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Discovery of 4-anilino-*N*-methylthieno[3,2-*d*]pyrimidines and 4-anilino-*N*-methylthieno[2,3-*d*]pyrimidines as potent apoptosis inducers

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ABSTRACT

We report the discovery of *N*-((benzo[*d*][1,3]dioxol-5-yl)methyl)-6-phenylthieno[3,2-*d*]pyrimidin-4-amine (**2a**) as an apoptosis inducer using our proprietary cell- and caspase-based ASAP HTS assay, and SAR study of HTS hit **2a** which led to the discovery of 4-anilino-*N*-methylthieno[3,2-*d*]pyrimidines and 4-anilino-*N*-methylthieno[2,3-*d*]pyrimidines as potent apoptosis inducers. Compounds **5d** and **5e** were the most potent with EC_{50} values of 0.008 and 0.004 μM in T47D human breast cancer cells, respectively. Compound **5d** was found to be highly active in the MX-1 breast cancer model. Functionally, compounds **5d** and **5e** both induced apoptosis through inhibition of tubulin polymerization.

Programmed cell death, or apoptosis, plays an important role in the development of cancers.[1] Excessive or improper inhibition of apoptosis could lead to unchecked cell proliferation, and resistance to cancer treatment.[2] In addition, it is known that many anticancer agents, including irinotecan[3] and vinblastine[4] kill tumors at least partially through induction of apoptosis.[5] Further exploration of novel approaches to activate and promote apoptosis in cancer cells,[6] therefore, could lead to the discovery of new anticancer agents.[7]

In our effort to discover and develop novel apoptosis inducers as potential anti-cancer drugs, we have created a cell-based high-throughput screening (HTS) assay for the identification of apoptosis inducers using our novel fluorescent caspase-3 substrate.[8,9] Since caspase-3 is known to be the key effector caspase that cleaves multiple protein substrates in cells leading to cell death,[10] our HTS assay can discover apoptosis inducers that interact with known or unknown targets through activation of any of the proapoptotic pathways upstream of caspase-3.

We have reported the discovery and structure–activity relationship (SAR) studies of several series of apoptosis inducers interacting with either known or novel molecular targets using our HTS assay.[11] These include gambogic acid (**1a**)[12,13] 4-aryl-4*H*-chromenes (**1b**),[14,15] and 3-aryl-5-aryl-1,2,4-oxadiazoles (**1c**)[16,17] (Chart 1). More recently, we have reported the discovery of 2-chloro-4-(4-methoxyanilino)-*N*-methylquinazoline (**1d**)[18] and 4-(4-methoxyanilino)-*N*,2-dimethylquinazoline (**1e**),[19] a clinical candidate, as potent apoptosis inducers with high in vivo efficacies in anticancer xenograft models.[20] Herein we report the discovery of *N*-((benzo[*d*][1,3]dioxol-5-yl)methyl)-6-phenylthieno[3,2-*d*]pyrimidin-4-amine (**2a**) as an apoptosis inducer using our HTS assay, and SAR study around **2a** which led to the discovery of a series of 4-anilino-*N*-methylthieno[3,2-*d*]pyrimidines and 4-anilino-*N*-methylthieno[2,3-*d*]pyrimidines as low nanomolar apoptosis inducers.

Many 4-anilinothieno[3,2-*d*]pyrimidines such as **1g**,[21] **1h**[22] and **1i**[23] (Chart 1) have been reported as potent kinase inhibitors. Structurally related compounds **3a**, **3d** and **3g–3h** were prepared as shown in Scheme 1 using previously reported procedures from reaction of commercially available 4-chlorothieno[3,2-*d*]pyrimidine (**6a**) with the corresponding substituted anilines in anhydrous isopropanol (IPA) in the presence of concentrated HCl.[18,19] *N*-((Benzo[*d*][1,3]dioxol-5-yl)methyl)thieno[3,2-*d*]pyrimidin-4-amine (**2b**) and *N*-(benzo[*d*][1,3]dioxol-6-yl)-6-phenylthieno[3,2-*d*]pyrimidin-4-amine (**2c**, Chart 2) were prepared similarly from reaction of **6a** with (benzo[*d*][1,3]dioxol-5-yl)methanamine, and 4-chloro-6-phenylthieno[3,2-*d*]pyrimidine (**6b**) with benzo[*d*]-[1,3]dioxol-5-amine, respectively. Compound **2a** was obtained from Maybridge. Compounds **3b–3c** and **3e–3f** were prepared in two steps via reaction of **6a** with substituted anilines to produce compounds **7a–7d**, followed by methylation using reported procedures (Scheme 2).[18,19] Compounds **4a**, **4c–4f** were prepared from reaction of the corresponding substituted 4-chlorothieno[3,2-*d*]pyrimidines (**6c–6g**) with *N*-methyl-4-methoxyaniline (Scheme 3). The thieno[2,3-*c*]pyridine analog **4g** (Table 2) was prepared under similar conditions from reaction of 7-chlorothieno[2,3-*c*]pyridine with *N*-methyl-4-methoxyaniline. Compound **4b** was prepared from

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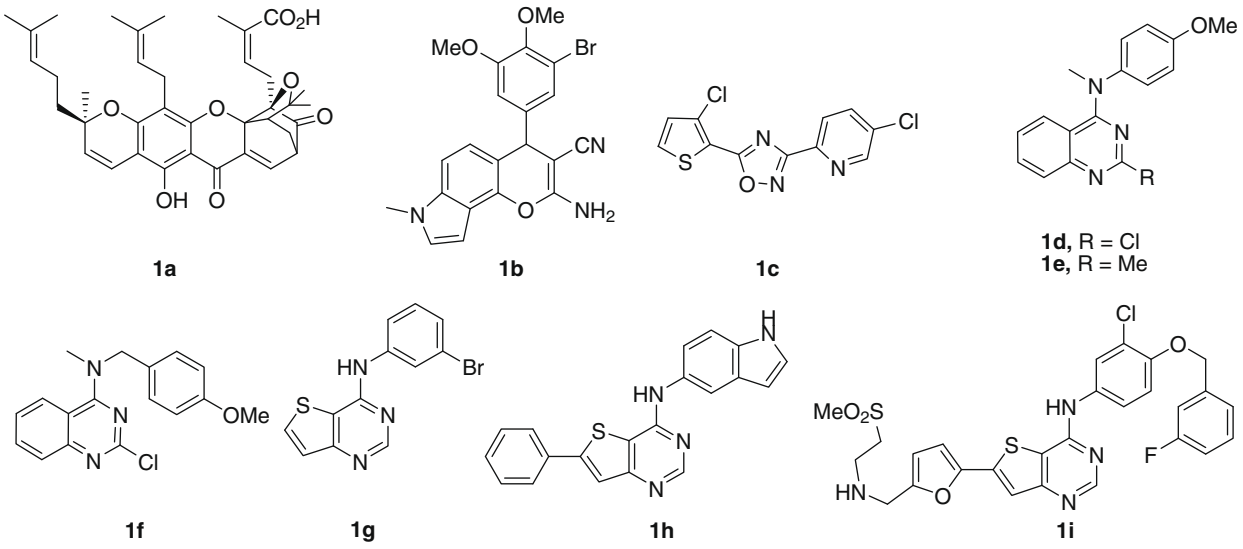
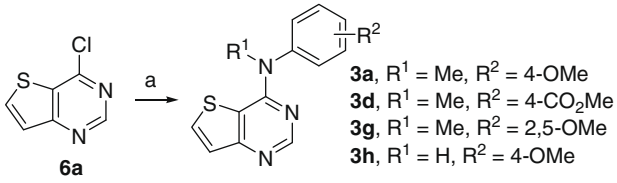


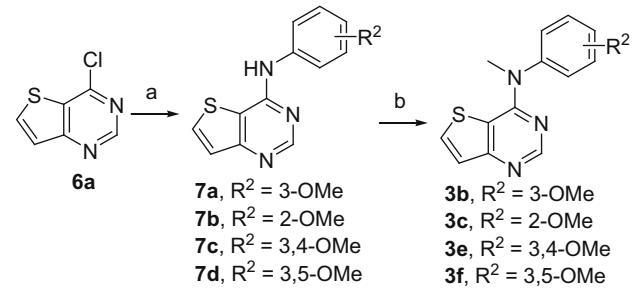
Chart 1.



Scheme 1. Reagents: (a) R^2-Ph-NHR^1, *i*-PrOH, HCl.

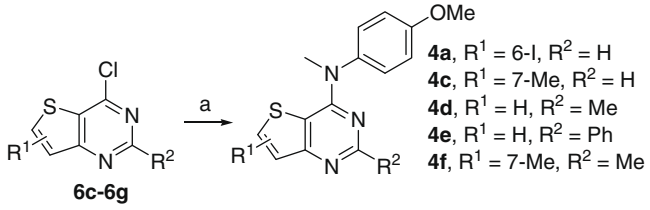
coupling of **4a** with pyridine-3-boronic acid using reported procedure (Scheme 4).[22] Compounds **5a–5h** were prepared from reaction of the commercial available substituted 4-chlorothieno[2,3-*d*]pyrimidines (**8a–8f**) with *N*-methyl-4-methoxyaniline or 4-methoxyaniline, respectively (Scheme 5).[24]

The apoptosis inducing activity of 4-anilino-*N*-methylthieno[3,2-*d*]pyrimidines, 4-anilino-*N*-methylthieno[2,3-*d*]pyrimidines and related compounds was measured by our cell- and caspase-based HTS assay[25] in two cell lines, human breast cancer cells T47D and human non-small cell lung cancer cells H1299, and the results are summarized in Tables 1–3. The screening hit **2a** had an EC_{50} value of 1 μM in T47D cells. Compound **2a** has a *C* log *P* value of 5.4, which is considered high for a drug candidate. We first explored the removal of the bulky 6-phenyl group. Unfortunately, compound **2b** was found to be inactive in T47D cells up to 10 μM. To make the molecule more rigid, we eliminated the methylene linker in **2a**, resulting in **2c** that also was inactive in T47D cells. We observed some structural similarity between **2b**–**2c** and **1d**–**1e** and decided to introduce a methyl group into the nitrogen of the anilino group (Chart 2), which was known to be important for the apoptotic activity of **1d** and **1e**.[18,19] Since compound **1f**, with an extra methylene linker, has been found



[a]Conditions: a) R^2-Ph-NH_2, *i*-PrOH, HCl; b) MeI, NaH, DMF

Scheme 2. Reagents: (a) R^2-Ph-NH_2, *i*-PrOH, HCl; (b) MeI, NaH, DMF.



Scheme 3. Reagents: (a) 4-OMe-Ph-NHMe, *i*-PrOH, HCl.

to be >30-fold less active than compound **1d**,[18] we decided to concentrate our SAR studies on compounds with an anilino and not with a benzylamino group.

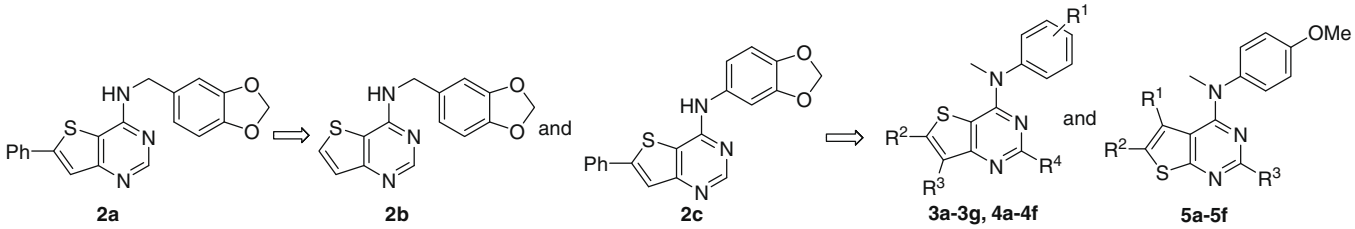
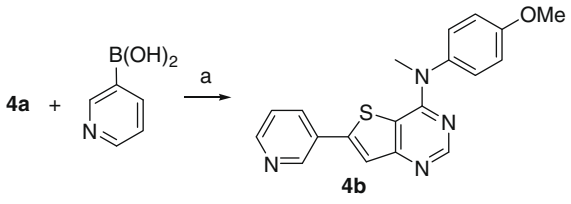
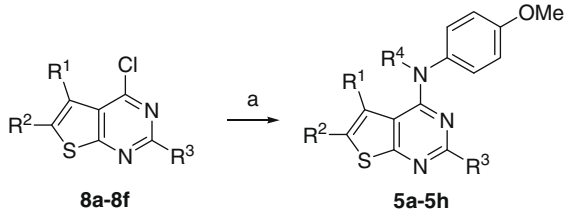


Chart 2.

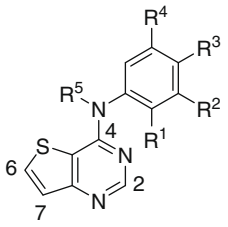


Scheme 4. Reagents: (a) DMF, K_2CO_3, DPPF, $(PhCN)_2PdCl_2$.



Scheme 5. Reagents: (a) 4-OMe-Ph-NHMe or 4-OMe-Ph-NH_2, *i*-PrOH, HCl.

Table 1
Activity of 4-anilinothieno[3,2-*d*]pyrimidines and related compounds in the caspase activation assay

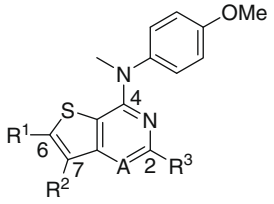


Compd #	R^1	R^2	R^3	R^4	R^5	EC_{50}[a] (μM)	
						T47D	H1299
2a	NA[b]	NA	NA	NA	NA	1.0 ± 0.1	0.70 ± 0.06
2b	NA	NA	NA	NA	NA	>10	>10
2c	NA	NA	NA	NA	NA	>10	>10
3a	H	H	OMe	H	Me	0.052 ± 0.004	0.24 ± 0.10
3b	H	OMe	H	H	Me	>10	>10
3c	OMe	H	H	H	Me	>10	>10
3d	H	H	CO_2Me	H	Me	0.19 ± 0.01	0.34 ± 0.01
3e	H	OMe	OMe	H	Me	1.7 ± 0.1	2.8 ± 0.2
3f	H	OMe	H	OMe	Me	>10	>10
3g	OMe	H	H	OMe	Me	2.6 ± 0.1	5.0 ± 0.1
3h	H	H	OMe	H	H	>10	>10

[a] Cells were treated with the test compounds for 48 h, data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).
[b] NA, not applied, please see Chart 2 for structure.

Table 1 shows that compound **3a**, with an *N*-methyl group and a 4-methoxy group at the 4-position of the anilino ring, was highly active with an EC_{50} value of 0.052 μM in T47D cells. In comparison, compound **3h**, without the *N*-methyl group, was inactive up to 10 μM, at least 200-fold less active than that of **3a**, indicting that the *N*-methyl group is critical for the apoptotic activity of 4-anilino-*N*-methylthieno[3,2-*d*]pyrimidines. Exploration of substitution at the anilino ring (**3b**–**3g**) showed that a small substitution at the 4-position was important for apoptotic activity, while substitutions at the 2- and 3-positions were not preferred. The SAR for substitutions at the anilino ring is similar to that of 4-anilino-*N*-methylquinazolines,[18,19] indicating that a thieno[3,2-*d*]pyrimidine ring system can be used to replace the quinazoline ring system in **1d** and **1e**.

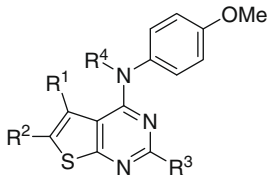
Table 2
Activity of 4-(4-methoxyanilino)-*N*-methylthieno[3,2-*d*]pyrimidines and related compound in the caspase activation assay



Compd #	R^1	R^2	R^3	A	EC_{50}[a] (μM)	
					T47D	H1299
4a	I	H	H	N	>10	>10
4b	3-Py[b]	H	H	N	>10	>10
4c	H	Me	H	N	0.56 ± 0.04	0.56 ± 0.04
4d	H	H	Me	N	0.014 ± 0.001	0.032 ± 0.008
4e	H	H	Ph	N	0.34 ± 0.01	0.63 ± 0.02
4f	H	Me	Me	N	0.24 ± 0.01	0.31 ± 0.03
4g	H	H	H	C	>10	>10

[a] Cells were treated with the test compounds for 48 h, data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).
[b] 3-Pyridyl.

Table 3
Activity of 4-(4-methoxyanilino)thieno[2,3-*d*]pyrimidines in the caspase activation assay



Compd #	R^1	R^2	R^3	R^4	EC_{50}[a] (μM)	
					T47D	H1299
5a	H	H	H	Me	0.022 ± 0.003	0.044 ± 0.004
5b	Me	H	H	Me	0.015 ± 0.003	0.025 ± 0.007
5c	Me	Me	H	Me	0.024 ± 0.004	0.053 ± 0.002
5d	H	H	Me	Me	0.008 ± 0.0002	0.016 ± 0.001
5e	Me	H	Me	Me	0.004 ± 0.0001	0.015 ± 0.003
5f	Me	Me	Me	Me	0.021 ± 0.005	0.091 ± 0.032
5g	Me	H	H	H	>10	>10
5h	Me	H	Me	H	>10	>10
1e	NA[b]	NA	NA	NA	0.002 ± 0.0001	0.006 ± 0.001
Colchicine	NA	NA	NA	NA	0.009 ± 0.001	0.05 ± 0.01

[a] Cells were treated with the test compounds for 48 h, data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).
[b] NA, not applied.

Keeping the preferred *N*-methyl-4-(4-methoxyanilino) group at the 4-position, we explored substitutions at the 2-, 6- and 7-positions of the thieno[3,2-*d*]pyrimidine ring (Table 2). Compounds **4a** and **4b**, with an iodo or 3-pyridyl group in the 6-position, were found to be inactive up to 10 μM in T47D cells, suggesting that substitution at the 6-position might not be tolerated. Compound **4c**, with a methyl group at the 7-position, had an EC_{50} value of 0.56 μM, about 10-fold less active than **3a**, indicating that substitution at the 7-position is better tolerated than the 6-position. The 2-methyl analog **4d** was found to be about fourfold more potent than **3a**, while the 2-phenyl analog **4e** was >20-fold less active than **4d**, indicating that a small group such as methyl at the 2-position might be preferred, which is similar to the reported 2-position

SAR of 4-anilino-*N*-methylquinazolines.[18,19] The 2,7-dimethyl analog **4f** was >2-fold more active than **4c**, confirming that the 2-methyl group contributed positively to the apoptotic activity. The thieno[2,3-*c*]pyridine analog **4g** was inactive up to 10 μM, indicating that the nitrogen at the 1-position is important for activity.

We then explored the replacement of the thieno[3,2-*d*]pyrimidine system in **3a** by the isosteric thieno[2,3-*d*]pyrimidine system (Table 3). Compound **5a** was >2-fold more potent than **3a**, suggesting that the thieno[2,3-*d*]pyrimidine system might be preferred over the thieno[3,2-*d*]pyrimidine system for apoptotic activity. By maintaining the *N*-methyl-4-(4-methoxyanilino) group at the 4-position, substitutions at the 2-, 5- and 6-positions of the thieno[2,3-*d*]pyrimidine ring were explored. The 5-methyl analog **5b** was slightly more active than **5a**, indicating that a small group is preferred at the 5-position. The 5,6-dimethyl analog **5c** was about twofold less active than **5b**, indicating that a small group at the 6-position is well tolerated, and suggesting that the SAR of the 6-position of 4-anilino-*N*-methylthieno[2,3-*d*]pyrimidines is not the same as that of 4-anilino-*N*-methylthieno[3,2-*d*]pyrimidines. The 2-methyl analog **5d** was about threefold more potent than **5a**, which is similar to the observed 2-position SAR of 4-anilino-*N*-methylthieno[3,2-*d*]pyrimidines as well as that of 4-anilino-*N*-methylquinazolines.[18,19] Combining the preferred methyl group at the 2- and 5-positions led to compound **5e**, which was highly active with an EC_{50} value of 0.004 μM in T47D cells, a potency approaching that of **1e**. The 2,5,6-trimethyl analog **5f** was several-fold less active than **5e**. Compounds **5g** and **5h**, without the *N*-methyl group, were both inactive up to 10 μM, >500-fold less active than the corresponding *N*-methyl analogs **5b** and **5e**, confirming that similar to the SAR of 4-anilino-*N*-methylquinazolines,[18,19] the *N*-methyl group is critical for apoptotic activity of 4-anilino-*N*-methylthieno[3,2-*d*]pyrimidines as well as that of 4-anilino-*N*-methylthieno[2,3-*d*]pyrimidines.

The activities of these compounds towards the human non-small cell lung cancer cell line H1299 roughly parallel the activity in T47D cells. The compounds that were inactive in T47D cells also were inactive in H1299 cells. Compounds **5d** and **5e**, the most active ones in this series against T47D cells, were also the most active ones against H1299 cells with EC_{50} values of 0.016 and 0.015 μM, respectively. In general, H1299 cells were slightly less sensitive (about two to fourfold less as indicated by the higher EC_{50} values) to the compounds than T47D cells in this assay.

We have found that **1d**, **1e** and related compounds are tubulin inhibitors that bind at or close to the colchicine site of β-tubulin.[18,19] Compounds **3a**, **4d** and **5a**–**5f**, which were highly active in the caspase activation assay, were tested in the tubulin polymerization assay.[26] All the compounds were found to inhibit tubulin polymerization with IC_{50} values of less than 1 μM, which is similar to that of compounds **1d** and **1e**. In comparison, colchicine, a well known tubulin inhibitor was found to be highly active in our caspase activation assay with an EC_{50} value of 9 nM against T47D cells (Table 3), and had a similar potency in the tubulin assay (0.5 μM). Therefore these 4-(4-methoxyanilino)-*N*-methylthieno[3,2-*d*]pyrimidines and 4-(4-methoxyanilino)-*N*-methylthieno[2,3-*d*]pyrimidines most probably induce apoptosis through inhibition of tubulin polymerization.

Compound **5d** was tested in a MX-1 xenograft breast cancer model. The MX-1 in vivo experiment was performed as described previously.[18] Compound **5d** inhibited tumor growth dose dependently and produced 90% tumor growth inhibition when dosed intravenous with a single administration at a dose of 75 mg/kg (Fig. 1), and is well tolerated with maximum body weight decrease of <10%.

In conclusion, we have discovered a series of 4-anilino-*N*-methylthieno[3,2-*d*]pyrimidines and 4-anilino-*N*-methylthieno[2,3-*d*]pyrimidines as potent apoptosis inducers. 4-(4-Methoxyanilino)-*N*,2,5-trimethylthieno[2,3-*d*]pyrimidine **5e** was found to be the most potent compound having an EC_{50} value of 0.004 μM in T47D cells, and to inhibit tubulin polymerization, which most probably is its main mechanism of action for apoptosis induction. Compound **5d**, one of the potent apoptosis inducers identified in the series, was highly efficacious in a MX-1 human xenograft model and could be a potential clinical candidate.

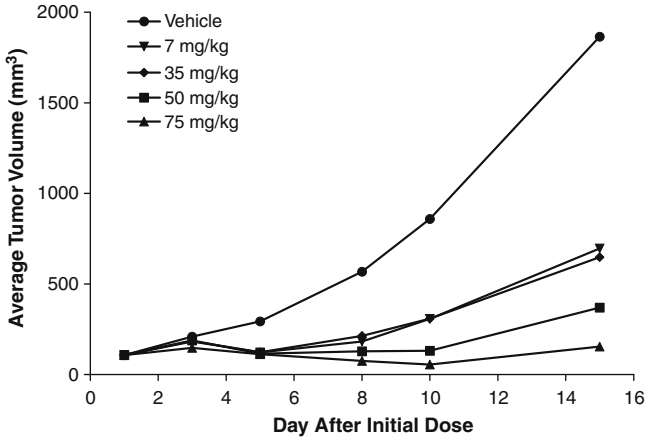


Figure 1. Compound **5d** inhibited the growth of established (~100 mm^3) MX-1 tumor xenografts in Crl:Nu/Nu-nuBR mice. Compound **5d** was dosed iv at 7 mg/kg intravenous days 1–5, or 35, 50 and 75 mg/kg single intravenous administrations at day 1. *P* value as calculated by Student's *t*-test is <0.001 for 75 mg/kg.

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24. Synthesis procedure for *N*-(4-methoxyphenyl)-*N*,2-dimethylthieno[2,3-*d*]pyrimidin-4-amine (**5d**). To an oven-dried one-neck reaction flask charged with a magnetic stir bar at room temperature under argon was added 4-chloro-2-methylthieno[2,3-*d*]pyrimidine (0.100 g, 0.542 mmol), isopropanol (2.7 mL), *N*-methyl-*p*-anisidine (0.082 g, 0.60 mmol) and 2.0 M HCl in ether (0.260 mL). The brown solution was heated at 80 °C for 4 h, cooled to rt and diluted with EtOAc (70 mL). The organic layer was washed with saturated $NaHCO_3$ (2 × 25 mL), brine (2 × 20 mL), dried over Na_2SO_4, filtered and concentrated to yield a brown oil. Purification by flash column chromatography (4 g pre-packed silica gel column, elution with EtOAc:hexanes, 1:9) gave 0.047 g (30%) of **5d** as a white solid: mp 87–88 °C; ^{1}H NMR ($CDCl_3$): 7.20–7.17 (m, 2H), 6.97–6.94 (m, 2H), 6.73 (dd, *J* = 6.0 and 0.5 Hz, 1H), 5.55 (dd, *J* = 6.0 and 0.8 Hz, 1H), 3.86 (s, 3H), 3.55 (s, 3H), 2.65 (s, 3H).
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