# Synthesis and evaluation of pyrido-thieno-pyrimidines as potent and selective Cdc7 kinase inhibitors ☆

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ABSTRACT

Cdc7 kinase plays a critical role in the regulation of DNA replication in eukaryotic cells and has been proposed as a target for cancer therapy. We have identified a class of Cdc7/Dbf4 inhibitors with a pyrido-thieno-pyrimidine core structure. Synthesis of a focused pyrido-thieno-pyrimidine library yielded potent and selective Cdc7 inhibitors with antiproliferative activity against cancer cells in vitro. Their synthesis and SAR data are presented herein.

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Cdc7 is a conserved serine/threonine kinase that plays an important role in initiation of DNA replication in eukaryotic cells.[1–5] It is expressed continuously during the cell cycle but activated during late-G1 and S phase by binding with its subunit Dbf4. Cell cycle-dependent expression of Dbf4 allows for a precise control of Cdc7 activity. Among the most important known substrates of Cdc7/Dbf4 are minichromosome maintenance proteins (MCM2-7) representing essential components of the origin initiation complex (ORC). Phosphorylation of one or more MCM proteins is thought to be a critical step in the initiation of DNA replication in eukaryotes.[6–8] The role of Cdc7 in this process has been supported by the observation that knockdown of its expression causes an abortive S phase, followed by cell death.[9] These results and the fact that Cdc7 has been found overexpressed in human cancer cell lines and tumor tissue samples[10] have suggested that Cdc7 could be a target for cancer therapy. Here, we report the synthesis and evaluation of pyrido-thieno-pyrimidines (Fig. 1) as potent and selective Cdc7 inhibitors.[11]

A high-throughput screen of Roche chemical library for inhibitors of Cdc7/Dbf4 identified 6h (Fig. 1, $R^1$ = CH$_3$, $R^2$ = H; $R^3$ = CH$_3$, $R^4$ = H, and $R^5$ = H) as a lead compound with IC$_{50}$ of 400 nM against Cdc7/Dbf4 and over 10-fold selectivity over the cyclin-dependent kinases (CDK1, CDK2, and CDK4), the closest human kinases in

structure. A focused library was synthesized using microwave-assisted chemistry outlined in Scheme 1.

Treatment of 2-chloro-nicotinonitrile 1 with sodium sulfide generated 2-mercapto-nicotinonitrile 2, which was reacted with 2-chloro-acetamide to give 3-amino-thieno-[2,3-b]-pyridine-2-carboxylic acid amide 3. Compound 4 was made by reacting 3 with trimethyl orthoformate with the catalysis of acid. Chlorination of 4 with phosphorus oxychloride followed by substitution of chlorine with amine NHR$^4$R$^5$ gave the desired product 6 in Table 2.[12] Micro-wave radiation has been used in all steps of the synthesis except the chlorination. All reactions were clean and no chromatography was needed for purifying intermediates.

Table 1 lists the effect of bases on the formation of intermediate 3. The IR spectrum of compound B revealed a typical CN absorption at 2220 cm$^{-1}$, which is consistent with the open-chain structure.

To compare the results of microwave-assisted reactions with the conventional synthetic protocol, two conventional reactions were run using the same stoichiometries as reactions I and II (Table 1) and refluxing with regular oil-bath heating. The microwave-as-
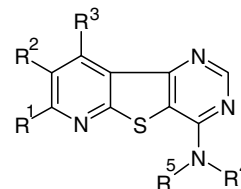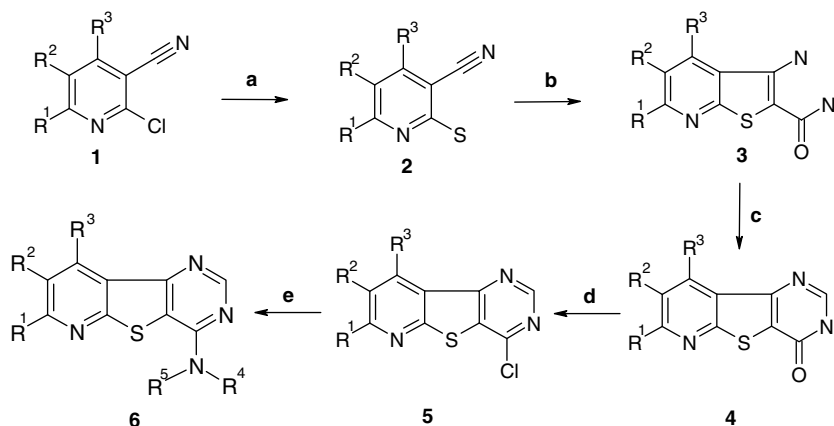


**Figure 1.** General structure of pyrido-thieno-pyrimidines.

**Scheme 1.** Reagents and conditions: (a) Na$_2$S, *t*-Butanol, microwave, 150 °C, 20 min; (b) chloroacetamide, K$_2$CO$_3$/EtOH, microwave, 120 °C, 10 min; (c) HC(OCH$_3$)$_3$, CH$_3$COOH, microwave, 150 °C, 10 min; (d) POCl$_3$, refluxing; (e) HNR$^4$R$^5$ microwave, 120 °C, 20 min (when R$^4$ and R$^5$ are H, the reaction was run in a sealed tube at 80 °C overnight).

**Table 1**
Effect of base to the formation of thieno ring
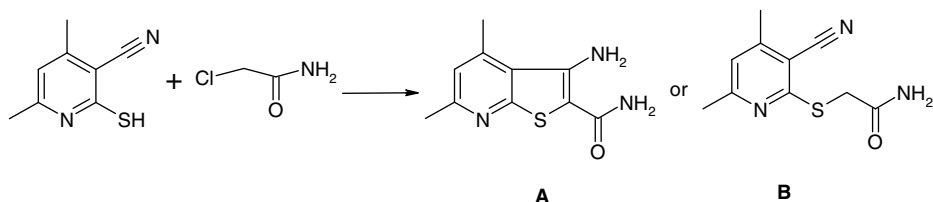


| Reaction No. | Base used | Product type: A or B | Reaction conditions (microwave) | Conversion rate[a] (%) |
|---|---|---|---|---|
| I | K$_2$CO$_3$/EtOH | A | 120 °C/10 min | 100 |
| II | NaOMe/MeOH | A | 120 °C/10 min | 100 |
| III | NaOAc/EtOH | B | 120 °C/10 min | 100 |
| IV | NaOH/MeOH | A | 120 °C/10 min | 100 |

[a] Determined by the LC–MS results of reaction mixtures.

sisted protocol offered one clear advantage, much shorter reaction time. While reactions I and II were completed within 10 min with micro-wave heating, their counterparts took more than 3 h to complete under regular heating conditions.[13]

To carry the reaction from **3** to **4**, both *p*-toluenesulfonic acid and acetic acid were used as acid catalysts, respectively (Scheme 2). While acetic acid produced the desired product, the *p*-toluene-

sulfonic acid-catalyzed reaction yielded a dark-colored solution with unclear LC–MS profile.

Synthesis of 2-methylated analog can be achieved using the same method. Cooking **3h** with neat acetic anhydride yielded clean result (Scheme 3).
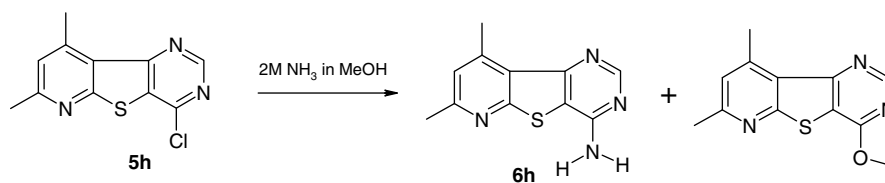
Although the crude product of chloride **5** (Scheme 1) was used for the next reaction without chromatography, the quick wash-up
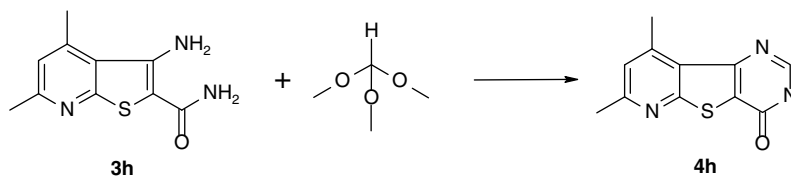


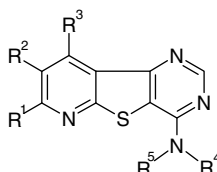**Scheme 2.** Building pyrimidinone ring by acid catalysis.
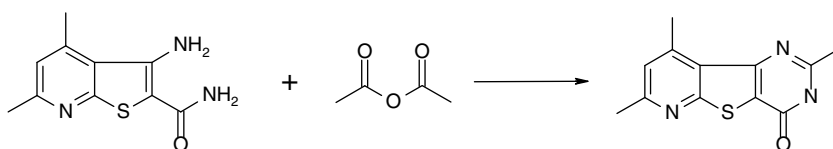


**Scheme 3.** Synthesize 2-methyl pyrido-thieno-pyrimidinone analog.

**Scheme 4.** Methanol as solvent caused the formation of by-product.

**Table 2**
Inhibition of Cdc7/Dbf4 activity in vitro



| Compound | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | Cdc7 ($IC_{50}$) (nM) | Ki (nM) |
|---|---|---|---|---|---|---|---|
| 6a | Me | $-(CH_2)_4-$ | | H | H | 5 | 0.5 |
| 6b | Me | Et | Me | H | H | 10 | 1 |
| 6c | $-(CH_2)_4-$ | | Me | H | H | 20 | 2 |
| 6d | Me | Allyl | Me | H | H | 20 | 2 |
| 6e | Me | $-(CH_2)_3-$ | | H | H | 30 | 3 |
| 6f | Me | Me | Me | H | H | 40 | 4 |
| 6g | $-(CH_2)_3-$ | | Me | H | H | 50 | 5 |
| 6h | Me | H | Me | H | H | 400 | 40 |
| 6i | Me | Benzyl | Me | H | H | 2000 | 200 |
| 6j | Cyclopropyl | H | $CF_3$ | H | H | 4000 | 400 |
| 6k | H | Ph | H | H | H | 9000 | 900 |
| 6l | Me | Et-O-C=O | H | H | H | 10,000 | 1000 |
| 6m | H | H | H | H | H | 12,000 | 1200 |
| 6n | Me | H | Me | H | Me | >30,000 | >3000 |
| 6o | Me | H | Me | Me | Me | >30,000 | >3000 |
| 6p | Me | H | Me | H | Ph | >30,000 | >3000 |

of residual phosphorus oxychloride and its by-product with cold aqueous sodium bicarbonate and water was critical for the success of reaction e.

For the transformation of compound **5** to **6**, the lower alcohols such as ethanol or isopropanol were used as solvents. It was found that methanol competed with ammonia for the replacement of chlorine, with the ratio of product to by-product about 1:1 for the following reaction (Scheme 4).

The test compounds were evaluated for inhibition of Cdc7/Dbf4 enzyme activity using a previously published Homogeneous Time-Resolved Fluorescence (HTRF) assay.[14] Table 2 shows the activity

and emerging structure–activity (SAR) relationship for compound **6**.

The following trends have emerged from the data in Table 2: using **6h** as a reference compound, adding small aliphatic groups at $R^2$ position dramatically increased the potency (compounds **6a**–**g**). Bigger groups such as benzyl at $R^2$ position decreased activity approximately 5-fold (**6i**). Unsubstituted pyrido showed a substantially lower activity (**6m**), suggesting that there might be a small hydrophobic cavity within Cdc7/Dbf4 complex interacting with this part of the molecule. Any substitution on the $R^4$ and $R^5$ positions led to loss of activity, which indicates a possible role of

**Table 3**
Compound **6d** was tested for activity against a panel of kinases using the IMAP assay platform

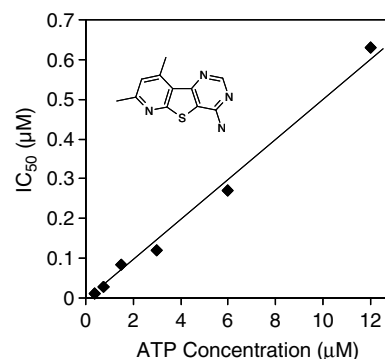| Kinase | $K_i$ (nM) |
|---|---|
| Cdc7/Dbf4 | 2 |
| CDK1/Cyclin B | >5000 |
| CDK2/Cyclin E | >5000 |
| CDK4/Cyclin D | >5000 |
| AKT | >5000 |
| PKA | 1100 |
| PKC-*a* | >5000 |
| PKC-*d* | >5000 |
| ERK2 | >5000 |
| SGK | >5000 |
| Fyn | >5000 |
| EphB$_3$ | >5000 |



**Figure 2.** Pyrido-thieno-pyrimidines are ATP-competitive inhibitors. The $IC_{50}$ of compound **6h** was determined as a function of the ATP concentration.
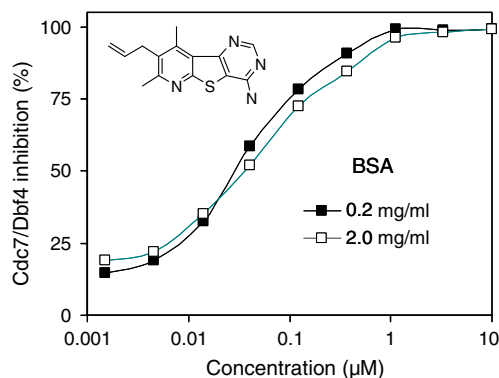
**Figure 3.** IC$_{50}$ of compound **6d** was determined in the presence of 0.2 and 2 mg/mL of bovine serum albumin.
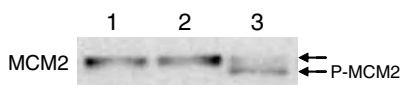


**Figure 4.** Inhibition of MCM2 phosphorylation in HCT 116 cancer cells. (1) G1 cells (control); (2) G1 cells + 20 μM compound **6d**; (3). G1 cells + DMSO (18 h, control).

the nitrogen proton as a H-bond donor. Interestingly, the intermediates (**3a–p**) showed similar inhibitory activity as the final products in the 1- to 20-μM range.

Compounds **6h** and **6d** were evaluated further. The ATP-dependence of **6h** activity indicated that these series of Cdc7 inhibitors are ATP-competitive (Fig. 2).

Compound **6d** demonstrated >500-fold selectivity[15] in vitro against a panel of 11 diverse human kinases including some of the cyclin-dependent kinases, CDK1, CDK2, and CDK4 (Table 3). This compound showed practically identical activity in the presence of 0.2 and 2.0 mg/mL bovine serum albumin suggesting that non-specific protein binding may not be a problem for this class of molecules (Fig. 3).

MCM2 is one of the important cellular targets of Cdc7/Dbf4. Therefore, compound **6d** was tested for inhibition of MCM2 phosphorylation in proliferating HT116 colon cancer cells (Fig. 4). Since Cdc7/Dbf4 complex phosphorylates MCM2 during S phase, cells were pre-synchronized in G1 phase before entry into S phase by mitotic shake-off.[16] They were then incubated in the presence of 20 μM compound **6d** for 18 h needed for entry into and transition through S phase. Western blot analysis of cell lysates revealed a dramatic decrease in the faster migrating form of the protein, previously shown to be hyperphosphotylated MCM2.[16,17] This was the

predominant form of MCM2 in the control cell that transitioned through S phase in the absence of Cdc7 inhibitor (Fig. 4). This indicated that compound **6d** penetrated the cells and inhibited intracellular Cdc7 activity. The MTT[18] assay for cell viability detected a dose-dependent antiproliferative activity in three human colon cancer cell lines (HCT116, RKO, and SW480) in vitro with IC$_{50}$ in the 6- to 10-μM range (Fig. 5). The relatively low $K_m$ for ATP of the Cdc7/Dbf4 complex (~500 nM)[14] is most likely the reason for the modest cellular potency of **6d** that has to compete with millimolar intracellular ATP concentrations.

During the preparation of this letter, two different classes of ATP-competitive Cdc7 inhibitors, indazoles[19] and pyrrolopyridinones,[20] have been reported. The most potent compounds in these series have shown single nanomolar $K_i$ values close to the $K_i$ of the most potent members of the pyrido-thieno-pyrimidines described here.

Pyrido-thieno-pyrimidine scaffold has relatively low molecular weight (<300) and good permeability (Caco-2 > 200 × 10$^{-7}$ cm/s). However, it showed limited solubility (<30 μg/mL at pH 6.5) and relatively high clearance in human liver microsomes (HLM) preparations in vitro. The poor solubility may also contribute to the moderate cellular activity. Interestingly, the intermediate **3h** was much more stable than its final product **6h** in HLM and the solubility of **3h** was about 2-fold higher than **6h**, suggesting that the pyrimidine part of pyrido-thieno-pyrimidine is prone to metabolism and the stacking effect of the highly conjugated system in pyrido-thieno-pyrimidines is detrimental to the solubility.

In conclusion, we have developed an efficient method for the synthesis of pyrido-thieno-pyrimidines. These analogs are potent and selective inhibitors of Cdc7/Dbf4 kinase in vitro. The representative compounds from these series can penetrate cultured cancer cells, inhibit MCM2 phosphorylation and show a moderate antiproliferative effect in cancer cells. Further improvement of potency and optimization for solubility and metabolic stability is needed to address the potential of Cdc7 as a cancer target in vivo.

## References and notes

1. Jiang, W.; McDoonald, D.; Hope, T. J.; Hunter, T. *EMBO J.* **1999**, *18*, 5703.
2. Kumagai, H.; Sato, N.; Yamada, M.; Mahony, D.; Seghezzi, W.; Lees, E.; Arai, K.; Masai, H. *Mol. Cell. Biol* **1999**, *280*, 5083.
3. Sclafani, R. A. *J. Cell Sci.* **2000**, *113*, 2111.
4. Lei, M.; Tye, B. K. *J. Cell Sci.* **2001**, *114*, 1447.
5. Montagnoli, A.; Bosotti, R.; Villa, F.; Rialland, M.; Brotherton, D.; Mercurio, C.; Berthelsen, J.; Santocanale, C. *EMBO J.* **2002**, *2112*, 3171.
6. Yoshizawa-Sugata, N.; Ishii, A.; Taniyama, C.; Matsui, E.; Arai, K.; Masai, H. *J. Biol. Chem.* **2005**, *19*, 13062.
7. Yanow, S. K.; Gold, D. A.; Yoo, H. Y.; Dunphy, W. G. *J. Biol. Chem.* **2003**, *278*, 41083.
8. Masai, H.; You, Z.; Arai, K. *Life* **2005**, 323.
9. Montagnoli, A.; Tenca, P.; Sola, F.; Carpani, D.; Brotherton, D.; Albanese, C.; Santocanale, C. *Cancer Res.* **2004**, *64*, 7110.
10. Hess, G.; Drong, R. F.; Weiland, K. L.; Slightom, J. L.; Sclafani, R. A.; Hollingsworth, R. E. *Gene* **1998**, *211*, 133.
11. The abstract of this letter has been presented at the following meeting: Zhao, C.; Chen, L.; Xu, Q.; Tovar, C.; Vassilev, L. T. *Abstracts of Papers*, 229th National Meeting of the American Chemical Society, San Diego, CA, USA, March 13–17, 2005; MEDI-141.
12. Spectroscopic data for 7,9-dimethyl-pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-4-ylamine (**6h**): white amorphous powder. HR-MS-EI (+) *m/z*: observed mass: 230.0629, calculated mass *m/z*: 230.0626 for C$_{11}$H$_{10}$N$_4$S. $^1$H NMR (300 MHz, in Me$_2$SO) δ 2.60 (s, 3H), 2.90 (s, 3H), 7.25 (s, 1H), 7.54 (s, 2H), 8.58 (s, 1H).
13. Youssefyeh, R. D.; Brown, R. E.; Wilson, J.; Shah, U.; Jones, H.; Love, B.; Khandwala, A.; Leibowitz, M. J.; Sonnino-Goldman, P. *J. Med. Chem.* **1984**, *27*, 1639.
14. Xu, K.; Stern, A. S.; Levin, W.; Chua, A.; Vassilev, L. T. *J. Biochem. Mol. Biol.* **2003**, *36*, 421.
15. Selectivity was measured against a panel of recombinant human kinases at 5 μM ATP concentration using the IMAP technology as described in Gaudet, E.
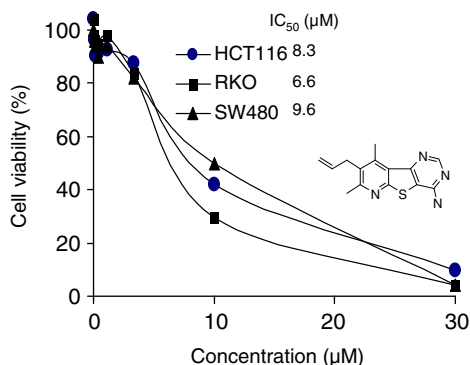
**Figure 5.** Antiproliferative activity of compound **6d** in HCT 116, RKO, and SW480 cancer cell lines.

A.; Huang, G.; Zhang, Y.; Huang, W.; Mark, D.; Sportsman, J. R. *J. Biomol. Screen.* **2003**, *8*, 164.

16. Procedure for evaluating compound **6d** for the inhibition of MCM2 phosphorylation in HCT 116 cell lines: HCT116 cells were treated with 100 nM of nocodazole for 16 h and mitotic cells were separated by shake-off, washed, and allowed to enter G1 phase. They were then treated with 20 μM of compound **6d** for 18 h and MCM2 protein was analyzed in cell lysates by Western blotting as previously described: Vassilev, L. T.; Tovar, C.; Chen, S.; Knezevic, D.; Zhao, X.; Sun, H.; Heimbrook, D. C.; Chen, L. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 10660.

17. Todorov, I. T.; Ataran, A.; Kearsey, S. E. *Cell Biol.* **1995**, *1296*, 1433.

18. Procedure for evaluating compound **6d** in MTT assay: exponentially growing cancer cells were incubated with compound **6d** for 5 days and cell mass/ viability was measured by the MTT assay as previously described: Vassilev, L. T.; Kazmer, S.; Marks, I. M.; Pezzoni, G.; Sala, F.; Mischke, S. G.; Foley, L.; Berthel, S. J. *Anti-cancer Drug Des.* **2001**, *16*, 7.

19. a Shafer, C.; Walter, A.; Lindvall, M. K.; Gesner, T.; Doyle, L. Patent WO2007/ 124288, A1.; (b) Shafer, C.; Lindvall, M. K.; Bellamacina, C.; Gesner, T.; Yabannavar, A.; Jia, W.; Lin, S.; Walter, A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4482.

20. Vanotti, E.; Amici, R.; Bargiotti, A.; Berthelsen, J.; Bosotti, R.; Ciavolella, A.; Cirla, A.; Cristiani, C.; D'Alessio, R.; Forte, B.; Isacchi, A.; Martina, K.; Menichincheri, M.; Molinari, A.; Montagnoli, A.; Orsini, P.; Pillan, A.; Roletto, F.; Scolaro, A.; Tibolla, M.; Valsasina, B.; Varasi, M.; Volpi, D.; Santocanale, C. *J. Med. Chem.* **2008**, *51*(3), 487.