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Discovery of novel thieno[2,3-*d*]pyrimidin-4-yl hydrazone-based inhibitors of Cyclin D1-CDK4: Synthesis, biological evaluation and structure–activity relationships. Part 2

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ABSTRACT

The design, synthesis and evaluation of novel thieno[2,3-*d*]pyrimidin-4-yl hydrazone analogues as cyclin-dependent kinase 4 (CDK4) inhibitor are described. Focusing on the optimization of the heteroaryl moiety at the hydrazone with substituted phenyl groups, 4-[(methylamino)methyl]benzaldehyde (**22**) and 5-iso-indolinecarbaldehyde (**24**) (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone derivatives have been identified. In this paper, the potency, selectivity profile and structure–activity relationships of our synthetic compounds are discussed.

1. Introduction

Cyclin-dependent kinases (CDKs) are a family of serine/threonine kinases which play a key role in the growth, development, proliferation, and death of eukaryotic cells.[1–3] CDKs are responsible for coordinating the events by which cells progress through the cell cycle and they become active at specific phases: G1, S, G2 and M.[4–6] In the G1 phase, the cyclin D1 increases to trigger the activation of CDK4/6 in early G1 and then cyclin E/CDK2 activates. Notably, CDK4 restricts the passage only through the G1 phase, whereas CDK2 controls the passage through not only the G1 but also the S phase with cyclin A.[7–9] CDK4/6 activities are negatively regulated by the tumor suppressor p16, a cyclin-dependent kinase inhibitor of the INK4 family. But many tumors have been reported to contain mutations, deletions or silencing of the p16 or the retinoblastoma tumor suppressor protein (Rb) gene.[10–12] Moreover, mutations in CDKs and abnormal expressions of their regulators have been found in a large percentage of melanoma patients.[13] In addition, Malumbres et al. have reported that knockdown of CDK4 in mammary tumor cells prevents tumor formation.[14] In contrast, McCormick and Tetsu have found that cancer cells proliferate despite CDK2 inhibition.[15] These results suggest that selective inhibition of CDK4 may restore normal cell activity and could be a more valuable approach to cancer therapy than CDK2, especially for those who have lost the INK4 family, such as p16.

Recently, several research groups have identified CDK inhibitors.[16–22] Above all, Flavopiridol,[19] UCN-01,[20] and R-roscovitine[21] have been developed and have advanced to phase II/III clinical trials as multi-CDK inhibitors. As well, PD0332991[22] is known to be a selective CDK4 inhibitor under active development.

In our previous paper, to improve the selectivity for CDK4 of HTS hit compound (**1**), we synthesized some thieno[2,3-*d*]pyrimidin-4-yl hydrazone analogues which modified both the C-2 and C-4 positions. As a result, we discovered the potent CDK4 (IC_{50} = 0.056 μg/mL) inhibitor (**2**) (Fig. 1), which had good selectivity for CDK2 (IC_{50} = 1.40 μg/mL) with moderate in vitro cytotoxic activity and limited aqueous solubility (44 μg/mL in pH 6.8 buffer solution).[23] To further explore the potential of the thieno[2,3-*d*]pyrimidine compounds, we introduced various substituted phenyl rings at the end of hydrazone. Herein, we report the synthesis and biological activity of several thieno[2,3-*d*]pyrimidin-4-yl hydrazone derivatives. In addition, we also describe a docking study of compound **2** with our homology model of CDK4.

2. Results and discussion

2.1. Molecular modeling

It has already been reported that many small molecule CDKs inhibitors are competitive with ATP for binding.[16] With this background, our early docking study with HTS hit compound (**1**) supported the hypothesis that the thieno[2,3-*d*]pyrimidin-4-yl hydrazone analogues are also competitive with ATP and probably

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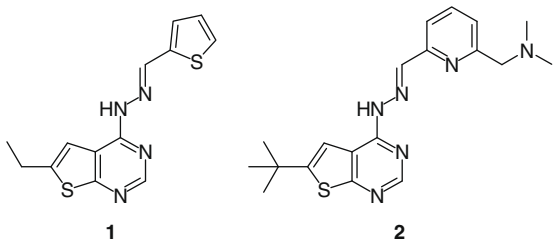


Figure 1. Lead compound **2** from in-house high-throughput screening hit compound **1**.

bind in the enzyme's ATP binding pocket. We first carried out an in silico docking study of **2** with the ATP binding pocket of our CDK4 homology model because no X-ray crystallographic information was available regarding the structure of CDK4 with active conformation. We have made these models using the known crystal structures of CDK2[24] and CDK6[25] as templates.

The analysis with our model suggested several features. The molecule (**2**) was buried toward the gatekeeper residue Phe93 in the ATP binding pocket. At the hinge region, NH of hydrazone and the nitrogen atom in the pyrimidine ring formed two backbone hydrogen bonds with Val 96 and CH–O interaction of the pyrimidine ring formed with the backbone carbonyl of Glu94. The other key hydrogen bond was formed between the ionizable nitrogen atom of the 6-(dimethylamino)methyl group and the side chain carbonyl of Asp99 at the solvent accessible region (Fig. 2). This interaction could explain the increase in potency of **2** relative to our reported pyridine analogues,[23] which lacked the substituents capable of forming this hydrogen bond. Moreover, it appeared that the nitrogen atom in the pyridine ring rarely contributes to increase CDK4 inhibitory activity in this case. To verify this assumption, we introduced various substituted phenyl rings at the end of hydrazone.

Recently, two research groups[26] have determined the crystal structure of CDK4/cyclin D. However, their structures are non-active conformations and the space of their ATP binding sites appeared to be smaller than that of our CDK4 homology model to insert molecule (**2**).

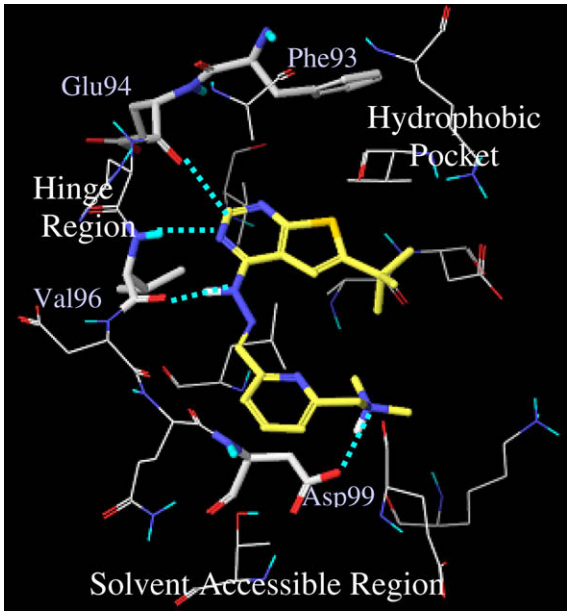


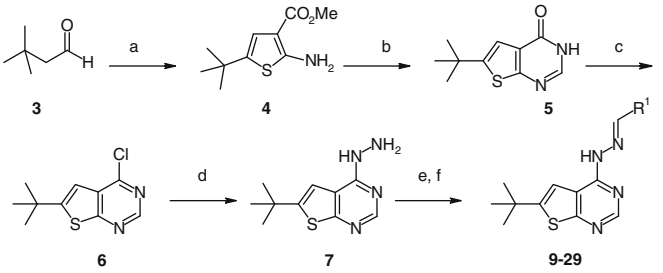
Figure 2. Predicted binding mode of compound **2** in the ATP binding site of our CDK4 homology model. The inhibitor is color-coded by atom type, where carbon is yellow, oxygen is red, sulfur is orange, and nitrogen is blue. The enzyme is color-coded by atom type in a similar fashion except that carbon is gray. Key enzyme residues are labeled and hydrogen bonds are shown as cyan dotted lines.

2.2. Synthesis

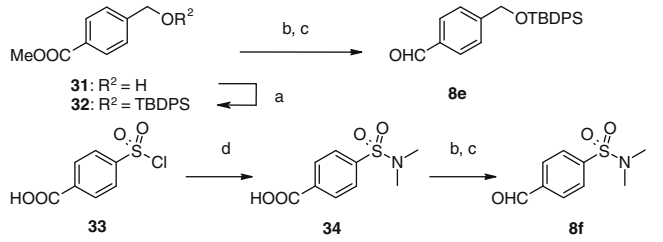
Preparation of all compounds **9–29** was accomplished using a general synthetic route as shown in Scheme 1. The thiophene intermediate (**4**) was synthesized from 3,3-dimethylbutanal (**3**) with methyl cyanoacetate in the presence of sulfur by the method of Tinney et al.[27] Cyclization of **4** with formamide gave 6-*tert*-butyl-4-oxothieno[2,3-*d*]pyrimidine (**5**). The hydrazine (**7**) was prepared from chlorination of the carbonyl group at the C-4 position of **5** with phosphorus oxychloride, followed by treatment with hydrazine monohydrate according to a reported procedure.[28] Finally, the hydrazone analogues (**9–29**) were produced by a condensation reaction with **7** and appropriate aldehydes (**8a–p**) in benzene, followed by deprotection in case of the compounds (**14**, **20**, **22**, **24**, **28** and **29**).

The aldehydes (**8e**, **f**) were prepared as shown in Scheme 2. Protection of the hydroxyl group of compound **31** with *tert*-butyldiphenylsilyl chloride followed by reduction of methyl ester using lithium aluminum hydride (LAH) and oxidation of the resulting alcohol with MnO_2 afforded **8e**. Compound **33** was converted to sulfonamide (**34**) with dimethylamine. Conversion of **34** to the aldehyde (**8f**) was carried out by the procedure utilized for the preparation of **8e** from compound **32**.

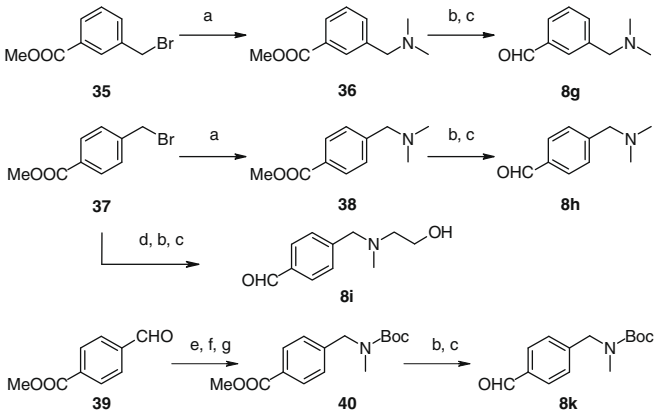
The aldehydes (**8g–i**, **k**) were synthesized as shown in Scheme 3. Amination of bromide (**35**, **37**) with dimethylamine produced the methyl esters (**36**, **38**) followed by the procedure described for compound **8f** gave **8g** and **8h**, respectively. Similarly, the aldehyde (**8i**) was obtained from compound **37** with *N*-methylethanolamine. Reductive amination of compound **39** with methylamine and $NaBH_4$ followed by standard Boc protection afforded the methyl ester (**40**). Finally, compound **40** was converted to **8k** in a similar manner.



Scheme 1. Reagents and conditions: (a) methyl cyanoacetate, S_8, Et_3N, DMF, 96%; (b) $HCONH_2$, 210 °C, 88%; (c) $POCl_3$, 110 °C, 65%; (d) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux, 55%; (e) aldehydes **8a–p**, benzene, reflux, 15–100%; (f) deprotection in case of **14**, **20**, **22**, **24**, **28** and **29**, 81–97%.



Scheme 2. Synthesis of aldehydes **8e** and **8f**. Reagents and conditions: (a) TBDPSCl, imidazole, THF; (b) LAH, THF, 0 °C; (c) MnO_2, CH_2Cl_2, reflux, three steps 81% (**8e** from **31**), two steps 58% (**8f** from **34**); (d) Me_2NH, THF, 57%.



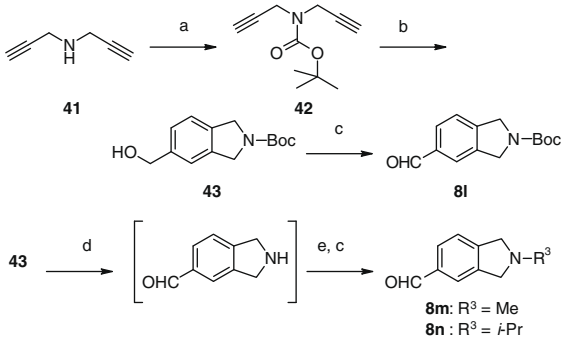
Scheme 3. Synthesis of aldehydes **8g–i** and **8k**. Reagents and conditions: (a) Me_2NH, THF, 98% (**35**), 78% (**38**); (b) LAH, THF, 0 °C; (c) MnO_2, CH_2Cl_2 or CCl_4, reflux, two steps 68% (**8g**), 86% (**8h**), 75% (**8i**), 58% (**8k**); (d) *N*-methylethanolamine, Et_3N, toluene, 70 °C, 74%; (e) $MeNH_2$, toluene; (f) $NaBH_4$, MeOH; (g) Boc_2O, DMAP, CH_2Cl_2, three steps 18%.

The isoindolinecarbaldehydes (**8l–n**) were prepared as shown in Scheme 4. Bicyclic compound (**8l**) was synthesized using the reported method of Middleton et al.[29] Protection of dipropargylamine (**41**), and successive reaction with propargyl alcohol and Wilkinson's catalyst gave the alcohol (**43**). Compound **43** was treated with MnO_2 to afford the aldehyde (**8l**). Deprotection of the Boc group of **43** with TFA followed by conversion to aldehydes (**8m**, **n**) in two steps was performed according to the above method.

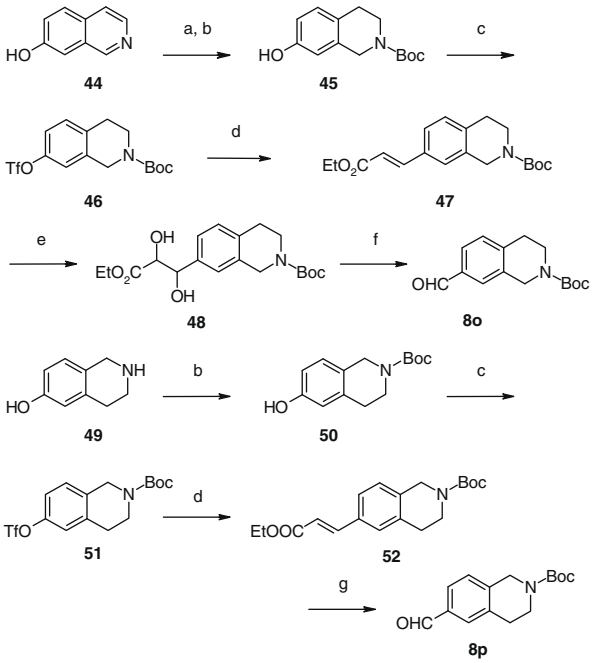
Dihydroisoquinoline intermediates (**8o**, **p**) were prepared as shown in Scheme 5. Compounds **8o** and **8p** were synthesized following a reported procedure.[29,30] Hydrogenation of isoquinolin-7-ol (**44**) using Pt_2O, followed by protection with $(Boc)_2O$ gave compound **45**, which was converted to the *O*-triflate (**46**) with *N*-phenyl-bis(trifluoromethanesulfonimide). The α,β-unsaturated ester (**47**) was derived from **46** with ethyl acrylate and $Pd(OAc)_2$. Oxidation of **47** with OsO_4 followed by cleavage of the resulting diol with $NaIO_4$ provided the aldehyde (**8o**). The aldehyde (**8p**) was synthesized from compound **49** in a similar manner.

2.3. Biological evaluation

To investigate our hypothesis that the nitrogen atom in the pyridine ring at the C-4 hydrazone part rarely contributes to increase CDK4 inhibitory activity, we tried to replace pyridine in the phenyl ring. Biological activities of the novel thieno[2,3-*d*]pyrimidin-4-yl hydrazone analogues were evaluated in two assay systems, that



Scheme 4. Synthesis of aldehydes **8l–n**. Reagents and conditions: (a) Boc_2O, Et_3N, CH_2Cl_2, 18%; (b) propargyl alcohol, Wilkinson's catalyst, EtOH, two steps 44%; (c) MnO_2, CCl_4, reflux, 66% (**8l**), 92% (**8m**), 33% (**8n**); (d) TFA, CH_2Cl_2; (e) HCHO or acetone, $NaBH(OAc)_3$, CH_2Cl_2, two steps 84% (R^3 = Me), 38% (R^3 = *i*-Pr).



Scheme 5. Synthesis of aldehydes **8o** and **8p**. Reagents and conditions: (a) PtO_2, AcOH, H_2 (40 psi); (b) $(Boc)_2O$, Et_3N, THF, H_2O, two steps 82% (**45**), 91% (**50**); (c) Tf_2NPh, Et_3N, CH_2Cl_2, quant. (**46**), quant. (**51**); (d) ethyl acrylate, $Pd(OAc)_2$, tri(*o*-tolyl)phosphine, CH_3CN, 61% (**47**), 18% (**52**); (e) OsO_4, NMO, THF/acetone/H_2O (1:1:1), 76%; (f) $NaIO_4$, THF/MeOH/H_2O (1:1:1), 91%; (g) OsO_4, $NaIO_4$, THF, H_2O, 46%.

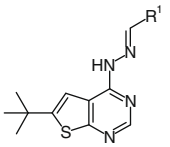
is, in vitro inhibitory activity (CDK4 and CDK2) and cytotoxicity against two cell lines (HCT116 human colon carcinoma and PC-6 human non-small cell lung carcinoma). The results are presented in Table 1.

Compound **9**, bearing non-substituted phenyl group at the C-4 position (R^1), showed moderate CDK4 inhibitory activity (CDK2: >20 μg/mL). 4-Carboxyphenyl (**11**) and 4-(hydroxymethyl)phenyl (**14**) derivatives retained the same CDK4 activity as **9**, but neither compound had selectivity against CDK2. On the other hand, 4-(methoxycarbonyl)phenyl (**10**) and 4-hydroxyphenyl (**12**) analogues were inactive. Surprisingly the sulfonamide analogue (**15**) exhibited superior activity to **2** against CDK2 (CDK4: >20 μg/mL). Introduction of the dimethylamino group (**16**) at the 3-position on the phenyl ring improved its CDK4 inhibitory activity with good selectivity (42.3-fold vs CDK2) as predicted by our docking study model. Moreover, we found that the *para*-substituted compounds, as exemplified by compounds **17**, **18**, **20** and **22**, showed potent CDK4 inhibitory activity. In addition, analogue (**22**) showed excellent activity to **2** against HCT116 and PC-6.

The biological activity data for thieno-pyrimidine analogues, bearing a bicyclic ring at the hydrazone moiety, are shown in Table 1, We then introduced dihydroindoline (**24–26**) or tetrahydroisoquinoline (**28**, **29**) rings. As a result, all the derivatives maintained relatively potent CDK4 inhibitory activity with selectivity against CDK2 (5.3–37.5-fold). Among these analogues, the compounds **24**, **25**, **28** and **29** had fairly good cytotoxicity against HCT116 and PC-6 (IC_{50} range: 0.046–0.111 μg/mL). These results support our hypothesis and it is apparent that the modification of the hydrazone moiety with a substituted phenyl ring increases CDK4 inhibitory activity and cytotoxicity.

For further characterization of our compounds, the cell cycle distribution analysis in HCT116 cells was carried out with the selected compounds **22**, **24** and **29**. Cells were collected after 16 h treatment of the tested compounds and the DNA content of the cells was assessed by flow cytometry analysis. After treating the

Table 1
Enzyme inhibitory and cytotoxic activity for substituted benzaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidine-4-yl)hydrazones



Compd	R^1	CDK4 IC_{50}[a] (μg/mL)	CDK2 IC_{50}[a] (μg/mL)	CDK4 selectivity[b]	HCT-116 IC_{50}[c] (μg/mL)	PC-6 IC_{50}[c] (μg/mL)	G1 arrest[d] (%)
2[23]	6-[(Dimethylamino)methyl]-2-pyridinyl	0.056	1.40	25.0	0.419	0.381	
9	Phenyl	0.61	>20	>33	NT[e]	6.540	
10	4-(Methoxycarbonyl)phenyl	>20	>20		NT[e]	NT[e]	
11	4-Carboxypheny	0.98	0.39	0.4	9.990	7.220	
12	4-Hydroxyphenyl	>20	11.0		0.844	>10	
14	4-(Hydroxymethyl)phenyl	0.40	0.26	0.7	0.665	0.556	
15	4-(*N*,*N*-Dimethylsulfonamide)phenyl	>20	1.22		1.420	2.120	
16	3-[(Dimethylamino)methyl]phenyl	0.026	1.10	42.3	0.405	0.598	
17	4-[(Dimethylamino)methyl]phenyl	0.077	0.60	7.8	0.128	0.107	
18	4-{[(2-Hydroxyethyl)(methyl)amino]methyl}phenyl	0.028	0.29	10.4	0.169	0.124	
20	4-(Aminomethyl)phenyl	0.021	1.60	76.2	0.334	0.214	
22	4-[(Methylamino)methyl]phenyl	0.038	0.68	17.9	0.056	0.044	57 (60)
24	Isoindolin-5-yl	0.083	0.52	6.3	0.111	0.071	62 (100)
25	2-Methylisoindolin-5-yl	0.096	1.00	10.4	0.088	0.050	
26	2-Isopropylisoindolin-5-yl	0.080	3.00	37.5	0.206	0.134	
28	1,2,3,4-Tetrahydro-isoquinolin-7-yl	0.034	0.18	5.3	0.107	0.069	
29	1,2,3,4-Tetrahydro-isoquinolin-6-yl	0.038	0.64	16.8	0.046	0.049	56 (50)

[a] Concentration (μg/mL) needed to inhibit the Rb phosphorylation by 50%, as determined from the dose–response curve. Values are the means of at least two determinations.
[b] The values are calculated by using the following equation (CDK2 IC_{50})/(CDK4 IC_{50}).
[c] Dose–response curves were determined at ten concentrations. The IC_{50} values are the concentrations needed to inhibit cell growth by 50%, as determined from these curves.
[d] Population of G1 phase in HCT116 cells after 16 h treatment with compounds (DMSO as control: 30–34%). Numbers in parentheses indicate the concentrations of compounds (ng/mL).
[e] NT = not tested.

cells with **24**, a significant increase in the G0/G1 population (62%) was observed and decreases in the S and G2/M populations at a concentration of 100 ng/mL. Compounds **22** and **29** also caused increases in the G1 population at the concentrations of 60 and 56 ng/mL, respectively (Table 1). In addition, the aqueous solubility of compound **22** was markedly improved (783 μg/mL), whereas those of bicyclic ring compounds **24** and **29** were very low (2.8 and 8.5 μg/mL, respectively), in comparison to the lead compound **2** (44 μg/mL).

To evaluate the antitumor effects in vivo, HCT116 cells were subcutaneously transplanted into nude mice and the synthetic compounds **22**, **24** and **29** were administered intravenously (iv) and/or orally (po) (Table 2). Compound **18** exhibited a tumor growth inhibition of 54% (iv, 300 mg/kg) and 57% (po, 350 mg/kg), respectively. Similarly, a 61% reduction was observed after iv administration of **24**. In contrast, tetrahydroisoquinoline analogue (**29**) had no effect against HCT116.

3. Conclusions

In summary, focusing on the optimization of the heteroaryl moiety at hydrazone with substituted phenyl groups, compounds **22**, **24** and **29** have been identified as potent, selective CDK4 inhibitors. These compounds have sufficient cytotoxic activities against HCT116 human colon carcinoma cell line with the ability to prevent cell progression. Moreover, compounds **22** and **24** showed sufficient efficacy in a xenograft model of the HCT116 cells. The aqueous solubility (783 μg/mL) of compound **22** also revealed significant improvement from that of compound **2**. These results provide valuable information for the design of a potent and water-soluble CDK4 inhibitor. Further work to improve selectivity and physical profile of CDK4 will be reported in due course.

4. Experimental

4.1. CDK4 Homology modeling

Our homology model of CDK4 was developed using the homology module of InsightII (Accelrys Software Inc.) with CDK2 (PDB code 1jst[24]; containing ATP-Mg^{2+} and sharing moderate sequence identity with CDK4) and CDK6 (1blx[25]; sharing 70% sequence identity with CDK4) as templates. Multiple sequence alignment was produced using CLUSTALW (1.4) with **P11802** of CDK4, 1jst, and

Table 2
Antitumor effects of **22**, **24**, and **29** against HCT116 solid tumors

Compd	Route	Dose/day (mg/kg)	Total dose (mg/kg)	Administration schedule	$IRTV_{max}$[a] (%) (day)	BWL_{max}[b] (%) (day)	T/D/N[c]
22	iv	300	1200	qdx4	54 (9)	9.7 (7)	0/1/5
22	po	350	1400	qdx4	57 (6)	16.0 (5)	0/0/5
24	iv	260	1040	qdx4	61 (5)	<0	0/0/5
29	iv	300	1200	qdx4	5 (7)	2.2 (1)	0/0/5

[a] Maximum inhibition rate of tumor volume. Numbers in parentheses indicate the day after initial drug administration on which $IRTV_{max}$ was reached.
[b] Maximum rate of body weight loss. Numbers in parentheses indicate the day of BWL_{max}.
[c] Number of mice which died of toxicity/number of mice which died of tumor/number of mice used.

1blx. Docking studies were performed to generate solutions for **1** using Glide (Schrödinger, Inc.).

4.2. Chemistry

4.2.1. General

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. The solvent and reagent names are abbreviated as follows: acetic acid (AcOH), ethyl acetate (EtOAc), di-*tert*-butyl dicarbonate $(Boc)_2O$, *N*,*N*-dimethylformamide (DMF), diethyl ether (Et_2O), lithium aluminum hydride (LAH), *N*-methylmorpholine-*N*-oxide (NMO), palladium acetate ($Pd(OAc)_2$), sodium triacetoxyborohydride ($NaBH(OAc)_3$), tetrabutylammonium fluoride (TBAF), *tert*-butyldiphenylsilyl chloride (TBDPSCl), triethylamine (Et_3N), trifluoroacetic acid (TFA), tetrahydrofuran (THF). Column chromatography was performed with a Merck Silica Gel 60 (particle size 0.060–0.200 or 0.040–0.063 mm). Flash column chromatography was performed with Biotage FLASH Si packed columns. Thin-layer chromatography (TLC) was performed on Merck pre-coated TLC glass sheets with Silica Gel 60 F_{254}, and compound visualization was effected with a 5% solution of phosphomolybdic acid in ethanol, UV lamp, iodine, or Wako ninhydrin spray. ^{1}H NMR spectra were recorded on a JEOL JNM EX400, and chemical shifts are given in ppm (δ) from tetramethylsilane as an internal standard. Significant ^{1}H NMR data are tabulated in the following order: number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constant(s) in hertz. Infrared (IR) spectra were recorded on a Hitachi 270-30 spectrometer and a JASCO FT/IR-6100. Electron spray ionization condition (ESI) mass spectra were recorded on an Agilent 1100 and SCIEX API-150EX spectrometer. Fast atom bombardment ionization condition (FAB) mass spectra were recorded on a JEOL JMS-HX110 spectrometer. Electron impact ionization condition (EI) mass spectra were recorded on a JEOL JMS-AX505W. High-resolution mass spectra were obtained on a JEOL JMS-100LP AccuTOF LC-plus spectrometer (ESI) or a JEOL JMS-700 mass spectrometer (EI). Elemental analysis was performed using a PerkinElmer CHNS/O 2400II or a Yokohama Analysis IC7000RS, and analytical results were within ±0.4% of the theoretical values unless otherwise noted.

4.2.1.1. 2-Amino-5-*tert*-butyl-3-methoxycarbonylthiophene (4).

3,3-Dimethylbutanal 3 (5.0 mL, 40 mmol) was added to a solution of methyl cyanoacetate (3.53 mL, 40 mmol), Et_3N (4.29 mL, 40 mmol) and DMF (6.19 mL, 80 mmol) under a N_2 atmosphere. After 10 min stirring, S8 (1.28 g, 40 mmol) was added and stirred at room temperature for 23 h. The reaction mixture was poured into water and extracted with EtOAc. The aqueous layer was extracted with EtOAc and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure to afford title compound **4** (8.21 g, 96%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.29 (9H, s), 3.79 (3H, s), 5.76 (2H, br), 6.62 (1H, s); MS (ESI) *m/z*: 214 (M^++H); HRMS (ESI) *m/z*: 214.08918 (calcd for $C_{10}H_{16}NO_2S$: 214.09017).

4.2.1.2. 6-*tert*-Butyl-4-oxothieno[2,3-*d*]pyrimidine (5).

A mixture of **4** (8.21 g, 38.5 mmol) in formamide (50 mL) was stirred under reflux for 2.5 h. After cooling, precipitate was formed, which was filtered off and washed with *n*-hexane to afford title compound **6** (5.03 g, 65%) as a pale yellow solid. ^{1}H NMR ($CDCl_3$) δ: 1.43 (9H, s), 7.21 (1H, s), 8.01 (1H, s), 12.15 (1H, br); IR (ATR) cm^{-1}: 2957, 2866, 1650, 1582, 1368, 1270, 1168; MS (ESI) *m/z*: 209 (M^++H); HRMS (ESI) *m/z*: 209.07370 (calcd for $C_{10}H_{13}N_2OS$: 209.07486); Anal. Calcd for $C_{10}H_{12}N_2OS$: C, 57.67; H, 5.81; N, 13.45; S, 15.40. Found: C, 57.88; H, 5.98; N, 13.46; S, 15.68.

4.2.1.3. 6-*tert*-Butyl-4-chlorothieno[2,3-*d*]pyrimidine (6).

Compound **5** (7.06 g, 33.9 mmol) in phosphorous oxychloride (140 mL) was stirred under reflux for 3.5 h. The reaction mixture was concentrated under reduced pressure. To the residue satd $NaHCO_3$ aq was added and extracted with EtOAc. The aqueous layer was extracted with EtOAc and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure to afford title compound **6** (5.03 g, 65%) as a brownish oil. ^{1}H NMR ($CDCl_3$) δ: 1.49 (9H, s), 7.11 (1H, s), 8.77 (1H, s); IR (KBr) cm^{-1}: 2963, 1491, 1413, 1363, 1137, 839; MS (EI) *m/z*: 226 (M^+); HRMS (ESI) *m/z*: 227.04013 (calcd for $C_{10}H_{12}ClN_2S$: 227.04097); Anal. Calcd for $C_{10}H_{11}ClN_2S{\cdot}0.25H_2O$: C, 51.94; H, 5.01; N, 12.11; Cl, 15.33; S, 13.87. Found: C, 51.81; H, 5.14; N, 11.95; Cl, 15.81; S, 7.57.

4.2.1.4. 6-*tert*-Butyl-4-hydrazinothieno[2,3-*d*]pyrimidine (7).

To a solution of **6** (5.03 g, 22.2 mmol) in EtOH (100 mL) was added hydrazine monohydrate (50 mL). The mixture was stirred under reflux for 5.5 h and cooled to room temperature. Water was added to the reaction mixture and extracted with EtOAc. The aqueous layer was extracted with EtOAc and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was recrystallized from Et_2O and *n*-hexane to afford title compound **7** (2.73 g, 55%) as a pale yellow solid. ^{1}H NMR (DMSO-d_6) δ: 1.37 (9H, s), 4.48 (2H, br), 7.37 (1H, s), 1.07 (1H, s), 8.78 (1H, s); IR (KBr) cm^{-1}: 3233, 2948,1576, 1440, 1345, 1305, 872; MS (EI) *m/z*: 222 (M^+); HRMS (ESI) *m/z*: 223.10077 (calcd for $C_{10}H_{15}N_4S$: 223.10174); Anal. Calcd for $C_{10}H_{14}N_4S{\cdot}0.1H_2O$: C, 53.59; H, 6.39; N, 25.00; S, 14.31. Found: C, 53.59; H, 6.29; N, 25.24; S, 14.30.

4.2.2. Benzaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (9)

A mixture of **7** (208 mg, 1.0 mmol) and benzaldehyde **8a** (112 μL, 1.1 mmol) in benzene (4 mL) was stirred under reflux for 3 h and cooled to room temperature. The precipitate was collected by filtration and washed with *n*-hexane to afford title compound **9** (262 mg, 84%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ: 1.50 (9H, s), 7.45 (3H, m), 7.70 (2H, m), 7.87 (1H, s), 7.88 (1H, s), 8.47 (1H, s), 8.98 (1H, br); IR (KBr) cm^{-1}: 3195, 2966, 1577, 1552, 1429; MS (EI) *m/z*: 310 (M^+); HRMS (ESI) *m/z*: 311.13045 (calcd for $C_{17}H_{19}N_4S$: 311.13304); Anal. Calcd for $C_{17}H_{18}N_4S$: C, 65.78; H, 5.84; N, 18.05; S, 10.33. Found: C, 65.81; H, 5.80; N, 18.06; S, 10.25.

4.2.3. Methyl 4-{[(6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazono]methyl}benzoate (10)

A mixture of **7** (223 mg, 1.00 mmol) and methyl 4-formylbenzoate **8b** (181 mg, 1.10 mmol) in benzene (10 mL) was stirred under reflux for 1 h and cooled to room temperature. The reaction mixture was concentrated under reduced pressure and the residue was recrystallized from Et_2O to afford title compound **10** (360 mg, 98%) as a pale yellow solid. ^{1}H NMR ($CDCl_3$) δ: 1.51 (9H, s), 3.96 (3H, s), 7.77 (2H, d, J = 7.5 Hz), 7.85 (1H, br), 7.92 (1H, br), 8.12 (2H, d, J = 7.5 Hz), 8.50 (1H, br); IR (KBr) cm^{-1}: 3253, 3228, 1698; MS (FAB) *m/z*: 369 (M^++H); HRMS (ESI) *m/z*: 369.13759 (calcd for $C_{19}H_{21}N_4O_2S$: 369.13852); Anal. Calcd for $C_{19}H_{20}N_4O_2S$: C, 61.94; H, 5.47; N, 15.21; S, 8.70. Found: C, 62.19; H, 5.53; N, 15.29; S, 8.81.

4.2.4. 4-{[(6-*tert*-Butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazono]methyl}benzoic acid (11)

Compound **11** was obtained from 4-formylbenzoic acid **8c** as a yellow solid (quant.) by following the procedure described for **9**. ^{1}H NMR (DMSO-d_6) δ: 1.46 (9H, s), 7.82 (2H, d, J = 8.3 Hz), 7.83 (1H, s), 8.02 (2H, d, J = 8.3 Hz), 8.29 (1H, s), 8.46 (1H, s), 11.95 (1H, br); IR

(KBr) cm^{-1}: 1701, 1558, 1358, 1273, 885; MS (FAB) *m/z*: 355 (M^++H); HRMS (ESI) *m/z*: 355.12227 (calcd for $C_{18}H_{19}N_4O_2S$: 355.12287); Anal. Calcd for $C_{18}H_{18}N_4O_2S$: C, 61.00; H, 5.12; N, 15.81; S, 9.05. Found: C, 60.65; H, 5.13; N, 15.00; S, 8.78.

4.2.5. 4-Hydroxybenzaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (12)

Compound **12** was obtained from 4-hydroxybenzaldehyde **8d** as a colorless solid (92%) by following the procedure described for **9**. ^1H NMR (DMSO-d_6) δ: 1.43 (9H, s), 6.84 (2H, d, J = 8.5 Hz), 7.54 (2H, d, J = 8.5 Hz), 7.82 (2H, br), 8.13 (1H, s), 8.36 (1H, s); IR (ATR) cm^{-1}: 1564, 1512, 1433, 1292, 1153; MS (FAB) *m/z*: 327 (M^++H); HRMS (ESI) *m/z*: 327.12799 (calcd for $C_{17}H_{19}N_4OS$: 327.12796); Anal. Calcd for $C_{17}H_{18}N_4OS$: C, 62.55; H, 5.56; N, 17.16; S, 9.82. Found: C, 62.42; H, 5.64; N, 17.08; S, 9.96.

4.2.5.1. 4-({[*tert*-Butyl(diphenyl)silyl]oxy}methyl)benzaldehyde (8e). To a mixture of methyl 4-(hydroxymethyl)benzoate **31** (1.0 g, 6.02 mmol) and imidazole (860 mg, 12.64 mmol) in THF (20 mL) was added TBDPSCl (1.64 mL, 6.32 mmol). The reaction mixture was stirred at room temperature for 7 h. After EtOAc and brine were added, the two layers were separated. The aqueous layer was extracted with EtOAc and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (5:1) to afford methyl 4-({[*tert*-butyl(diphenyl)silyl]oxy}methyl)benzoate **32**. The residue was added to a suspension of LAH (246 mg, 6.48 mmol) in THF (25 mL) at 0 °C, and stirred at room temperature for 5 h. After cooling at 0 °C, MeOH (0.55 mL), water (0.25 mL), 15% NaOH aq (0.25 mL) and water (0.75 mL) were added to a reaction mixture successively. The precipitate was filtered through Celite. The filtrate was concentrated under reduced pressure and the residue was dissolved in $CHCl_3$ (46 mL). To this solution was added Mn_2O (4.6 g) and the mixture was stirred under reflux for 6 h. The reaction mixture was filtered through Celite. The filtrate was concentrated under reduced pressure and the residue was chromatographed on silica gel eluted with $CHCl_3$ to afford title compound **8e** (1.86 g, 81%) as a yellow oil. ^1H NMR ($CDCl_3$) δ: 1.12 (9H, s), 4.84 (2H, s), 7.39–7.44 (6H, m), 7.51 (2H, d, J = 8.3 Hz), 7.68 (4H, dd, J = 1.7, 7.8 Hz), 7.85 (2H, d, J = 8.3 Hz), 10.00 (1H, s); MS (FAB) *m/z*: 375 (M^++H).

4.2.5.2. 4-({[*tert*-Butyl(diphenyl)silyl]oxy}methyl)benzaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (13). Compound **13** was obtained from **8e** as a colorless solid (90%) by following the procedure described for **9**. ^1H NMR ($CDCl_3$) δ: 1.12 (9H, s), 1.50 (9H, s), 4.81 (2H, s), 7.37–7.46 (8H, m), 7.67–7.71 (6H, m), 7.89 (1H, br), 7.96 (1H, br), 8.44 (1H, br); IR (KBr) cm^{-1}: 1558, 1429, 1115, 1074, 702; MS (FAB) *m/z*: 579 (M^++H); HRMS (ESI) *m/z*: 579.25812 (calcd for $C_{34}H_{39}N_4OSSi$: 579.26138); Anal. Calcd for $C_{34}H_{38}N_4OSSi$: C, 70.55; H, 6.62; N, 9.68; S, 5.54. Found: C, 70.61; H, 6.63; N, 9.70; S, 5.81.

4.2.5.3. 4-(Hydroxymethyl)benzaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (14). To a solution of **13** (345 mg, 0.60 mmol) in THF (7 mL) was added TBAF (1 M THF solution, 1.2 mL, 1.19 mmol), and the mixture was stirred at 60 °C for 4 h. After EtOAc and brine were added to the reaction mixture, the two layers were separated. The aqueous layer was extracted with EtOAc and the combined organic layer was dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was recrystallized from MeOH, EtOAc, and *n*-hexane to afford title compound **14** (191 mg, 94%) as a colorless solid. ^1H NMR ($CDCl_3$) δ: 1.50 (9H, s), 4.76 (2H, s), 7.45 (2H, d, J = 8.1 Hz), 7.70 (2H, d, J = 8.1 Hz), 7.87 (1H, s), 7.90 (1H, s), 8.46 (1H, s); IR (KBr) cm^{-1}: 1577, 1556, 1512, 1431, 1360, 1344; MS (FAB) *m/z*: 341 (M^++H). HRMS (ESI) *m/z*: 341.14399 (calcd for $C_{18}H_{21}N_4OS$: 341.14361); Anal. Calcd for $C_{18}H_{20}N_4OS{\cdot}0.1EtOAc$: C, 63.28; H, 6.00; N, 16.04; S, 9.18. Found: C, 63.61; H, 6.26; N, 15.84; S, 9.04.

4.2.5.4. 4-[(Dimethylamino)sulfonyl]benzoic acid (34). To a solution of 4-chlorosulfonylbenzoic acid **33** (1.0 g, 4.53 mmol) in THF (20 mL) was added dimethylamine (2 M THF solution, 4.76 mL, 9.52 mmol) at 0 °C. The mixture was stirred at room temperature for 16 h. After EtOAc and 1 N NaOH aq were added, the two layers were separated. The aqueous layer was acidified with 6 N HCl aq and the precipitate was collected by filtration to afford title compound **34** (589 mg, 57%) as a colorless solid. ^1H NMR (DMSO-d_6) δ: 2.63 (6H, s), 7.86 (2H, d, J = 6.9 Hz), 8.16 (2H, d, J = 7.3 Hz); MS (FAB) *m/z*: 230 (M^++H).

4.2.5.5. 4-Formyl-*N*,*N*-dimethylbenzenesulfonamide (8f). To a suspension of LAH (192 mg, 5.06 mmol) in Et_2O (6 mL) was added **34** (580 mg, 2.53 mmol) at 0 °C, and stirred at room temperature for 1.5 h. After cooling at 0 °C, MeOH (0.55 mL), water (0.25 mL), 15% NaOH aq (0.25 mL) and water (0.75 mL) were added to a reaction mixture successively. The precipitate was filtered through Celite. The filtrate was concentrated under reduced pressure and the residue was dissolved in $CHCl_3$ (10 mL). To this solution was added Mn_2O (1.0 g) and the mixture was stirred under reflux for 4 h. The reaction mixture was filtered through Celite. The filtrate was concentrated under reduced pressure and the residue was recrystallized from EtOAc and *n*-hexane to afford title compound **8f** (309 mg, 62%) as a pale yellow solid. ^1H NMR ($CDCl_3$) δ: 2.76 (6H, s), 7.95 (2H, d, J = 8.0 Hz), 8.06 (2H, d, J = 8.0 Hz), 10.12 (1H, s); MS (FAB) *m/z*: 214 (M^++H).

4.2.5.6. 4-{[(6-*tert*-Butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazono]methyl}-*N*,*N*-dimethylbenzenesulfonamide (15). Compound **15** was obtained from **8f** as a pale yellow solid (quant.) by following the procedure described for **9**. ^1H NMR ($CDCl_3$) δ: 1.51 (9H, s), 2.76 (6H, s), 7.81 (1H, s), 7.86 (4H, s), 7.99 (1H, s), 8.49 (1H, s); IR (KBr) cm^{-1}: 1560, 1550, 1435, 1342, 1157, 1128, 750; MS (FAB) *m/z*: 418 (M^++H); HRMS (ESI) *m/z*: 418.13540 (calcd for $C_{19}H_{24}N_5O_2S_2$: 418.13714); Anal. Calcd for $C_{19}H_{23}N_5O_2S_2$: C, 54.65; H, 5.55; N, 16.77; S, 15.36. Found: C, 54.67; H, 5.53; N, 16.83; S, 15.42.

4.2.5.7. Methyl 3-[(dimethylamino)methyl]benzoate (36). To a solution of methyl 4-(bromomethyl)benzoate **35** (2.3 g, 10.0 mmol) in THF (13 mL) was added dimethylamine (2 M THF solution, 10 mL, 20 mmol) at 0 °C. The mixture was stirred at room temperature for 16 h. The reaction mixture was separated with satd $NaHCO_3$ aq and EtOAc. The aqueous layer was extracted with EtOAc and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure to afford title compound **36** (1.89 g, 98%) as a yellow oil. ^1H NMR ($CDCl_3$) δ: 2.24 (6H, s), 3.46 (2H, s), 3.91 (3H, s), 7.40 (1H, t, J = 7.6 Hz), 7.53 (1H, d, J = 7.6 Hz), 7.94 (1H, d, J = 7.6 Hz), 7.97 (1H, s); MS (EI) *m/z*: 193 (M^+).

4.2.5.8. 3-[(Dimethylamino)methyl]benzaldehyde (8g). Compound **8g** was obtained from **36** as a yellow oil (68%) by following the procedure described for **8f**. ^1H NMR ($CDCl_3$) δ: 2.24 (6H, s), 3.50 (2H, s), 7.49 (1H, t, J = 7.6 Hz), 7.59 (1H, d, J = 7.6 Hz), 7.78 (1H, d, J = 7.6 Hz), 7.83 (1H, s), 10.02 (1H, s); MS (FAB) *m/z*: 164 (M^++H).

4.2.5.9. 3-[(Dimethylamino)methyl]benzaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (16). Compound **16** was obtained from **8g** as a colorless solid (67%) by following the procedure described for **9**. ^1H NMR ($CDCl_3$) δ: 1.52 (9H, s), 2.27 (6H, s),

3.48 (2H, s), 7.35–7.42 (2H, m), 7.57 (1H, d, J = 7.1 Hz), 7.73 (1H, s), 7.87 (1H, s), 7.92 (1H, s), 8.48 (1H, s); IR (KBr) cm^{-1}: 2958, 2775, 1558, 1434, 1346, 1157, 1120, 773; MS (FAB) m/z: 368 (M^++H). HRMS (ESI) m/z: 368.19011 (calcd for $C_{20}H_{26}N_5S$; 368.19089); Anal. Calcd for $C_{20}H_{25}N_5S{\cdot}0.25H_2O$: C, 65.36; H, 6.86; N, 19.06; S, 8.37. Found: C, 64.90; H, 6.84; N, 18.96.

4.2.5.10. Methyl 4-[(dimethylamino)methyl]benzoate (38). Compound **38** was obtained from methyl 4-(bromomethyl)benzoate **37** as a yellow oil (78%) by following the procedure described for **36**. ^1H NMR ($CDCl_3$) δ: 2.25 (6H, s), 3.47 (2H, s), 3.91 (3H, s), 7.38 (2H, d, J = 8.3 Hz), 7.99 (2H, d, J = 8.3 Hz); MS (FAB) m/z: 194 (M^++H).

4.2.5.11. 4-[(Dimethylamino)methyl]benzaldehyde (8h). Compound **8h** was obtained from **38** as a yellow oil (86%) by following the procedure described for **8f**. ^1H NMR ($CDCl_3$) δ: 2.26 (6H, s), 3.50 (2H, s), 7.49 (2H, d, J = 8.1 Hz), 7.84 (2H, d, J = 8.1 Hz), 10.00 (1H, s); MS (FAB) m/z: 164 (M^++H).

4.2.5.12. 4-[(Dimethylamino)methyl]benzaldehyde(6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (17). Compound **17** was obtained from **8h** as a colorless solid (54%) by following the procedure described for **9**. ^1H NMR ($CDCl_3$) δ: 1.50 (9H, s), 2.30 (6H, s), 3.50 (2H, s), 7.41 (2H, d, J = 8.1 Hz), 7.68 (2H, d, J = 8.1 Hz), 7.87 (1H, s), 7.89 (1H, s), 10.00 (1H, s); IR (KBr) cm^{-1}: 1558, 1427, 1348, 773; MS (FAB) m/z: 368 (M^++H). HRMS (ESI) m/z: 368.19245 (calcd for $C_{20}H_{26}N_5S$: 368.19089); Anal. Calcd for $C_{20}H_{25}N_5S$: C, 65.36; H, 6.86; N, 19.06; S, 8.73. Found: C, 65.14; H, 6.86; N, 19.00; S, 8.60.

4.2.5.13. 4-{[(2-Hydroxyethyl)(methyl)amino]methyl}benzaldehyde (8i). To a solution of methyl 4-(bromomethyl)benzoate **37** (1.0 g, 4.37 mmol) in toluene (10 mL) was added *N*-methyl ethanolamine (420 μL, 5.24 mmol). After stirring at room temperature for 1 h, Et_3N (3.0 mL, 21.8 mmol) was added to the reaction mixture, and stirred at 70 °C for 19.5 h. The mixture was separated with EtOAc and water. The aqueous layer was extracted with EtOAc and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was dissolved in THF (7 mL) and was added to a suspension of LAH (122 mg, 3.21 mmol) in THF (7 mL) at 0 °C. The reaction mixture was stirred at room temperature for 5 h. After cooling at 0 °C, MeOH (0.29 mL), water (0.13 mL), 15% NaOH aq (0.13 mL) and water (0.39 mL) were added to a reaction mixture successively. The precipitate was filtered through Celite. The filtrate was concentrated under reduced pressure and the residue was dissolved in $CHCl_3$ (10 mL). To this solution was added Mn_2O (1.4 g) and the mixture was stirred under reflux for 3.5 h. The reaction mixture was filtered through Celite. The filtrate was concentrated under reduced pressure and the residue was chromatographed on silica gel eluted with $CHCl_3$/MeOH (10:1) to afford title compound **8i** (468 mg, 55%) as a pale yellow oil. ^1H NMR ($CDCl_3$) δ: 2.26 (3H, s), 2.63 (2H, t, J = 5.4 Hz), 3.65 (4H, s), 7.49 (2H, d, J = 8.1 Hz), 7.85 (2H, dd, J = 1.7, 8.1 Hz), 10.00 (1H, s); MS (FAB) m/z: 194 (M^++H); HRMS (ESI) m/z: 194.11582 (calcd for $C_{11}H_{16}NO_2$: 194.11810).

4.2.5.14. 4-{[(2-Hydroxyethyl)(methyl)amino]methyl}benzaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (18). Compound **18** was obtained from **8i** as a pale yellow solid (80%) by following the procedure described for **9**. ^1H NMR (DMSO-d_6) δ: 1.46 (9H, s), 2.17 (3H, s), 2.50 (2H, d, J = 1.2 Hz), 3.54 (4H, br), 4.41 (1H, t, J = 5.4 Hz), 7.42 (2H, d, J = 7.8 Hz), 7.67 (2H, d, J = 7.8 Hz), 7.86 (1H, br), 8.23 (1H, s), 8.42 (1H, s), 11.77 (1H, br); IR (KBr) cm^{-1}: 1581, 1558, 1433, 1356; MS (FAB) m/z: 398 (M^++H); HRMS (ESI) m/z: 398.20146 (calcd for $C_{21}H_{28}N_5OS$: 398.20235); Anal. Calcd for $C_{21}H_{27}N_5OS$: C, 63.45; H, 6.85; N, 17.62; S, 8.07. Found: C, 63.97; H, 6.79; N, 17.52; S, 8.01.

4.2.5.15. *tert*-Butyl (4-{[(6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazono]methyl}benzyl)carbamate (19). Compound **19** was obtained from *tert*-butyl (4-formylbenzyl)carbamate **8j** as a pale yellow solid (90%) by following the procedure described for **9**. ^1H NMR ($CDCl_3$) δ: 1.48 (9H, s), 1.50 (9H, s), 4.37 (2H, d, J = 5.6 Hz), 4.90 (1H, br), 7.36 (1H, s), 7.37 (2H, d, J = 8.1 Hz), 7.68 (2H, d, J = 8.1 Hz), 7.86 (1H, s), 7.87 (1H, s), 8.48 (1H, s); IR (ATR) cm^{-1}: 2972, 1712, 1558, 1157; MS (FAB) m/z: 440 (M^++H); HRMS (ESI) m/z: 440.21149 (calcd for $C_{23}H_{30}N_5O_2S$: 440.21202); Anal. Calcd for $C_{23}H_{29}N_5O_2S$: C, 62.84; H, 6.65; N, 15.93; S, 7.29. Found: C, 63.02; H, 6.53; N, 15.75; S, 7.44.

4.2.5.16. 4-(Aminomethyl)benzaldehyde (6-*tert*-butylthieno-[2,3-*d*]pyrimidin-4-yl)hydrazone (20). To a solution of **19** (100 mg, 0.23 mmol) in 1,4-dioxane (1.0 mL) was added 4 N HCl in 1,4-dioxane (1.0 mL) at 0 °C. The mixture was stirred at room temperature for 10.5 h and concentrated under reduced pressure. The residue was recrystallized from EtOAc and *n*-hexane to afford title compound **20** (82 mg, 87%) as a pale yellow solid. ^1H NMR (DMSO-d_6) δ: 1.46 (9H, s), 3.68 (2H, br), 4.08 (2H, s), 7.60 (2H, d, J = 8.3 Hz), 7.83 (2H, d, J = 8.3 Hz), 7.95 (1H, s), 8.46 (1H, s), 8.48 (1H, s); IR (ATR) cm^{-1}: 1641, 1587, 1086; MS (FAB) m/z: 340 (M^++H); HRMS (ESI) m/z: 340.15959 (calcd for $C_{18}H_{22}N_5S$: 340.15952); Anal. Calcd for $C_{18}H_{21}N_5S{\cdot}2HCl$: C, 52.43; H, 5.62; Cl, 17.19; N, 16.98; S, 7.78. Found: C, 52.09; H, 5.74; Cl, 17.13; N, 16.65; S, 7.82.

4.2.5.17. Methyl 4-{[(*tert*-butoxycarbonyl)(methyl)amino]methyl}-benzoate (40). To a solution of methyl 4-formylbenzoate **39** (2.0 g, 12.18 mmol) in toluene (20 mL) was added methylamine (2 M THF solution, 6.09 mL, 12.18 mmol). The solution was stirred at room temperature for 30 min and concentrated under reduced pressure. The residue was diluted with MeOH (200 mL) and $NaBH_4$ (510 mg, 13.40 mmol) was added and stirred at room temperature for 2 h. After being acidified with 1 N HCl aq, the mixture was concentrated under reduced pressure. The residue was diluted with $CHCl_3$ and washed with 10% NaOH aq. The two layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure. To a solution of the residue in CH_2Cl_2 (50 mL) were added Boc_2O (2.8 mL, 12.2 mmol) and a catalytic amount of DMAP. The reaction mixture was stirred for 4 h at room temperature. After evaporation of the solvent, the residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (10:1) to afford title compound **40** (600 mg, 18%) as a colorless oil. ^1H NMR ($CDCl_3$) δ: 1.45–1.49 (9H, br), 2.81–2.88 (3H, br), 3.91 (3H, s), 4.47 (2H, s), 7.30 (2H, d, J = 8.1 Hz), 8.00 (2H, d, J = 8.1 Hz); MS (FAB) m/z: 280 (M^++H).

4.2.5.18. *tert*-Butyl 4-(formylbenzyl)methylcarbamate (8k). Compound **8k** was obtained from **40** as a colorless oil (58%) by following the procedure described for **8f**. ^1H NMR ($CDCl_3$) δ: 1.45–1.50 (9H, br), 2.83–2.89 (3H, br), 4.50 (2H, br), 7.38 (2H, d, J = 8.1 Hz), 7.86 (2H, d, J = 8.1 Hz), 10.01 (1H, s); MS (ESI) m/z: 272 (M^++Na); HRMS (ESI) m/z: 272.12570 (calcd for $C_{14}H_{19}NNaO_3$: 272.12626).

4.2.5.19. *tert*-Butyl (4-{[(6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazono]methyl}benzyl)methylcarbamate (21). A mixture of **7** (81 mg, 0.37 mmol) and **8k** (100 mg, 0.40 mmol) in benzene (3 mL) was stirred under reflux for 3 h and cooled to room temperature. The precipitate was collected by filtration and recrystallized with EtOAc and *n*-hexane to afford title compound **21** (61 mg, 37%)

as a pale yellow solid. ^{1}H NMR ($CDCl_3$) δ: 1.51 (18H, s), 2.88 (3H, br), 4.47 (2H, s), 7.30 (1H, s), 7.32 (1H, s), 7.68 (2H, d, J = 8.3 Hz), 7.88 (2H, s), 8.49 (1H, s), 9.12 (1H, br); IR (KBr) cm^{-1}: 2962, 1703, 1680, 1556, 1437, 1126; MS (FAB) m/z: 454 (M^++H); HRMS (ESI) m/z: 454.22882 (calcd for $C_{24}H_{32}N_5O_2S$: 454.22767); Anal. Calcd for $C_{24}H_{31}N_5O_2S$: C, 63.55; H, 6.89; N, 15.44; S, 7.37. Found: C, 63.26; H, 6.81; N, 15.35; S, 7.01.

4.2.5.20. 4-[(Methylamino)methyl]benzaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (22). Compound **22** was obtained from **21** as a pale yellow solid (81%) by following the procedure described for **20**. ^{1}H NMR (DMSO-d_6) δ: 1.46 (9H, s), 2.55–2.57 (3H, m), 4.18 (2H, m), 7.64 (1H, s), 7.66 (1H, s), 7.98 (2H, br), 8.53 (1H, br), 9.19 (2H, br); IR (ATR) cm^{-1}: 3396, 2738, 2667, 1651, 1595; MS (FAB) m/z: 354 (M^++H); HRMS (ESI) m/z: 354.17533 (calcd for $C_{19}H_{23}N_5S$: 354.17524); Anal. Calcd for $C_{19}H_{23}N_5S{\cdot}2HCl{\cdot}H_2O$: C, 51.35; H, 6.12; Cl, 15.95; N, 15.76; S, 7.22. Found: C, 51.63; H, 6.04; Cl, 15.96; N, 15.73; S, 7.28.

4.2.5.21. *N*-*tert*-Butoxycarbonyl-dipropargylamine (42). To a mixture of dipropargylamine **41** (5.45 g, 58.5 mmol) and Et_3N (10 mL, 72 mmol) in CH_2Cl_2 (60 mL) was added Boc_2O (15 mL, 65 mmol). The reaction mixture was stirred overnight at room temperature. After evaporation of the solvent, the residue was partitioned between EtOAc and water. The organic layer was washed with brine and dried over Na_2SO_4. The solvent was removed under reduced pressure to afford title compound **42** (11.5 g, quant.) as a brown oil. ^{1}H NMR ($CDCl_3$) δ: 1.48 (9H, s), 2.22 (2H, t, J = 2.5 Hz), 4.17 (4H, br).

4.2.5.22. *tert*-Butyl 5-(hydroxymethyl)-1,3-dihydro-2*H*-isoindole-2-carboxylate (43). To a solution of *N*-*tert*-butoxycarbonyl-dipropargylamine **42** (7.35 g, 38 mmol) in EtOH (160 mL) was dropwised propargylalcohol (9.0 mL, 0.155 mol) followed by the addition of chlorotris(triphenylphosphine)rhodium(I) (1.0 g, 1.1 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 24 h, and concentrated under reduced pressure. The residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (2:1). The eluate was concentrated under reduced pressure, and the residue was recrystallized from Et_2O and *n*-hexane to afford title compound **43** (4.17 g, 44%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.52 (9H, s), 4.62 (4H, br), 4.69 (2H, br), 7.21–7.27 (3H, m); IR (KBr) cm^{-1}: 3421, 2863, 1673; MS (FAB) m/z: 250 (M^++H); Anal. Calcd for $C_{14}H_{19}NO_3$: C, 67.45; H, 7.68; N, 5.62. Found: C, 67.53; H, 7.58; N, 5.58.

4.2.5.23. *tert*-Butyl 5-formyl-1,3-dihydro-2*H*-isoindole-2-carboxylate (8l). To a solution of **43** (500 mg, 2.00 mmol) in CCl_4 (15 mL) was added MnO_2 (2.0 g) and the mixture was stirred for 30 min at room temperature. The reaction mixture was filtered through Celite and washed with $CHCl_3$. The filtrate was concentrated under reduced pressure and the residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (4:1) to afford title compound **8l** (325 mg, 66%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.53 (9H, s), 4.72 (2H, br), 4.76 (2H, br), 7.38–7.45 (1H, m), 7.75–7.81 (2H, m), 10.00 (1H, s); IR (KBr) cm^{-1}: 3050, 1693; MS (EI) m/z: 247 (M^+); Anal. Calcd for $C_{14}H_{17}NO_3{\cdot}0.1H_2O$: C, 67.70; H, 6.98; N, 5.64. Found: C, 67.51; H, 6.98; N, 5.45.

4.2.5.24. *tert*-Butyl 5-{[(6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazono]methyl}-2*H*-isoindole-2-carboxylate (23). Compound **23** was obtained from **8l** as a pale yellow solid (96%) by following the procedure described for **9**. ^{1}H NMR ($CDCl_3$) δ: 1.50 (9H, s), 1.54 (9H, s), 4.70 (2H, br), 4.74 (2H, br), 7.31–7.36 (1H, m), 7.56 (1H, s), 7.70 (1H, s), 7.85 (1H, s), 7.94 (1H, s), 8.46 (1H, s); IR (KBr) cm^{-1}: 2968, 1689, 1564, 1433, 1396, 1107; MS (FAB) m/z: 452 (M^++H); HRMS (ESI) m/z: 452.21125 (calcd for $C_{24}H_{30}N_5O_2S$: 452.21202); Anal. Calcd for $C_{24}H_{29}N_5O_2S$: C, 63.83; H, 6.47; N, 15.51; S, 7.10. Found: C, 63.91; H, 6.44; N, 15.48; S, 7.36.

4.2.5.25. 5-Isoindolinecarbaldehyde (6-*tert*-butylthieno[2,3-*d*]-pyrimidin-4-yl)hydrazone (24). Compound **24** was obtained from **23** as a colorless solid (97%) by following the procedure described for **20**. ^{1}H NMR (DMSO-d_6) δ: 1.44 (9H, s), 4.55 (4H, br), 7.53 (1H, d, J = 7.8 Hz), 7.85 (1H, br), 7.90 (1H, s), 8.49 (1H, s), 9.80 (2H, br); IR (KBr) cm^{-1}: 3388, 2698, 2582, 1643; MS (FAB) m/z: 352 (M^++H); HRMS (ESI) m/z: 352.15884 (calcd for $C_{19}H_{22}N_5S$: 352.15959); Anal. Calcd for $C_{19}H_{21}N_5S{\cdot}2HCl{\cdot}0.75H_2O$: C, 52.11; H, 5.64; N, 15.99; Cl, 16.19; S, 7.32. Found: C, 52.28; H, 5.55; N, 15.88; Cl, 16.45; S, 7.27.

4.2.5.26. 2-Methylisoindoline-5-carbaldehyde (8m). To a solution of **43** (200 mg, 0.80 mmol) in CH_2Cl_2 (0.4 mL) was added TFA (1 mL) and the mixture was stirred for 2 min at room temperature. After evaporation of the solvent, CH_2Cl_2 (2.4 mL) and Et_3N (223 μL, 1.60 mmol) were added to the residue. The reaction mixture was stirred at room temperature for 15 min followed by the addition of AcOH (115 μL, 2.00 mmol), 35% aq formaldehyde (138 μL, 1.60 mmol), and $NaBH(OAc)_3$ (272 mg, 1.28 mmol). The mixture was stirred at room temperature for 15 h. After EtOAc and water were added to the reaction mixture, the two layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was diluted with CCl_4 (2 mL). To this solution was added Mn_2O (220 mg) and the mixture was stirred under reflux for 7 h. The reaction mixture was filtered through Celite. The filtrate was concentrated under reduced pressure to afford title compound **8m** (100 mg, 92%) as a pale yellow solid. ^{1}H NMR ($CDCl_3$) δ: 2.60 (3H, s), 3.91 (4H, d, J = 2.9 Hz), 7.16 (2H, d, J = 1.7 Hz), 7.71 (1H, s), 9.97 (1H, s); MS (FAB) m/z: 162 (M^++H).

4.2.5.27. 2-Methylisoindoline-5-carbaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (25). A mixture of **7** (125 mg, 0.56 mmol) and **8m** (100 mg, 0.62 mmol) in benzene (4.5 mL) was stirred under reflux for 2.5 h and cooled to room temperature. The reaction mixture was concentrated under reduced pressure. The residue was chromatographed on silica gel eluted with $CHCl_3$/MeOH (10:1) to afford title compound **25** (31 mg, 15%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ: 1.50 (9H, s), 2.64 (3H, s), 3.97 (4H, s), 7.28 (1H, s), 7.50 (1H, d, J = 7.1 Hz), 7.61 (1H, s), 7.86 (1H, s), 7.88 (1H, s), 8.48 (1H, s); IR (KBr) cm^{-1}: 2954, 1562, 1429, 1354, 1344; MS (FAB) m/z: 366 (M^++H); HRMS (ESI) m/z: 366.17483 (calcd for $C_{20}H_{24}N_5S$: 366.17524); Anal. Calcd for $C_{20}H_{23}N_5S$: C, 65.72; H, 6.34; N, 19.16; S, 8.77. Found: C, 65.34; H, 6.40; N, 19.02; S, 8.91.

4.2.5.28. 2-Isopropylisoindoline-5-carbaldehyde (8n). Compound **8n** was obtained from **43** as a yellow oil (33%) by following the procedure described for **8m** with acetone instead of formaldehyde. ^{1}H NMR ($CDCl_3$) δ: 1.21 (6H, d, J = 6.3 Hz), 2.75–2.80 (1H, m), 3.95 (2H, d, J = 8.1 Hz), 4.02 (2H, s), 7.16 (1H, s), 7.36 (1H, d, J = 8.1 Hz), 7.72 (1H, d, J = 3.4 Hz), 9.98 (1H, s); MS (FAB) m/z: 190 (M^++H); HRMS (ESI) m/z: 190.12308 (calcd for $C_{12}H_{16}NO$: 190.12319).

4.2.5.29. 2-Isopropylisoindoline-5-carbaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (26). A mixture of **7** (47 mg, 0.21 mmol) and **8n** (60 mg, 0.32 mmol) in benzene (2 mL) was stirred under reflux for 19.5 h and cooled to room temperature. The reaction mixture was concentrated under reduced pressure and the residue was separated with water and EtOAc.

The aqueous layer was extracted with EtOAc and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel eluted with $CHCl_3$/MeOH (20:1). The eluate was concentrated under reduced pressure, and the residue was recrystallized from *n*-hexane to afford title compound **26** (18 mg, 21%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ: 1.22 (6H, d, J = 6.3 Hz), 1.50 (9H, s), 2.81 (1H, m), 4.02 (4H, s), 7.29 (1H, d, J = 7.8 Hz), 7.52 (1H, d, J = 7.8 Hz), 7.60 (2H, s), 7.86 (1H, s), 7.88 (1H, s), 8.47 (1H, s); IR (KBr) cm^{-1}: 2964, 1556, 1427, 1354, 1107; MS (FAB) *m/z*: 394 (M^++H). HRMS (ESI) *m/z*: 394.20572 (calcd for $C_{22}H_{28}N_5S$: 394.20654); Anal. Calcd for $C_{22}H_{27}N_5S{\cdot}0.5H_2O$: C, 65.64; H, 7.01; N, 17.40; S, 7.97. Found: C, 65.68; H, 6.80; N, 17.00; S, 8.25.

4.2.5.30. *tert*-Butyl 7-hydroxy-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (45). Isoquinolin-7-ol **44** (1.50 g, 10.3 mmol) was hydrogenated over PtO_2 (100 mg) in AcOH (15 mL) at 40 psi for overnight. The catalyst was filtered off and the filtrate was concentrated under reduced pressure. The residue was diluted with the mixture of THF (15 mL) and water (5 mL). To the solution were added Et_3N (1.67 mL, 12 mmol) and Boc_2O (2.50 mL, 10.9 mmol). The reaction mixture was stirred for 2 h at room temperature. The mixture was partitioned between EtOAc and water. The organic layer was washed with satd NH_4Cl aq and brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (4:1) to afford title compound **45** (2.05 g, 82%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.50 (9H, s), 2.75 (2H, t, J = 6.0 Hz), 3.62 (2H, t, J = 6.0 Hz), 4.53 (2H, s), 6.74–6.63 (2H, m), 6.98 (2H, t, J = 6.0 Hz); IR (ATR) cm^{-1}: 3337, 1654, 1435, 1159; MS (FAB) *m/z*: 250 (M^++H); HRMS (ESI) *m/z*: 272.12681 (calcd for $C_{14}H_{19}NNaO_3$: 272.12626); Anal. Calcd for $C_{14}H_{19}NO_3{\cdot}0.1H_2O$: C, 66.96; H, 7.71; N, 5.58. Found: C, 66.85; H, 7.58; N, 5.46.

4.2.5.31. *tert*-Butyl 7-(trifluoromethanesulfonyl)oxy-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (46). To a solution of **45** (2.00 g, 8.00 mmol) was added *N*-phenyl-bis(trifluoromethanesulfonimide) (3.15 g, 8.8 mmol) was added in CH_2Cl_2 (100 mL) at 0 °C. The reaction mixture was stirred at room temperature for 24 h and concentrated. The residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (9:1). The eluate was concentrated under reduced pressure, and the residue was recrystallized from *n*-hexane to afford title compound **46** (3.05 g, quant.) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.49 (9H, s), 2.83 (2H, t, J = 6.0 Hz), 3.65 (2H, t, J = 6.0 Hz), 4.58 (2H, s), 7.02 (1H, d, J = 2.4 Hz), 7.06 (1H, dd, J = 2.4, 8.5 Hz), 7.19 (1H, d, J = 8.5 Hz); IR (ATR) cm^{-1}: 2987, 1670, 1423, 1208; MS (ESI) *m/z*: 404 (M^++Na); HRMS (ESI) *m/z*: 404.07421 (calcd for $C_{15}H_{18}F_3NNaO_5S$: 404.07555).

4.2.5.32. *tert*-Butyl 7-[(1*E*)-3-ethoxy-3-oxoprop-1-en-1-yl]-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (47). To a solution of **46** (3.00 g, 7.86 mmol) in CH_3CN (30 mL) were added ethyl acrylate (1.1 mL, 10 mmol), $Pd(OAc)_2$ (176 mg, 10 mol%), tri(*o*-tolyl)phosphine (530 mg, 1.74 mmol), and Et_3N (2.2 mL, 15.8 mmol). The reaction mixture was stirred under reflux for 18 h. After EtOAc was added to the reaction mixture, the two layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layer was washed with satd NH_4Cl aq, brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (10:1–3:1) to afford title compound **47** (1.59 g, 61%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.34 (3H, t, J = 7.2 Hz), 1.49 (9H, s), 2.85 (2H, t, J = 5.9 Hz), 3.65 (2H, t, J = 5.9 Hz), 4.26 (2H, q, J = 7.2 Hz), 4.58 (2H, s), 6.40 (1H, d, J = 16.1 Hz), 7.15 (1H, q, J = 8.1 Hz), 7.27–7.42 (2H, m), 7.65 (1H, d, J = 4.3, 16.1 Hz); MS (ESI) *m/z*: 354 (M^++Na), 276 (M^+−Boc).

4.2.5.33. *tert*-Butyl 7-(3-ethoxy-1,2-dihydroxy-3-oxopropyl)-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (48). To a solution of **47** (1.50 g, 4.52 mmol) in a mixture of THF (10 mL), acetone (10 mL), and H_2O (10 mL) were added NMO (1.06 g, 9.0 mmol) and OsO_4 (115 mg, 10 mol %), and the mixture was stirred at room temperature for 7 h. After EtOAc and satd $Na_2S_2O_3$ aq were added to the reaction mixture, the two layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layer was washed with satd $NaHCO_3$ aq, brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (2:1) to afford title compound **48** (1.26 g, 76%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 1.30 (3H, t, J = 7.0 Hz), 1.49 (9H, s), 2.71 (1H, d, J = 7.3 Hz), 2.82 (2H, t, J = 5.5 Hz), 3.11 (1H, d, J = 5.5 Hz), 3.63 (2H, t, J = 5.5 Hz), 4.29 (2H, q, J = 7.0 Hz), 4.34 (1H, dd, J = 5.5, 3.0 Hz), 4.58 (2H, s), 4.99 (1H, dd, J = 7.3, 3.0 Hz), 7.13–7.22 (3H, m); MS (ESI) *m/z*: 388 (M^++Na).

4.2.5.34. *tert*-Butyl 7-formyl-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (8o). To a solution of **48** (1.20 g, 3.28 mmol) in a mixture of THF (12 mL), MeOH (12 mL), and H_2O (12 mL) were added $NaIO_4$ (1.40 g, 6.55 mmol), and the mixture was stirred at room temperature for 1 h. After EtOAc and water were added to the reaction mixture, the two layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (4:1) to afford title compound **8o** (777 mg, 91%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 1.50 (9H, s), 2.92 (2H, t, J = 5.5 Hz), 3.68 (2H, t, J = 5.5 Hz), 4.65 (2H, s), 7.30 (1H, d, J = 7.5 Hz), 7.63 (1H, s), 7.68 (1H, d, J = 7.5 Hz), 9.97 (1H, s); IR (KBr) cm^{-1}: 3366, 2976, 1690, 1415, 1365, 1159; MS (FAB) *m/z*: 284 (M^++Na).

4.2.5.35. *tert*-Butyl 7-{[2-(6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazono]methyl}-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (27). Compound **27** was obtained from **8o** as a colorless solid (77%) by following the procedure described for **9**. ^{1}H NMR ($CDCl_3$) δ: 1.51 (18H, s), 2.88 (2H, br), 3.68 (2H, br), 4.63 (2H, s), 7.21 (1H, d, J = 7.8 Hz), 7.46–7.50 (2H, m), 7.87 (2H, s), 8.45 (1H, s); IR (KBr) cm^{-1}: 2962, 1695, 1562, 1425, 1163; MS (FAB) *m/z*: 466 (M^++H). HRMS (ESI) *m/z*: 466.22667 (calcd for $C_{25}H_{32}N_5O_2S$: 466.22767); Anal. Calcd for $C_{25}H_{31}N_5O_2S$: C, 64.49; H, 6.71; N, 15.04; S, 6.89. Found: C, 63.74; H, 6.66; N, 14.38; S, 6.93.

4.2.5.36. 1,2,3,4-Tetrahydro-7-isoquinolinecarbaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (28). Compound **28** was obtained from **27** as a colorless solid (97%) by following the procedure described for **20**. ^{1}H NMR (CD_3OD) δ: 1.52 (9H, s), 3.21 (2H, t, J = 6.3 Hz), 3.56 (2H, t, J = 6.3 Hz), 4.46 (2H, s), 7.41 (1H, d, J = 7.8 Hz), 7.80 (1H, s), 7.88 (1H, s), 7.91 (2H, d, J = 7.8 Hz), 8.55 (1H, s), 8.59 (1H, s); IR (KBr) cm^{-1}: 2627, 1641, 1587; MS (FAB) *m/z*: 366 (M^++H). HRMS (ESI) *m/z*: 366.17704 (calcd for $C_{20}H_{24}N_5S$: 366.17524); Anal. Calcd for $C_{20}H_{23}N_5S{\cdot}2HCl{\cdot}0.75H_2O$: C, 53.15; H, 5.91; N, 15.50; Cl, 15.69; S, 7.10. Found: C, 53.71; H, 5.85; N, 14.69; Cl, 14.93; S, 7.16.

4.2.5.37. *tert*-Butyl 6-hydroxy-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (50). To a mixture of 6-hydroxy-1,2,3,4-tetrahydroisoquinoline **49** (500 mg, 2.17 mmol) and Et_3N (0.40 mL, 2.85 mmol) in THF (7.5 mL) and water (2.5 mL) was added Boc_2O (569 mg, 2.61 mmol). The reaction mixture was stirred overnight at room temperature, and concentrated under reduced pressure.

The residue was partitioned between EtOAc and H_2O. The organic layer was washed with satd NH_4Cl aq, brine and dried over Na_2SO_4 and concentrated. The residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (1:1) to afford title compound **50** (493 mg, 91%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.49 (9H, s), 2.77 (2H, t, J = 5.9 Hz), 3.61 (2H, t, J = 5.9 Hz), 4.49 (2H, s), 4.95 (1H, s), 6.62 (1H, s), 6.67 (1H, t, J = 8.3 Hz), 6.96 (1H, t, J = 8.3 Hz); MS (ESI) *m/z*: 272 (M^++Na).

4.2.5.38. *tert*-Butyl 6-(trifluoromethanesulfonyl)oxy-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (51). Compound **51** was obtained from **50** as a colorless oil (quant.) by following the procedure described for **46**. ^{1}H NMR ($CDCl_3$) δ: 1.49 (9H, s), 2.85 (2H, t, J = 6.0 Hz), 3.64 (2H, t, J = 6.0 Hz), 4.57 (2H, s), 7.05 (1H, d, J = 2.4 Hz), 7.08 (1H, dd, J = 2.4, 8.5 Hz), 7.15 (1H, d, J = 8.5 Hz); IR (ATR) cm^{-1}: 2979, 1693, 1663, 1418, 1205, 1130; MS (ESI) *m/z*: 404 (M^++Na), 326 (M^+-tBu); HRMS (ESI) *m/z*: 404.07534 (calcd for $C_{15}H_{18}F_3NNaO_5S$: 404.07555).

4.2.5.39. *tert*-Butyl 6-[(1*E*)-3-ethoxy-3-oxoprop-1-en-1-yl]-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (52). Compound **52** was obtained from **51** as a yellowish oil (18%) by following the procedure described for **47**. ^{1}H NMR ($CDCl_3$) δ: 1.34 (3H, t, J = 7.3 Hz), 1.49 (9H, s), 2.85 (2H, m), 3.65 (2H, m), 4.26 (2H, q, J = 7.3 Hz), 4.58 (2H, s), 6.41 (1H, d, J = 16.1 Hz), 7.12 (1H, d, J = 8.1 Hz), 7.29 (1H, s), 7.35 (1H, d, J = 8.1 Hz), 7.64 (1H, d, J = 16.1 Hz); MS (FAB) *m/z*: 332 (M^++H).

4.2.5.40. *tert*-Butyl 6-formyl-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (8p). To a solution of **52** (309 mg, 0.93 mmol) in a mixture of THF (4 mL) and H_2O (2 mL) were added OsO_4 (2 mg, 1 mol %) and $NaIO_4$ (1.40 g, 6.55 mmol) slowly, and the mixture was stirred at 50 °C for 3.5 h. After EtOAc and satd $Na_2S_2O_3$ aq were added to the reaction mixture, the two layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layer was washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was chromatographed on silica gel eluted with *n*-hexane/EtOAc (4:1) to afford title compound **8p** (112 mg, 46%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.50 (9H, s), 2.92 (2H, t, J = 5.8 Hz), 3.68 (2H, t, J = 5.8 Hz), 4.65 (2H, s), 7.28 (1H, d, J = 7.3 Hz), 7.66 (1H, s), 7.69 (1H, d, J = 7.3 Hz), 9.96 (1H, s); MS (FAB) *m/z*: 262 (M^++H).

4.2.5.41. 1,2,3,4-Tetrahydroisoquinoline-6-carbaldehyde (6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazone (29). A mixture of **7** (86 mg, 0.39 mmol) and **8p** (112 mg, 0.43 mmol) in benzene (2 mL) was stirred under reflux for 2 h and cooled to room temperature. The reaction mixture was concentrated under reduced pressure and the residue was recrystallized from EtOAc and *n*-hexane to afford *tert*-butyl 6-{[(6-*tert*-butylthieno[2,3-*d*]pyrimidin-4-yl)hydrazono]methyl}-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (87 mg, 49%). To this solution isoquinolinecarboxylate in 1,4-dioxane (1.0 mL) was added 4 N HCl/1,4-dioxane (0.5 mL) at 0 °C. The mixture was stirred at room temperature for 12 h and concentrated under reduced pressure. The residue was recrystallized from EtOAc and *n*-hexane to afford title compound **29** (65 mg, 72%) as an orange solid. ^{1}H NMR (CD_3OD) δ: 1.53 (9H, s), 3.24 (2H, t, J = 6.3 Hz), 3.57 (2H, t, J = 6.3 Hz), 4.45 (2H, s), 7.38 (1H, d, J = 7.8 Hz), 7.84 (1H, s), 7.88 (1H, d, J = 7.8 Hz), 7.89 (1H, s), 8.54 (1H, s), 8.60 (1H, br); IR (ATR) cm^{-1}: 2790, 1635, 1587, 1091; MS (FAB) *m/z*: 366 (M^++H); HRMS (ESI) *m/z*: 366.17526 (calcd for $C_{20}H_{24}N_5S$: 366.17524); Anal. Calcd for $C_{20}H_{23}N_5S\cdot2.25HCl\cdot2H_2O$: C, 49.68; H, 6.10; Cl, 16.50; N, 14.48; S, 6.63. Found: C, 49.89; H, 5.79; Cl, 16.68; N, 14.40; S, 6.66.

4.3. Kinase inhibition assays

CDK4, CDK2, cyclin D, and cyclin E proteins were purified from baculovirus infected Sf-9 insect cells. Glutathione S-transferase Rb (GST-Rb) protein was expressed and purified from bacteria using glutathione-sepharose beads by the standard procedure. Enzyme assays to determine the concentration of compounds that cause 50% of kinase inhibition (IC_{50}) were performed in 96-well filter plates (Whatmann, GF/C filter). The assay mixture (30 μL of 10 mM ATP contained 0.2 mCi [^{33}P]ATP, 30 μL of enzymes (Cdk4/D1 or Cdk2/E), 30 μL of 25% GST-Rb with glutathione sepharose beads in kinase assay buffer (50 mM Hepes pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 5 mg/mL AMSF, 5 mg/mL aprotinine, 0.1 mM NaF, 10 mL β-glycerophosphate, and 0.1 mM sodium-*o*-vanadate) and 10 μL of the tested compound in a final volume of 100 μL) was incubated on the plate for 30 min at 30 °C. The plate was washed for four times, and the kinase activities were measured. The IC_{50} values were determined by analyzing the dose–response inhibition curves.

4.4. Cytotoxicity assay

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay was performed against HCT116 and PC-6 cell lines to examine the growth inhibitory effects of our compounds. HCT116 cell line was purchased from American Type Culture Collection (ATCC, USA). PC-6 cell line was obtained from Immuno-Biological Laboratories (Gunma, Japan). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were plated in 96-well micro plates on day 0, and stock solutions serially diluted in DMSO were added to each well on day 1. After 3 days of culture (day 4), the number of viable cells was determined by the MTT assay. The concentration of compounds that cause 50% of growth inhibition was calculated by the following equation $100 \times [(T - T_0)/(C - T_0)] = 50$ (C: control optical density; T: test optical density; T_0: optical density at time zero).

4.5. Cell cycle analysis

HCT116 cells were treated with test compounds for 16 h and were stained with propidium iodide using a Cycle Test Kit (Becton Dickinson). Then the cells were analyzed using a FACscan flow cytometer with CellQuest software (Becton Dickinson) in accordance with the manufacturer's recommendations. The percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle were quantified using Modifit software (Verity Software House, Inc.).

4.6. Antitumor activity assay

For in vivo studies, HCT116 tumor cells were subcutaneously transplanted into the right hind limb of male BALB/c-nu/nu mice (Japan SLC, Inc.) aged 5 or 6 weeks. After the subcutaneous tumors averaged 80–100 mm^3 in size, the tested compounds dissolved in 20% Captisol® (β-cyclodextrin sulfobutylether sodium salt, CyDex, Inc.) solution were administered intravenously or orally once a day for 4 days at the doses indicated in Table 2. The tumor volume was measured with a caliper periodically.

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