H, m). Anal. Calcd for $C_{16}H_{19}N_5OS$: C, 58.34; H, 5.81; N, 21.26. Found: C, 58.32; H, 5.80; N, 21.07.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-morpholin-4-ylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (13e). Compound **13e** was prepared in 58% yield from **12e** by a procedure similar to that described for **13b** as a pale brown solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.33–2.47 (3H, m), 3.50–3.61 (4H, m), 3.61–3.72 (4H, m), 7.11 (1H, s), 7.83 (0.6H, br s), 8.19 (0.4H, br s), 11.35 (1H, br s), 12.79–13.07 (1H, m). Anal. Calcd for $C_{14}H_{15}N_5O_2S \cdot 0.5H_2O$: C, 51.52; H, 4.94; N, 21.46. Found: C, 51.33; H, 4.98; N, 21.18.

6-Bromo-2-(chloromethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (14). A mixture of **10** (15 g, 62.7 mmol), chloroacetonitrile (12 mL, 188 mmol) and 4 M HCl in cyclopentylmethylether (100 mL) was stirred at room temperature for 2 h, and stirred at 70 °C for 1 h. The mixture was concentrated under reduced pressure, and saturated $NaHCO_3$ aq. was added to the residue. The precipitate was collected by filtration and washed with water. The obtained solid was dried at 80 °C for 8 h under reduced pressure to give **14** (18 g, yield 100%) as a pale brown solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.51 (2H, s), 7.56 (1H, s), 13.00 (1H, br s).

2-[(Benzylamino)methyl]-6-bromothieno[3,2-*d*]pyrimidin-4(3*H*)-one (15a). To a suspension of 1-phenylmethanamine (0.586 mL, 5.37 mmol), K_2CO_3 (495 mg, 3.58 mmol), sodium iodide (27 mg, 0.179 mmol) and DMF (5.0 mL) was added **14** (500 mg, 1.79 mmol). The mixture was stirred at 70 °C for 1 h. The mixture was purified by column chromatography on amino silica gel (*n*-hexane/EtOAc, 85:15 to 0:100, v/v, then EtOAc/MeOH, 100:0 to 93:7, v/v) to give **15a** (234 mg, yield 37%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.67 (2H, s), 3.72 (2H, s), 7.17–7.40 (5H, m), 7.47–7.87 (2H, m). 1H of amide or amine portion was not observed independently.

6-Bromo-2-(pyrrolidin-1-ylmethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (15c). Compound **15c** was prepared in 84% yield by a procedure similar to that described for **15a** as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.65–1.77 (4H, m), 2.52–2.60 (4H, m), 3.57 (2H, s), 7.60 (1H, s), 12.24 (1H, br s).

6-Bromo-2-(piperidin-1-ylmethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (15d). Compound **15d** was prepared in 83% yield by a procedure similar to that described for **15a** as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.31–1.42 (2H, m), 1.45–1.56 (4H, m), 2.35–2.46 (4H, m), 3.40 (2H, s), 7.61 (1H, s), 12.20 (1H, br s).

6-Benzyl-2-bromo-6,7-dihydroimidazo[1,5-*a*]thieno[3,2-*d*]pyrimidin-9(5*H*)-one (17). A mixture of **15a** (185 mg, 0.528 mmol), 37% aqueous formaldehyde (1 mL) and THF (2 mL) was stirred at room temperature for 1 h. The precipitate was collected by filtration, and washed with water and EtOAc to give **17** (104 mg, yield 54%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.87 (2H, s), 4.04 (2H, s), 4.84 (2H, s), 7.24–7.45 (5H, m), 7.63 (1H, s).

6-Benzyl-2-(3-methyl-1*H*-pyrazol-4-yl)-6,7-dihydroimidazo[1,5-*a*]thieno[3,2-*d*]pyrimidin-9(5*H*)-one trifluoroacetic acid (16a). A mixture of **17** (100 mg, 0.276 mmol), *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate (255 mg, 0.828 mmol), $PdCl_2$(dppf) (23 mg, 0.028 mmol), Na_2CO_3 (66 mg, 1.10 mmol), DME (3.0 mL) and water (1.5 mL) was stirred at 100 °C for 1.5 h. The mixture was diluted with water and extracted with EtOAc. The combined extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 85:15 to 0:100, v/v) to give a crude product of **18** as a pale yellow solid (89 mg). A mixture of the crude product of **18** (80 mg), MeOH (2 mL) and TFA (2 mL) was stirred with heating at 70 °C for 5 h. The reaction mixture was concentrated under reduced pressure, and the residue was triturated with MeOH–EtOAc. The precipitate was collected by filtration to afford **16a** (43 mg, yield 44%) as a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.46 (3H, br s), 4.20 (2H, s), 4.31 (2H, s), 7.40 (1H, s), 7.42–7.58 (5H, m), 8.05 (1H, br s), 9.15–10.22 (2H, m), 13.04 (1H, br s). Anal. Calcd for $C_{20}H_{18}N_5O_3SF_3 \cdot 0.25H_2O$: C, 51.11; H, 3.97; N, 14.90. Found: C, 51.10; H, 3.81; N, 14.89.

2-[(Dimethylamino)methyl]-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one

dihydrochloride (16b'). A mixture of **14** (140 mg, 0.50 mmol), dimethylamine hydrochloride (200 mg, 1.54 mmol) and DMF (3 mL) was stirred at 100 °C for 1 h in a sealed tube by using microwave reactor. The mixture was concentrated under reduced pressure. The residue was extracted with EtOAc–THF. The combined extracts were washed with saturated $NaHCO_3$ aq., dried over $MgSO_4$, and concentrated in vacuo to give crude product of **15b**. A mixture of the crude product of **15b**, *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate (380 mg, 1.23 mmol), Na_2CO_3 (265 mg, 2.50 mmol), $PdCl_2$(dppf) (41 mg, 0.050 mmol), DME (4.0 mL) and water (2.0 mL) was stirred at reflux for 1 h under Ar. The mixture was diluted with brine and extracted with EtOAc–THF. The combined extracts were dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography on amino silica gel (EtOAc/MeOH, 100:0 to 80:20, v/v). The residue was dissolved with MeOH (3 mL) and 10% HCl in MeOH (1 mL). The mixture was concentrated under reduced pressure. The residue was triturated with EtOAc–MeOH and the precipitate was collected by filtration to afford **16b'** (24 mg, yield 13%) as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.47 (3H, s), 2.95 (6H, s), 4.40 (2H, s), 7.40 (1H, s), 8.10 (1H, s), 10.40 (1H, br s), 12.82 (1H, br s). HRMS: Calcd for $C_{13}H_{16}N_5OS$ $[M–2HCl+H]^+$: 290.1070. Found: 290.1058.

6-(3-methyl-1*H*-pyrazol-4-yl)-2-(pyrrolidin-1-ylmethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (16c). A mixture of **15c** (100 mg, 0.318 mmol), *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate (294 mg, 0.954 mmol), Na_2CO_3 (95 mg, 1.59 mmol), $PdCl_2$(dppf) (26 mg, 0.032 mmol), DME (3.0 mL) and water (1.5 mL) was stirred for 3 h at 100 °C under Ar. Then 8 M NaOH (1.0 mL) was added, and the mixture was stirred further 30 min at same temperature. The mixture was extracted with EtOAc–THF. The combined extracts were dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography on amino silica gel (*n*-hexane/EtOAc, 80:20 to 0:100, v/v, then EtOAc/MeOH, 100:0 to 90:10, v/v). The residue was triturated with EtOAc–MeOH, and the precipitate was collected by filtration to afford **16c** (44 mg, yield 44%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.65–1.78 (4H, m), 2.45 (3H, s), 2.53–2.59 (4H, m), 3.57 (2H, s), 7.37 (1H, s), 8.00 (1H, br s), 11.84–13.16 (2H, m). Anal. Calcd for $C_{15}H_{17}N_5OS$: C, 57.12; H, 5.43; N, 22.21. Found: C, 56.96; H, 5.24; N, 22.04.

6-(3-Methyl-1*H*-pyrazol-4-yl)-2-(piperidin-1-ylmethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (16d). A mixture of **15d** (175 mg, 0.533 mmol), *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole-1-carboxylate (361 mg, 1.17 mmol), Na_2CO_3 (128 mg, 2.13 mmol), $PdCl_2$(dppf) (44 mg, 0.053 mmol), DME (4.0 mL) and water (2.0 mL) was stirred at 100 °C for 1 h under Ar. The mixture was extracted with EtOAc–THF. The combined extracts were dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography on amino silica gel (EtOAc/MeOH, 100:0 to 90:10, v/v). The residue was triturated with EtOAc–MeOH–*n*-hexane, and the precipitate was collected by filtration to afford **16d** (56 mg, yield 32%) as a pale brown solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.30–1.44 (2H, m), 1.44–1.60 (4H, m), 2.40–2.48 (7H, m), 3.42 (2H, s), 7.38 (1H, s), 8.04 (1H, br s), 12.61 (1H, br s), 12.61 (1H, br s). Anal. Calcd for $C_{16}H_{19}N_5OS·0.2H_2O$: C, 57.71; H, 5.87; N, 21.03. Found: C, 58.02; H, 5.89; N, 20.67.

6-(1*H*-Pyrazol-4-yl)-2-(pyrrolidin-1-ylmethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (16e). Compound **16e** was prepared in 50% yield from **15c** by a procedure similar to that described for **16d** as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.69–1.73 (4H, m), 2.54–2.58 (4H, m), 3.57 (2H, s), 7.48 (1H, s), 8.16 (2H, br s), 12.90 (1H, br s), 1H of pyrazole or amide portion was not observed independently. Anal. Calcd for $C_{14}H_{15}FN_5OS$: C, 55.80; H, 5.02; N, 23.24. Found: C, 55.68; H, 5.13; N, 22.95.

6-(3-Ethyl-1*H*-pyrazol-4-yl)-2-(pyrrolidin-1-ylmethyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride (16f'). Compound **16f'** was prepared in 75% yield from **15c** by a procedure similar to that described for **16d** and followed removal of dimethylsulfamoyl group and salt formation with HCl in Et_2O–MeOH as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.26 (3H, t, J = 7.5Hz), 1.82–2.08 (4H, m), 2.84–2.92 (2H, m), 3.17–3.25 (2H, m), 3.64–3.77 (2H, m), 4.51 (2H, s), 7.38 (1H, s), 8.09 (1H, s), 10.61 (1H, br s), 12.80 (1H, br s). HRMS: Calcd for $C_{16}H_{20}N_5OS$ $[M–2HCl+H]^+$: 330.1383. Found: 330.1355.

***tert*-Butyl (2*S*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)azetidine-1-carboxylate (17a).** 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 **11** (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 **17a** (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 (17b).** 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 **11** (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 **17b** (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 (17c).** Compound **2c** (24.8 g) was prepared from **11** (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 **17a** 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 (17d) and *tert*-butyl (2*R*)-2-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)piperidine-1-carboxylate (17e). 17c** (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 **17d** (13.1 g, 66%, 99.9% ee) and **17e** (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 (17f).** Compound **17f** (474 mg) was prepared from **11** (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 **17a** 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 (17g). 17f** (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 **17g** (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)

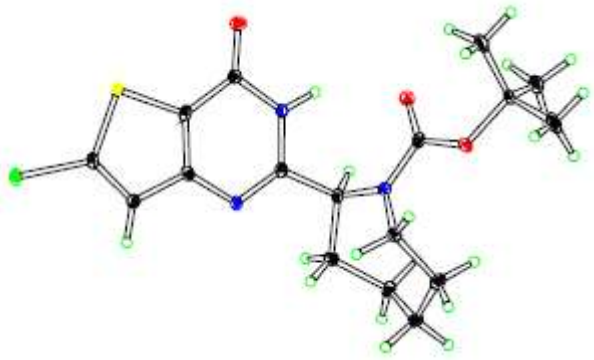

Figure 17. ORTEP of **17g** (CCDC 1918343). Thermal ellipsoids are drawn at 30% probability.

X-ray structure analysis of 17g

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

Crystal data for **17g**: $C_{17}H_{22}BrN_3O_3S$, *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[23] = –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[24] and was refined using full-matrix least-squares on F^2 with SHELXL-97.[25] 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.

2-((2*S*)-Azetidin-2-yl)-6-(3-methyl-1*H*-pyrazol-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (18a). A mixture of **17a** (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 **18a** (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 (18b). Compound **17b** (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 (18d). Compound **18d** (1.77 g) was prepared from **17d** (3.25 g, 7.84 mmol) in 58% yield by a procedure similar to that described for **18a** 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 (18d'). To a suspension of **18d** (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 **18d'** (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 (18e). Compound **18e** (60.8 mg) was prepared from **17e** (120 mg, 0.290 mmol) in 54% yield by a procedure similar to that described for **18a** 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 (18g). Compound **18g** (92.7 mg) was prepared from **17g** (310 mg, 0.724 mmol) in 39% yield by a procedure similar to that described for **18a** 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.

***tert*-Butyl 1-(6-bromo-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)-2-azabicyclo[2.1.1]hexane-2-carboxylate (17h).** Compound **17h** (180 mg) was prepared in 29% yield from **11** (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 **17a**. ^{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 (17i). To a mixture of **11** (921 mg, 4.17 mmol) and benzyl 1-(chlorocarbonyl)-7-azabiciclo[2.2.1]heptanes-7-carboxylate **30** (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 **17i** (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-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 (18h). Compound **18h** (98 mg) was prepared from **17h** (150 mg, 0.364 mmol) in 70% yield by a procedure similar to that described for **18a** 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 (18i). A mixture of **17i** (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 **18i** (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.

1-(1-(4-Methoxybenzyl)-5-methyl-1*H*-pyrazol-4-yl)ethanone (20). 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 **20** (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 (21). 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 **20** (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 **21** (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 (22). 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, **21** (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 **22** (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 (23). A mixture of quinuclidine-2-carboxylic acid **37** (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), **22** (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 **23** (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 (24).** A mixture of **23** (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 **24** (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 (25). To a suspension of **24** (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 **25** (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

(26a). A mixture of **25** (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 **26a** (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 (26b) 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 (26c). 26a** (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 = **26c**, tR2 = **26b**]. The obtained crude **26b** (9.53 g) was recrystallized from EtOH–water (780 mL, 100/1, v/v) to give **26b** (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 **26c** (9.40 g) was recrystallized from EtOH–water (820 mL, 100:1, v/v) to give **26c** (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 $C_{17}H_{19}N_5OS·0.5H_2O$: 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 (26b')

A mixture of **26b** (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 **26b'** (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 **26b''** as described below (Figure 5, CSD ID: 1918344).

X-ray structure analysis of 26b''

Preparation of single crystal 26b'' A solution of **26b'** (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 **26b''** (Figure 14): $C_{17}H_{20}N_5OS^{+}\cdot$ 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[23] = 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[24] and was refined using full-matrix least-squares on F^2 with SHELXL-97.[25] 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.

(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 (27). To a stirred mixture of **18d** (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 **27** (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 18). 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 **27**.

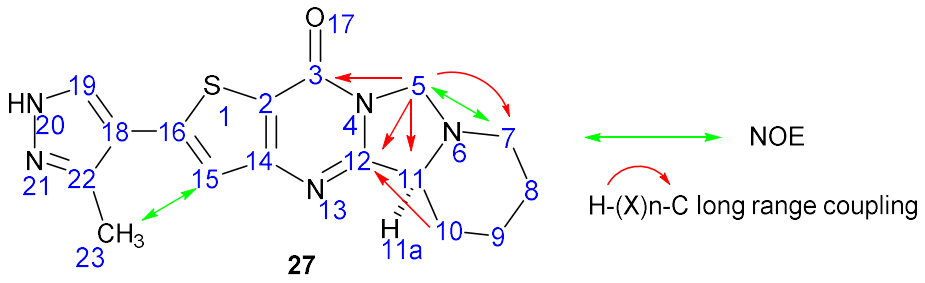

Figure 18. Long range coupling and NOE observed in compound **27**.

7-[(Benzyloxy)carbonyl]-7-azabiciclo[2.2.1]heptane-1-carboxlic acid (29). A mixture of methyl 7-benzoyl-7-azabiciclo[2.2.1]heptane-1-carboxylate **28**[19] (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 **29** (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 (30). To a mixture of **29** (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 (32).** 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 **32** (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 (33).** To a solution of **32** (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 **33** (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 (34).** To a mixture of **33** (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 **34** (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 (35).** To a solution of **34** (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 **35** (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 (36). To a solution of **35** (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 **36** (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 (37). A mixture of **36** (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 **37** (45.3 g, ca.135 mmol, 65%) as a white solid. Content rate of **37** 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).

Docking study

Docking model of **26b** 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 **26b** was determined by scoring with the MM/PBSA (Molecular Mechanics/Poisson Boltzmann Surface Area) approach.

生物活性に関する実験

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 **18d** 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 **18d'**, **26b**, and **18i** (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.

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