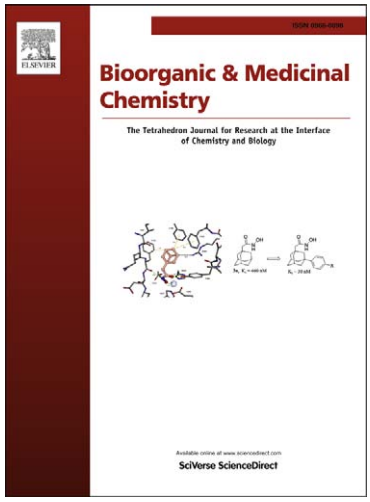

Design, synthesis and biological evaluation of novel 3,4,5-trisubstituted aminothiophenes as inhibitors of p53-MDM2 interaction. Part 2

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Design, synthesis and biological evaluation of novel 3,4,5-trisubstituted aminothiophenes as inhibitors of p53-MDM2 interaction. Part 2

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Abstract:

Five series of novel 3,4,5-trisubstituted aminothiophene derivatives and analogs were designed and synthesized based on our previous studies. All target compounds were evaluated for their p53-MDM2 binding inhibitory activities and anti-proliferation activities against A549 and PC3 tumor cell lines. Twelve compounds displayed comparable p53-MDM2 binding inhibitory activities to that of Nutlin-3. Among them, compound **7a** exhibited marked binding affinity (IC_{50} = 0.086μM). In addition, most target compounds showed potent anti-proliferation activities with IC_{50} values at low micromolar level. A good selective profile for wild-type p53 expression cell line was also observed. Molecular docking analysis was performed as well to predict possible binding modes of target compounds with MDM2.

Keywords: 3,4,5-trisubstituted aminothiophenes, P53-MDM2 interaction, Anti-cancer, SARs.

1. Introduction

P53, the "guardian of the genome", is a transcriptional regulated protein which is activated by a number of genotoxic factors[1]. In response to stress, p53 promotes the transcription of target genes responsible for cell cycle arrest, DNA repair and apoptosis[2, 3]. However, in approximately 50% of all human tumors, p53 is inactivated by mutation or deletion[4]. The remaining half of human tumors expresses wild-type p53, which is often down-regulated by other mechanisms. One of the main reasons involves the oncoprotein MDM2 (murine double minute 2, also frequently referred to as HDM2 in human), a master regulator of p53[5-7]. In the last decade, the identification of several kinds of potent small molecule p53-MDM2 binding inhibitors has demonstrated that reactivation of p53 by disrupting the p53-MDM2 interaction is a promising anticancer therapeutic strategy[8-10].

We have reported the identification of a 3,4,5-trisubstituted aminothiophene derivative MCL0527 as a lead compound (Fig. 1)[11]. And the previous chemical modification and structure-activity relationship studies of MCL0527, focusing on the 2-amino and 3-carboxy groups of thiophene ring, have yielded several inhibitors with improved potency[12]. Herein, with the attempt to find better p53-MDM2 binding inhibitors, the continuous chemical modification and optimization of MCL0527 was carried out. Since the 4'-chlorophenyl at the 4-position of thiophene ring, serving as a mimic of Trp23 residue, is a privileged structure available in many kinds of p53-MDM2 binding inhibitors, our modification is focused on the optimization of 5-substituent, 3-ester on thiophene ring and core structure. Firstly, retaining the 3,4,5-trisubstituted aminothiophene scaffold, the chlorine atom on the 4'-chlorophenyl at the 5-position of thiophene ring was replaced. Secondly, in view of the wide application of amide or thioamide in drug discovery, the amide or thioamide derivatives were prepared based on the principle of bioisosterism. Thirdly, the core structure thiophene ring was changed into pyrrole ring or fused rings to investigate corresponding impacts on potency. All synthesized compounds were evaluated *in vitro* for their p53-MDM2 binding inhibitory activities and anti-proliferation activities against two human cancer cell lines. Besides, molecular docking analysis was performed to determine possible binding modes of target compounds with MDM2.

2. Results and discussion

2.1. Chemistry

3,4,5-trisubstituted aminothiophenes were prepared using the method described previously[11]. The appropriate phenylacetic acids were converted into acyl chlorides (**1a-e**), which reacted with chlorobenzene to give corresponding Friedel–Crafts acylation products (**2a-e**). Condensation of acylation compounds (**2a-e**) with methyl cyanoacetate or 2-cyanoacetamide derivatives in the presence of $TiCl_4$ afforded olefin intermediates (**3**)[13]. Cyclization of olefin intermediates (**3)** with elemental sulfur and diethylamine yielded target compounds (**4a-y**, Scheme 1). Some of the potent compounds (**4f**, **4g**, **5** and **6**)[11,12] were treated with Lawesson's Reagent to afford thioamide derivatives (**7a-d,** Scheme 2).

The ketone **2b** was brominated to give bromoketone **8**[14], which subsequently reacted with ethyl 3-amino-3-iminopropanoate or 3-amino-N-cyclopropyl-3-iminopropanamide[15] to yield the 3,4,5-trisubstituted aminopyrroles (**10a**, **b**, Scheme 3).

Hydrolysis of **4e** with 2N NaOH aqueous solution afforded the carboxylic acid derivative **11**, which subsequently reacted with Meldrum's acid and corresponding aldehydes to give

dihydrothieno[2,3-b]pyridones (**12a-c**). Alkylation of **12a, b** with bromochloropropane, followed by reaction with morpholine or piperidine, yielded the target compounds **13a, b** (Scheme 4).

Reaction of **14** with formic acid and acetic anhydride gave thieno[2,3-d]pyrimidone (**15**). Treatment of **15** with phosphorus oxychloride yielded chlorinated intermediate **16**, which subsequently reacted with corresponding amines to afford thieno[2,3-d]pyrimidine derivatives (**17a-d**, Scheme 5).

2.2. p53-MDM2 binding inhibitory activities

All synthesized target compounds were evaluated for their p53-MDM2 binding inhibitory activities by fluorescence-polarization based binding assay (FP assay)[16, 17]. Nutlin-3 was used as positive control. The results are summarized in Table 1-3.

As shown in Table 1, in the series of 3,4,5-trisubstituted aminothiophenes, 4'-chloro (**4e-k**), 4'-fluoro (**4l-o**), 4'-bromo (**4p-t**) or no substituents (**4a-d**) on the phenyl at 5-position of thiophene ring generally imparted equally positive effect to the binding affinities. Introducing nitro group (**4u-y**) to the 4'-position of the phenyl led to a sudden drop of potency. Retaining the 4'-chlorophenyl group at the 5-position of thiophene ring, replacing 3-carboxamide with thioamide yielded an additive effect on potency (**7a-d**). For example, the thioamide derivative **7a** (*Ki* = 0.086 μM) is 6 times more potent than parent compound **4f** (*Ki* = 0.54 μM). Replacing the thiophene ring of compound **4f** with pyrrole ring, compound **10b** (*Ki* = 0.53 μM) still possessed significant binding inhibitory activities. However, most of dihydrothieno[2,3-b]pyridone and thieno[2,3-d]pyrimidine derivatives showed poor p53-MDM2 binding inhibitory activities (Table 2 and 3).

2.3. Tumor cell growth inhibition studies

The obtained target compounds were tested for their tumor cell growth inhibitory activity *in vitro* against two human cancer cell lines, including human lung adenocarcinoma epithelial cells A549 (wild-type p53) and human pancreatic carcinoma cells PC3 (p53 null). The results are summarized in Table 1-3.

In general, most of the tested compounds showed good inhibitory selectivity for wild-type p53 expression cell line A549 over p53 null cell line PC3. Particularly, compound **4l**, **4r**, **4s** and **10a** displayed more than 5-fold inhibitory selectivity for A549 over PC3, better than that of Nutlin-3. Over 10 compounds exhibited comparable anti-proliferation activities to that of Nutlin-3 against A549 cell line. Especially, compound **10a** showed the best growth inhibitory activity (IC_{50} = 2.53 μM). Most of the thioamide derivatives (**7a-d**) did not exhibit satisfactory anti-proliferation activities, possibly due to the poor solubility. Replacing amide linkage with thioamide led to an increase in liposolubility. For instance, the *C*lo*P* value of thioamide **7a** (*C*lo*P* = 6.19) is much higher than that of amide **4f** (*C*lo*P* = 5.38). On the other hand, dihydrothieno[2,3-b]pyridones **12a-c** and thieno[2,3-d]pyrimidines **17a-d** displayed weak *in vitro* anti-proliferation activities, which were consistent with the MDM2 binding assay results. Interestingly, 3,4,5-trisubstituted aminopyrroles (**10a**, **b**) showed good performance in not only p53-MDM2 binding inhibition but also tumor cell anti-proliferation . Therefore, they can be utilized as novel leads for further investigation.

2.4. Molecular docking studies and structural alignment analysis

To examine possible binding modes of target compounds (e.g., **7a** and **10b**) with MDM2, molecular docking studies were conducted utilizing CDOCKER programme within Discovery Studio 2.1 software package. The published X-ray crystal structure of MDM2 (PDB ID:1YCR) was used for docking calculation. The docking models (Fig. 2) demonstrated that thioamide derivative **7a** and 3,4,5-trisubstituted aminopyrrole derivative **10b** could mimic the p53 peptide to interact with MDM2 protein. Generally, the interactions are nonspecific van der Waals contacts. The binding modes involved three hydrophorbic pockets (Phe19, Trp23 and Leu26) that were filled by one cyclopropyl and two 4'-chlorophenyl groups of **7a** and **10b**, respectively. The pyrrole ring served as a core structure, just like the thiophene ring, which played the role of projecting three hydrophobic substituents into the MDM2 binding cleft at proper directions. The docking model also illustrated that the thioamide moiety, as a bioisostere of amide, was easier to be exposed to the solvents, which may explain the fact that the binding affinity of compound **7a** is more potent than that of **4f**. In addition, the NH of pyrrol ring (**10b**) was found exposed to the solvents in the molecular docking results, where hydrosoluble side chains could be attached to adjust the molecular drug-like properties.

With the attempt to explain the sudden drop of potency observed in 2,3,4-trisubstituted dihydrothieno[2,3-b]pyridones and 4,5,6-trisubstituted thieno[2,3-d]pyrimidines, molecular alignment was carried out. Structural energy minimization of thioamide derivative **7a**, dihydrothieno[2,3-b]pyridone derivative **12c** (*S* and *R* isomers) and thieno[2,3-d]pyrimidine derivative **17a** was performed. The minimized conformers of **12c** and **17a** were aligned with the most potent compound **7a**, respectively, by fitting with thiophene ring skeleton as a common structure. As shown in Fig. 3, although the bis-chloro-phenyl structures could match well, the furane of **(*S*)-12c** and **(*R*)-12c** and the cyclopropyl of **17a** displayed dramatically different spatial orientation compared to the cyclopropyl group of **7a**. It means that both core structures of **12c** and **17a** may not be able to project the furane ring or cyclopropyl group into the MDM2 binding pocket at proper direction, even leading to surface contact with MDM2, which are responsible for the loss of potency.

3. Conclusion

Five series of novel 3,4,5-trisubstituted aminothiophene derivatives and analogs were designed and synthesized as novel p53-MDM2 binding inhibitors. *In vitro* biological evaluation for their p53-MDM2 binding inhibitory activities and anti-proliferation activities revealed that twelve compounds displayed comparable p53-MDM2 binding inhibitory activities to that of Nutlin-3 and most tested compounds showed potent tumor cell anti-proliferation activities with IC_{50} values at low micromolar level. Meanwhile, a good selective profile for wild-type p53 expression cell line was observed. Compound **7a,** a thioamide derivative, exhibited the most potent MDM2 binding affinity with a *Ki* value of 0.086 μM. Interestingly, 3,4,5-trisubstituted aminopyrroles (**10a**, **b**) showed good performance not only in p53-MDM2 binding inhibition but also in tumor cell anti-proliferation. Therefore, they can be utilized as novel leads for further investigation. Molecular docking analysis further suggested the binding modes of **7a** and **10b** with MDM2. This study might be useful in future development of novel p53-MDM2 binding inhibitors.

4. Experimental

4.1. Chemistry

Melting points were determined with a B-540 Büchi apparatus and are uncorrected. NMR spectra were recorded on a Brüker 500 (500 MHz) spectrometer (chemical shifts are given in ppm(δ) relative to TMS as internal standard, coupling constants (J) are in hertz (Hz), and signals are using the following abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet, etc. Mass spectra (MS), ESI (positive) were recorded on an Esquire-LC-00075 spectrometer. Thin layer chromatography was carried out using plate silica gel F254 Merck. Reagents and solvents were purchased from common commercial suppliers and were used without further purification. All yields are unoptimized and generally represent the result of a single experiment.

*4.1.1. Synthesis of 1-(4-chlorophenyl)-2-phenylethanone derivatives (**2a-e**) and 2-cyanoacetamide derivatives were exactly following the procedures of reference 18 and 19*[18, 19].

*4.1.2. General procedure for the synthesis of 3,4,5-trisubstituted aminothiophene derivatives (**4a-y**)*

A mixture of 1-(4-chlorophenyl)-2-phenylethanone (**2**, 1.1 mmol) and 2-cyanoacetamide (2.2 mmol) in THF (10 mL) was slowly added to a solution of $TiCl_4$ (5.5 mmol) in dry THF (20 mL) at 0℃. After removing the ice bath, pyridine (0.2 mL) was added. And the reaction mixture was stirred at room temperature overnight. The progress of the reaction was monitored by TLC. After completion of the reaction, THF was removed under reduced pressure and the residue was dissolved in ethyl acetate (30 mL), washed with water (2 x 30 mL) and brine (2 x 30 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude olefin intermediate (**3**) was used for the next step without purification. To a stirred solution of the crude product (**3**) in THF (20 mL), elemental sulfur (2.2 mmol) and diethylamine (0.2 mL) were added. The reaction mixture was stirred at room temperature for 2~4h. THF was removed under reduced pressure and the residue was dissolved in ethyl acetate (20 mL), washed with water (2 x 20 mL) and brine (2 x 20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude products were purified by column chromatography in gradient elution to give the target compounds (**4a-y**).

*4.1.2.1. 2-Amino-4-(4-chlorophenyl)-N-cyclopropyl-5-phenylthiophene-3-carboxamide (**4a**).* Yellow solid (81%), mp: 183 ~ 185 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.20 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.16 (m, 3H, Ar-H), 7.00 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.19 (br, 2H, NH_2), 4.88 (s, 1H, NH), 2.60 (m, 1H, CH), 0.63 (m, 2H, CH_2), 0.04 (m, 2H, CH_2). ESI-MS: m/z = 369 $[M+1]^+$.

*4.1.2.2. (2-Amino-4-(4-chlorophenyl)-5-phenylthiophen-3-yl)(pyrrolidin-1-yl)methanone (**4b**).* Yellow solid (72%), mp: 187 ~ 189 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 (m, 5H, Ar-H), 7.16 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.10 (d, *J* = 8.0 Hz, 2H, Ar-H), 3.50 (m, 4H, $2CH_2$), 1.60 (m, 4H, $2CH_2$). ESI-MS: m/z = 383 $[M+1]^+$.

*4.1.2.3. (2-Amino-4-(4-chlorophenyl)-5-phenylthiophen-3-yl)(morpholino)methanone (**4c**).* Yellow solid (70%), mp: 203 ~ 205 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.24 (d,*J* = 8.5 Hz, 2H, Ar-H), 7.21(m, 3H, Ar-H), 7.10 (d,*J* = 8.4 Hz, 2H, Ar-H), 7.09 (d,*J* = 6.6 Hz, 2H, Ar-H), 3.36 (m, 8H, $4CH_2$). ESI-MS: m/z = 399 $[M+1]^+$.

*4.1.2.4. 2-Amino-N-benzyl-4-(4-chlorophenyl)-5-phenylthiophene-3-carboxamide (**4d**).* Yellow

solid (66%), mp: 186 ~ 188 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.28 (m, 4H, Ar-H), 7.16 (m, 6H, Ar-H), 6.98 (dd, *J* = 7.6, 1.8 Hz, 2H, Ar-H), 6.94 (dd, *J* = 7.6, 1.8 Hz,, 2H, Ar-H), 6.34 (br, 2H, NH_2), 5.08 (t, *J* = 5.2 Hz, 1H, NH), 4.28 (d, *J* = 5.2 Hz, 2H, CH_2). ESI-MS: m/z = 419 $[M+1]^+$.

4.1.2.5. 2-Amino-4-(4-chlorophenyl)-N-cyclopropyl-5-(4-fluorophenyl)thiophene-3-carboxamide ***(4l)***. White solid (63%), mp: 175 ~ 176 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.18 (d, *J* = 6.4 Hz, 2H, Ar-H), 6.94 (d, *J* = 6.5 Hz, 2H, Ar-H), 6.87 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.11 (br, 2H, NH_2), 4.88 (s, 1H, NH), 2.58 (dt,*J* = 10.4, 3.4 Hz, 1H, CH), 0.68 (m, 2H, CH_2), 0.06 (m, 2H, CH_2). ESI-MS: m/z = 387 $[M+1]^+$.

4.1.2.6. (2-Amino-4-(4-chlorophenyl)-5-(4-fluorophenyl)thiophen-3-yl)(pyrrolidin-1-yl)methanone ***(4m)***. Yellow solid (61%), mp: 185 ~ 186 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.20 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.11 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.05 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.90 (d, *J* = 8.4 Hz, 2H, Ar-H), 3.42 (m, 4H, $2CH_2$), 1.58 (m, 4H, $2CH_2$). ESI-MS: m/z = 401 $[M+1]^+$.

4.1.2.7. (2-Amino-4-(4-chlorophenyl)-5-(4-fluorophenyl)thiophen-3-yl)(morpholino)methanone ***(4n)***. White solid (78%), mp: 190 ~ 191 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.08 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.03 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.90 (d, *J* = 8.6 Hz, 2H, Ar-H), 3.60 (m, 8H, $4CH_2$). ESI-MS: m/z = 417 $[M+1]^+$.

4.1.2.8. 2-Amino-N-benzyl-4-(4-chlorophenyl)-5-(4-fluorophenyl)thiophene-3-carboxamide ***(4o)***. White solid (81%), mp: 192 ~ 194 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 (m, 3H, Ar-H), 7.18 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.12 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.93 (m, 4H, Ar-H), 6.84 (m, 2H, Ar-H), 5.07 (t, *J* = 5.1 Hz, 1H, NH), 4.27 (d, *J* = 5.2 Hz, 2H, CH_2). ESI-MS: m/z = 437 $[M+1]^+$.

4.1.2.9. 2-Amino-5-(4-bromophenyl)-4-(4-chlorophenyl)-N-cyclopropylthiophene-3-carboxamide ***(4p)***. Yellow solid (56%), mp: 182 ~ 184 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.12 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.11 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.92(d, *J* = 8.5 Hz, 2H, Ar-H), 5.97 (br, 2H, NH_2), 4.87 (s, 1H, NH), 2.58 (dt,*J* = 10.4, 3.4 Hz, 1H, CH), 0.65 (m, 2H, CH_2), 0.04 (m, 2H, CH_2). ESI-MS: m/z = 447 $[M+1]^+$.

4.1.2.10. 2-Amino-5-(4-bromophenyl)-4-(4-chlorophenyl)-N-cyclohexylthiophene-3-carboxamide ***(4q)***. Yellow solid (59%), mp: 192 ~ 194 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.16 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.12 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.91 (d, *J* = 8.6 Hz, 2H, Ar-H), 4.80 (d, *J* = 7.5 Hz, 1H, NH), 3.72 (m, 1H, CH), 1.61 (m, 2H, CH_2), 1.45 (m, 1H, CH_2), 1.35 (m, 2H, CH_2), 1.25 (m, 2H, CH_2), 1.09 (m, 1H, CH_2), 0.75 (m, 2H, CH_2). ESI-MS: m/z = 489 $[M+1]^+$.

4.1.2.11.

(2-Amino-5-(4-bromophenyl)-4-(4-chlorophenyl)thiophen-3-yl)(pyrrolidin-1-yl)methanone ***(4r)***. Yellow solid (50%), mp: 195 ~ 197 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36 (d,*J* = 8.4 Hz, 2H, Ar-H), 7.17 (d,*J* = 8.5 Hz, 2H, Ar-H), 7.06 (d,*J* = 8.4 Hz, 2H, Ar-H), 6.99 (d,*J* = 8.5 Hz, 2H, Ar-H), 3.69 (m, 4H, $2CH_2$), 1.59 (m, 4H, $2CH_2$). ESI-MS: m/z = 461 $[M+1]^+$.

4.1.2.12. (2-Amino-5-(4-bromophenyl)-4-(4-chlorophenyl)thiophen-3-yl)(morpholino)methanone ***(4s)***. Yellow solid (61%), mp: 200 ~ 201 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42 (d,*J* = 8.1 Hz, 2H, Ar-H), 7.17 (d,*J* = 8.2 Hz, 2H, Ar-H), 7.03 (d,*J* = 8.2 Hz, 2H, Ar-H), 6.98 (d,*J* = 8.2 Hz, 2H, Ar-H), 3.70 (m, 8H, $4CH_2$). ESI-MS: m/z = 477 $[M+1]^+$.

4.1.2.13. 2-Amino-N-benzyl-5-(4-bromophenyl)-4-(4-chlorophenyl)thiophene-3-carboxamide ***(4t)***. Yellow solid (55%), mp: 223 ~ 225 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.31 (m, 3H, Ar-H), 7.10 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.06 (d, *J* = 8.3 Hz, 2H, Ar-H), 6.93 (m, 2H, Ar-H), 6.88 (d, *J* = 8.5 Hz, 2H, Ar-H), 5.05 (t, *J* = 4.7 Hz, 1H, NH), 4.26 (d, *J* = 5.0 Hz, 2H, CH_2). ESI-MS: m/z = 497 $[M+1]^+$.

*4.1.2.14. 2-Amino-4-(4-chlorophenyl)-N-cyclopropyl-5-(4-nitrophenyl)thiophene-3-carboxamide (**4u**).* Red solid (75%), mp: 203 ~ 204 °C.^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.44 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.20 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.05 (d, *J* = 8.7 Hz, 2H, Ar-H), 4.84 (s, 1H, NH), 2.59 (m, 1H, CH), 0.63 (m, 2H, CH_2), 0.02 (m, 2H, CH_2). ESI-MS: m/z = 414 $[M+1]^+$.

*4.1.2.15. 2-Amino-4-(4-chlorophenyl)-N-cyclohexyl-5-(4-nitrophenyl)thiophene-3-carboxamide (**4v**).* Red solid (71%), mp: 182 ~ 183 °C.^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (d,*J* = 8.7 Hz, 2H, Ar-H), 7.45 (d,*J* = 8.2 Hz, 2H, Ar-H), 7.25 (d,*J* = 8.3 Hz, 2H, Ar-H), 7.06 (d,*J* = 8.7 Hz, 2H, Ar-H), 6.51 (br, 2H, NH_2), 4.77 (d,*J* = 6.8 Hz, 1H, NH), 3.72 (m, 1H, CH), 1.62 (m, 2H, CH_2), 1.45 (m, 1H, CH_2), 1.37 (m, 2H, CH_2), 1.26 (m, 2H, CH_2), 1.14 (m, 1H, CH_2), 0.73 (m, 2H, CH_2). ESI-MS: m/z = 456 $[M+1]^+$.

*4.1.2.16. (2-Amino-4-(4-chlorophenyl)-5-(4-nitrophenyl)thiophen-3-yl)(pyrrolidin-1-yl)methanone (**4w**).* Red solid (78%), mp: 207 ~ 209 °C.^{1}H NMR (500 MHz, $CDCl_3$) δ 8.04 (d, *J* = 8.9 Hz, 2H, Ar-H), 7.26 d, *J* = 8.9 Hz, 2H, Ar-H), 7.17 (m, 4H, Ar-H), 3.41 (m, 2H, CH_2), 2.79 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 1.54 (m, 2H, CH_2). ESI-MS: m/z = 428 $[M+1]^+$.

*4.1.2.17. (2-Amino-4-(4-chlorophenyl)-5-(4-nitrophenyl)thiophen-3-yl)(piperidin-1-yl)methanone (**4x**).* Red solid (69%), mp: 185 ~ 187 °C.^{1}H NMR (500 MHz, $CDCl_3$) ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.03 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.28 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.17 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.13 (d, *J* = 8.3 Hz, 2H, Ar-H), 3.57 (m, 2H, CH_2), 3.40 (m, 2H, CH_2), 1.67 (m, 4H, $2CH_2$), 1.59 (m, 2H, CH_2). ESI-MS: m/z = 442 $[M+1]^+$.

*4.1.2.18. (2-Amino-4-(4-chlorophenyl)-5-(4-nitrophenyl)thiophen-3-yl)(morpholino)methanone (**4y**).* Red solid (81%), mp: 199 ~ 201 °C.^{1}H NMR (500 MHz, $CDCl_3$) δ 8.04 (d, *J* = 8.9 Hz, 2H, Ar-H), 7.31 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.17 (d, *J* = 8.9 Hz, 2H, Ar-H), 7.12 (d, *J* = 8.4 Hz, 2H, Ar-H), 3.36 (m, 8H, $4CH_2$). ESI-MS: m/z = 444 $[M+1]^+$.

*4.1.3. General procedure for the synthesis of aminothiophene thioamide derivatives (**7a-d**)*

Lawesson reagent (1.0 mmol) was added to a solution of 3,4,5-Trisubstituted aminothiophene (**4f**, **4g**, **5** and **6**, 1.0 mmol) in toluene (15 mL), and then the mixture was stirred for 1h at 80℃. After cooling to room temperature, the reaction mixture was evaporated under reduced pressure. The residue was extracted with ethyl acetate (2 x 20 mL). The organic layer was washed with water (2 x 20 mL) and brine (2 x 20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude products were purified by column chromatography (PE: EtOAc=20:1, v/v) to give the target compounds (**7a-d**).

*4.1.3.1. 2-Amino-4,5-bis(4-chlorophenyl)-N-cyclopropylthiophene-3-carbothioamide (**7a**).* Yellow solid (39%), mp: 190 ~ 192 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.15 (d, *J* = 10.0 Hz, 2H, Ar-H), 7.12 (d, *J* = 10.0 Hz, 2H, Ar-H), 6.92 (d, *J* = 8.0 Hz, 2H, Ar-H), 6.60 (br, 2H, NH_2), 6.25 (s, 1H, NH), 3.02 (m, 1H, CH), 0.74 (m, 2H, CH_2), 0.00 (m, 2H, CH_2). ESI-MS: m/z = 419 $[M+1]^+$.

*4.1.3.2. 2-Amino-4,5-bis(4-chlorophenyl)-N-cyclopentylthiophene-3-carbothioamide (**7b**).* Yellow solid (43%), mp: 204 ~ 206 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.17(m, 4H, Ar-H), 6.89 (d, *J* = 8.5 Hz, 2H, Ar-H), 6.38 (br, 2H, NH_2), 6.32 (d, *J* = 6.8 Hz, 1H, NH), 4.58 (m, 1H, CH), 1.85 (m, 2H, CH_2), 1.49 (m, 2H, CH_2), 1.30 (m, 2H, CH_2), 0.95 (m, 2H, CH_2). ESI-MS: m/z = 447 $[M+1]^+$.

*4.1.3.3. 2-Amino-4,5-bis(4-chlorophenyl)-N-cyclohexylthiophene-3-carbothioamide (**7c**).* Yellow

solid (35%), mp: 202 ~ 204 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.16 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.13 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.89 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.24 (d, *J* = 7.4 Hz, 1H, NH), 4.27 (m, 1H, CH), 1.69 (m, 2H, CH_2), 1.49 (m, 2H, CH_2), 1.32 (m, 3H, CH_2), 1.06 (m, 1H, CH_2), 0.68 (m, 2H, CH_2). ESI-MS: m/z = 461 $[M+1]^+$.

*4.1.3.4. 2-Amino-N-(tert-butyl)-4,5-bis(4-chlorophenyl)thiophene-3-carbothioamide (**7d**).* Yellow solid (32%), mp: 201 ~ 204 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.18 (m, 4H, Ar-H), 6.89 (d, *J* = 8.5 Hz, 2H, Ar-H), 6.26 (s, 1H, NH), 6.14 (br, 2H, NH_2), 1.19 (s, 9H, $3CH_3$). ESI-MS: m/z = 435 $[M+1]^+$.

*4.1.4. 2-Bromo-2,3-bis(4-chlorophenyl)ethanon (**8**) and ethyl 3-amino-3-iminopropanoate/3-amino-N-cyclopropyl-3-iminopropanamide (**9a-b**) were exactly following the procedures of reference 14 and 15*[14, 15].

*4.1.5 General procedure for the synthesis of 3,4,5-trisubstituted aminopyrrole derivatives (**10a** and **10b**)*

A mixture of 2-Bromo-2,3-bis(4-chlorophenyl)ethanon (**8**, 1.0mmol), ethyl 3-amino-3-iminopropanoate/3-amino-N-cyclopropyl-3-iminopropanamide (**9a-b**, 1.0mmol) and $NaHCO_3$ (2.5 mmol) in 5 mL ethanol was refluxed for 1.5 h. After cooling to room temperature, the reaction mixture was evaporated under reduced pressure. The residue was extracted with ethyl acetate (2 x 20 mL). The organic layer was washed with water (2 x 20 mL) and brine (2 x 20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude products were purified by column chromatography (PE: EtOAc=3:1, v/v) to give the target compounds (**10a-b**).

*4.1.5.1. Ethyl 2-amino-4,5-bis(4-chlorophenyl)-1H-pyrrole-3-carboxylate (**10a**).* White solid (32%), mp: 253 ~ 255 °C. ^{1}H NMR (500 MHz, DMSO) δ 10.83 (s, 1H, NH), 7.32 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.24 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.15 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.00 (d, *J* = 8.5 Hz, 2H, Ar-H), 5.83 (s, 2H, NH_2), 3.89 (q, *J* = 7.0 Hz, 2H, CH_2), 0.92 (t, *J* = 7.1 Hz, 3H, CH_3). ESI-MS: m/z = 375 $[M+1]^+$.

*4.1.5.2. 2-Amino-4,5-bis(4-chlorophenyl)-N-cyclopropyl-1H-pyrrole-3-carboxamide (**10b**).* White solid (18%), mp: 230 ~ 232 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.88 (s, 1H, NH), 7.38 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.22 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.13 (d, *J* = 8.3 Hz, 2H, Ar-H), 6.92 (d, *J* = 8.1 Hz, 2H, Ar-H), 5.02 (s, 1H, NH-amide), 2.53 (m, 1H, CH), 0.55 (m, 2H, CH_2), 0.08 (m, 2H, CH_2). ESI-MS: m/z = 386 $[M+1]^+$.

4.1.6. General procedure for the synthesis of 2,3,4-trisubstituted 4,5-dihydrothieno[2,3-b]pyridin-6(7H)-ones *(**12a-c**)*

A mixture of 2-amino-4,5-bis(4-chlorophenyl)thiophene-3-carboxylic acid[12] (**11,** 1.0 mmol), malonic acid cyclic isopropylidene ester (1.1 mmol) and corresponding aldehydes (1.05 mmol) in 5 mL AcOH was refluxed for 2 h. After cooling to room temperature, the reaction mixture was evaporated under reduced pressure. The residue was extracted with ethyl acetate (2 x 20 mL). The organic layer was washed with water (2 x 20 mL) and brine (2 x 20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude products were purified by column chromatography to give the target compounds (**12a-c**).

*4.1.6.1. 2,3-Bis(4-chlorophenyl)-4-phenyl-4,5-dihydrothieno[2,3-b]pyridin-6(7H)-one (**12a**).* White solid (61%), mp: 247 ~ 248 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.51 (s, 1H, NH), 7.28 (m, 3H, Ar-H), 7.18 (m, 4H, Ar-H), 7.03 (m, 4H, Ar-H), 6.82 (d, *J* = 8.1 Hz, 2H, Ar-H), 3.97 (d, *J* = 7.2 Hz, 1H, CH), 3.11 (dd, *J* = 16.1, 7.5 Hz, 1H, CH_2), 2.78 (d, *J* = 16.1 Hz, 1H, CH_2). ESI-MS: m/z = 450 $[M+1]^+$.

*4.1.6.2. 2,3-Bis(4-chlorophenyl)-4-(thiophen-2-yl)-4,5-dihydrothieno[2,3-b]pyridin-6(7H)-one (**12b**).* Yellow solid (65%), mp: 240 ~ 241 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.50 (s, 1H, NH), 7.25 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.19 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.17 (d, *J* = 5.0 Hz 1H, Ar-H), 7.05 (d, *J* = 8.5 Hz, 2H, Ar-H), 6.99 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.90 (dd, *J* = 5.0, 3.6 Hz, 1H, Ar-H), 6.70 (d, *J* = 3.4 Hz, 1H, Ar-H), 4.26 (d, *J* = 6.3 Hz, 1H, CH), 3.10 (dd, *J* = 16.1, 6.9 Hz, 1H, CH_2), 2.91 (d, *J* = 16.0 Hz, 1H, CH_2). ESI-MS: m/z = 456 $[M+1]^+$.

*4.1.6.3. 2,3-Bis(4-chlorophenyl)-4-(furan-2-yl)-4,5-dihydrothieno[2,3-b]pyridin-6(7H)-one (**12c**).* Yellow solid (45%), mp: 238 ~ 239 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (s, 1H, NH), 7.31 (d, *J* = 1.5 Hz, 1H, Ar-H), 7.29 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.20 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.07 (m, 4H, Ar-H), 6.24 (dd, *J* = 3.1, 1.9 Hz, 1H, Ar-H), 5.89 (d, *J* = 3.2 Hz, 1H, Ar-H), 4.04 (t, *J* = 4.5 Hz, 1H, CH), 2.97 (d, *J* = 4.5 Hz, 2H, CH_2). ESI-MS: m/z = 440 $[M+1]^+$.

*4.1.7. General procedure for the synthesis of 2,3,4-trisubstituted 4,5-dihydrothieno[2,3-b]pyridin-6(7H)-ones (**13a-b**)*

Compound **12a, b** (1.0 mmol) and K_2CO_3 (1.5 mmol) were dissolved in DMF (5 mL) and stirred for 30 min. Then 1-bromo-3-chloropropane (1.2 mmol) was slowly added with vigorous stirring at 80℃ for 45 min. After cooling to room temperature, the reaction mixture was washed with water (2 x 10 mL) and extracted with ethyl acetate (2 x 10 mL). The organic layer was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Then, the obtained residue was dissolved in 5 mL dioxane, to which was added triethylamine (5.0 mmol) and correspongding amines (10.0 mmol), refluxed for 24h. After cooling to room temperature, the reaction mixture was evaporated under reduced pressure. The residue was extracted with ethyl acetate (2 x 10 mL). The organic phase was washed with water (2 x 10 mL) and brine (2 x 10 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The obtained residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate = 1:1, v/v) to give the target compounds **13a-b**.

4.1.7.1.

*2,3-Bis(4-chlorophenyl)-7-(3-morpholinopropyl)-4-phenyl-4,5-dihydrothieno[2,3-b]pyridin-6(7H)-one (**13a**).* White solid (31%), mp: 105 ~ 107 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.28 (d, *J* = 7.1 Hz, 2H, Ar-H), 7.22 (m, 1H, Ar-H), 7.17 (m, 4H, Ar-H), 7.04 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.99 (d, *J* = 7.4 Hz, 2H, Ar-H), 6.82 (d, *J* = 8.0 Hz, 2H, Ar-H), 4.16 (m, 1H, CH), 3.90 (d, *J* = 7.0 Hz, 1H, CH_2), 3.74 (m, 4H, $2CH_2$), 3.66 (dt, *J* = 13.9, 6.8 Hz, 1H, CH_2), 3.09 (dd, *J* = 15.9, 7.5 Hz, 1H, CH_2), 2.82 (d, *J* = 15.9 Hz, 1H, CH_2), 2.45 (m, 6H, $3CH_2$), 1.98 (d, *J* = 32.0 Hz, 2H, CH_2). ESI-MS: m/z = 577 $[M+1]^+$.

4.1.7.2.

*2,3-Bis(4-chlorophenyl)-4-(furan-2-yl)-7-(3-(pyrrolidin-1-yl)propyl)-4,5-dihydrothieno[2,3-b]pyridin-6(7H)-one (**13b**).* Yellow oil (35%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 (m, 1H, Ar-H), 7.25 (m, 2H, Ar-H), 7.18 (dd, *J* = 8.6, 2.5 Hz, 2H, Ar-H), 7.05 (dd, *J* = 8.5, 2.2 Hz, 4H, Ar-H), 6.23 (m, 1H, Ar-H), 5.89 (m, 1H, Ar-H), 4.25 (m, 1H, CH), 3.95 (m, 1H, CH_2), 3.57 (m, 1H, CH_2), 3.49 (m, 1H, CH_2), 3.42 (m, 1H, CH_2), 2.96 (m, 2H, CH_2), 2.54 (m, 4H, $2CH_2$), 1.91 (m, 2H, CH_2), 1.81 (m, 4H, $2CH_2$). ESI-MS: m/z = 581 $[M+1]^+$.

*4.1.8. Synthesis of 2-amino-4,5-bis(4-chlorophenyl)thiophene-3-carbonitrile (**14**)*

Same as **4a-y** above, except with malononitrile. Target compound **14** was obtained as a yellow solid (53%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.26 (m, 4H, Ar-H), 7.06 (d, *J* = 8.3 Hz, 2H, Ar-H), 6.11 (br, 2H, NH_2). ESI-MS: m/z = 345 $[M+1]^+$.

*4.1.9. Synthesis of 5,6-bis(4-chlorophenyl)thieno[2,3-d]pyrimidin-4(3H)-one (**15**)*

A mixture of 2-amino-4,5-bis(4-chlorophenyl)thiophene-3-carbonitrile **14** (1.0 mmol), formic acid (4 mL) and acetic anhydride (4 mL) was heated at 110 ℃ for 16 h. The solvent was removed under reduced pressure and the residue was extracted with ethyl acetate (2 x 20 mL) and organic layer was washed with water (2 x 20 mL) and brine (2 x 20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by column chromatography (petroleum ether/ethyl acetate = 10:1, v/v) to give the target compounds (**15**). White solid (86%), mp: > 250 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.58 (s, 1H, Ar-H), 8.01 (s, 1H, NH), 7.45 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.30 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.17 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.00 (d, *J* = 8.3 Hz, 2H, Ar-H). ESI-MS: m/z = 373 $[M+1]^+$.

*4.1.10. Synthesis of 4-chloro-5,6-bis(4-chlorophenyl)thieno[2,3-d]pyrimidine (**16**)*

A solution of 5,6-bis(4-chlorophenyl)thieno[2,3-d]pyrimidin-4(3H)-one (**15**, 1.0 mmol) in $POCl_3$ (3 mL) was heated at 70℃ for 5 h. After cooling to room temperature, the reaction mixture was quenched with ice water, and then extracted with ethyl acetate (2 x 20 mL). The organic layer was washed with water (2 x 20 mL) and brine (2 x 20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by column chromatography (petroleum ether/ethyl acetate = 15:1, v/v) to give the target compounds (**16**). Yellow solid (95%), mp: 200 ~ 202 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.89 (s, 1H, Ar-H), 7.62 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.56 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.51 (m, 4H, Ar-H). ESI-MS: m/z = 391 $[M+1]^+$.

4.1.11. General procedure for the synthesis of 4,5,6-trisubstituted thieno[2,3-d]pyrimidine derivatives (**17a-d**).

A mixture of 4-chloro-5,6-bis(4-chlorophenyl)thieno[2,3-d]pyrimidine (**16**, 1.0 mmol) and corresponding amines (3.0 mmol) in *n*-butanol (4ml) was refluxed for 1h. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was extracted with ethyl acetate (2 x 20 mL). The organic layer was washed with water (2 x 20 mL) and brine (2 x 20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by column chromatography to give the target compounds (**17a-d**).

*4.1.11.1. 5,6-Bis(4-chlorophenyl)-N-cyclopropylthieno[2,3-d]pyrimidin-4-amine (**17a**).* White solid (61%), mp: 152 ~ 153 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.88 (s, 1H, Ar-H), 7.38 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.22 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.09 (m, 2H, Ar-H), 6.89 (m, 2H, Ar-H), 5.02 (s, 1H, NH), 1.26 (m, 1H, CH), 0.55 (m, 2H, CH_2), 0.09 (m, 2H, CH_2). ESI-MS: m/z = 412 $[M+1]^+$.

*4.1.11.2. 5,6-Bis(4-chlorophenyl)-N-cyclohexylthieno[2,3-d]pyrimidin-4-amine (**17b**).* White solid (69%), mp: 153 ~ 154 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.40 (s, 1H, Ar-H), 7.55 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.43 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.42 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.26 (d, *J* = 8.2 Hz, 2H, Ar-H), 4.58 (s, 1H, NH), 4.07 (m, 1H, CH), 1.88 (m, 2H, CH_2), 1.52 (m, 2H, CH_2), 1.40 (m, 2H, CH_2), 1.23 (m, 2H, CH_2), 1.03 (m, 2H, CH_2). ES I-MS: m/z = 454 $[M+1]^+$.

*4.1.11.3. 5,6-Bis(4-chlorophenyl)-4-(piperidin-1-yl)thieno[2,3-d]pyrimidine (**17c**).* White solid (49%), mp: 173 ~ 175 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.47 (s, 1H, Ar-H), 7.46 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.36 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.33 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.26 (d, *J* = 8.8 Hz, 2H, Ar-H), 3.23 (m, 4H, $2CH_2$), 1.47 (m, 2H, CH_2), 1.33 (m, 4H, $2CH_2$). ESI-MS: m/z = 440 $[M+1]^+$.

*4.1.11.4. 4-(5,6-Bis(4-chlorophenyl)thieno[2,3-d]pyrimidin-4-yl)morpholine (**17d**).* White solid (53%), mp: 203 ~ 205 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52 (s, 1H, Ar-H), 7.48 (d, *J* = 8.1 Hz, 2H,

Ar-H), 7.37 (dd, *J* = 14.3, 8.4 Hz, 4H, Ar-H), 7.28 (d, *J* = 8.9 Hz, 2H, Ar-H), 3.43 (m, 4H, $2CH_2$), 3.22 (m, 4H, $2CH_2$). ESI-MS: m/z = 442 $[M+1]^+$.

4.2. Biological evaluation

4.2.1. MDM2 protein expression and purification

MDM2 (1-118) plasmid was provided by Dr. Shaomeng Wang's group, and transformed into Escherichia coli BL-21 (DE3). Cultures were grown at 37℃ in TB medium, and induced by 0.4 mM IPTG at an OD600 of 0.6 at 18℃ for 20 h. Cells were lysed in 50 mM Tries, pH 7.5 buffer containing 500 mM NaCl and 10% glycerol. MDM2 (1-118) was purified from the soluble fraction using Ni-NTA resin, and desalted in PBS buffer pH 7.5, 150 mM NaCl and 10% glycerol. The protein was purified to >95% as judged SDS–PAGE.

4.2.2. Fluorescence polarization competitive binding assay

Measurements were made with a Beckmancoulter DTX880 Multilabel Plate Reader using a 485 nM excitation filter and a 535 nM emission filter. Assays were performed in Microtiter 96-Well black and round bottom plates. Nutlin-3 was used as a positive control, while DMSO was used as a negative control. Assays were performed in duplicate and repeated at least three times on separate days. Competition experiments were carried out in a total volume of 20 μL 40 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT, 4% DMSO. Probe peptide was present at a final concentration of 1 nM, and MDM2 was present at a final concentration of 10nM. Plates were allowed to incubate at room temperature for 1 h prior to measurement. The Ki values for inhibitors were calculated by a web-based computer program[20].

4.2.3. Cell proliferation assay

Cell proliferation was assessed by sulforhodamin B (SRB) assay. Briefly, A549 and PC3 cells were seeded into 96-well plates and cultured overnight; exposed to serial concentrations of compounds for 72 h. Cells were then washed with PBS and fixed with 10% (w/v) trichloroacetic acid at 4 oC for an hour. After washing, the cells were stained for 30 min with 0.4% SRB dissolved in 1% acetic acid. Then the cells were washed by 1% acetic acid for 5 times, and protein-bound dye was extracted with 10 mmol unbuffered Tris base. The absorbance was measured at 515 nm using a multiscan spectrum (Thermo Electron Co., Vantaa, Finland). The inhibition rate on cell proliferation of each well was calculated as (A515 control cells – A515 treated cells)/ A515 control cells×100%. The average IC_{50} values were determined by Logit method from at least two independent tests.

4.3. Molecular docking and structural alignment

Docking simulations were carried out by using CDOCKER module (Discovery Studio, version 2.1; Accelrys, San Diego, CA, USA, 2008). The X-ray crystal structure of MDM2 bound to the transactivation domain of p53 (PDB ID:1YCR) was used for the docking calculation. After removing the ligand and solvent molecules, the CHARMm-force field was applied to the protein.

And the area around the p53 peptide was chosen as the active site with a radius set as 10 Å. Each compound was generated random conformations using CHARMm-based molecular dynamics (1000 steps), and then docked into the defined MDM2 binding site. The other parameters were set as default. The final binding conformation of **7a** and **10b** was determined based on the calculated CDOCKING ENERAGE. The most stable binding mode among the top 10 docking poses of each

Structural alignment analysis was carried out by using Fit atoms module in SYBYL 6.9 molecular modeling software. Structural energy minimization was performed using the standard Tripos molecular mechanics force field and Gasteiger-Hückel charge, the max iterations for the minimization was set to 2000. The minimization was terminated when the energy gradient convergence criterion of 0.01 kcal/mol Å was reached. The minimized conformers of **(*S*)-12c**, **(*R*)-12c** and **17a** were aligned with the most potent compound **7a**, respectively, by fitting with thiophene ring skeleton as a common structure. The aligned molecules were shown in Fig. 3.

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Figure captions.

Fig. 1. Structure of the lead compound MCL0527.

Fig. 2. Molecular docking analysis of **7a** and **10b** with MDM2. (A) Stereoview of **7a** bound to the p53-binding site of MDM2, displayed with selected residues. (B) Stereoview of **10b** bound to the p53-binding site of MDM2, displayed with selected residues. (C) Overlay of **7a** (green) with the p53's 9-mer peptide backbone and side chains Phe 19, Trp 23, and Leu 26 (pink) in a surface show of MDM2. (D) Overlay of **10b** (yellow) with the p53's 9-mer peptide backbone and side chains Phe 19, Trp 23, and Leu 26 (pink) in a surface show of MDM2.

Fig. 3. Structural alignment analysis of **(*S*)-12c**, **(*R*)-12c**, **17a** with **7a**. (A) Superposition of **(*S*)-12c** (green) and **7a** (white). (B) Superposition of **(*R*)-12c** (magenta) and **7a** (white). (C) Superposition of **17a** (orange) and **7a** (white).

Scheme 1. Synthetic route of 3,4,5-trisubstituted aminothiophenes (**4a-y**). Reagents and conditions: (a) oxalyl chloride, CH_2Cl_2, rt; (b) $AlCl_3$, CH_2Cl_2, rt; (c) methyl cyanoacetate or 2-cyanoacetamide derivatives, $TiCl_4$, pyridine, THF, rt; (d) sulfur, $NHEt_2$, THF, rt.

Scheme 2. Synthetic route of 3,4,5-trisubstituted aminothiophene thioamides (**7a-d**). Reagents and conditions: (a) Lawesson's Reagent, toluene, 80℃.

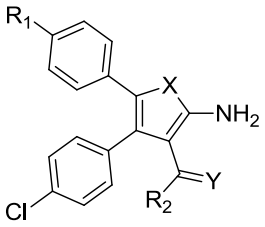
Scheme 3. Synthetic route of 3,4,5-trisubstituted aminopyrroles (**10a**, **b**). Reagents and conditions: (a) Br_2, $CHCl_3$, Et_2O, reflux; (b) $NaHCO_3$, EtOH, reflux.

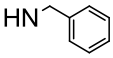
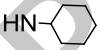
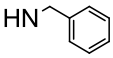
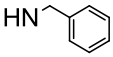
Scheme 4. Synthetic route of 2,3,4-trisubstituted dihydrothieno[2,3-b]pyridones (**12a-c, 13a, b**). Reagents and conditions: (a) 2N NaOH aqueous solution, EtOH, reflux; (b) Meldrum's acid, aldehydes, AcOH, reflux; (c) K_2CO_3, DMF, 1-bromo-3-chloropropane, 80℃; TEA, amines, dioxane, reflux.

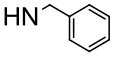
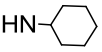
Scheme 5. Synthetic route of 4,5,6-trisubstituted thieno[2,3-d]pyrimidines (**17a-d**). Reagents and conditions: (a) HCOOH, Ac_2O, reflux; (b) PO_3Cl, 70℃; (c) amines, *n*-butanol, reflux.

Table 1

P53–MDM2 binding inhibitory activities and i*n vitro* tumor cell anti-proliferation activities of 3,4,5-trisubstituted aminothiophenes (**4a-y**, **5**, **6** and **7a-d**) and 3,4,5-trisubstituted aminopyrroles (**10a**, **b**).



Compd.	R_1	R_2	X	Y	*Ki*[a] (μM)	SRB IC_{50} (μM)	
						A549[a]	PC3[a]
4a	H		S	O	0.63	4.3	17.09
4b	H		S	O	1.96	5.8	22.24
4c	H		S	O	0.28	14.23	29.27
4d	H		S	O	1.14	8.51	22.76
4e[d]	Cl	OCH_3	S	O	1.52	5.04	2.78
4f[d]	Cl		S	O	0.54	1.95	3.68
4g[d]	Cl		S	O	0.23	2.41	7.3
4h[d]	Cl		S	O	0.74	0.89	6.22
4i[e]	Cl		S	O	1.21	4.62	14.33
4j[e]	Cl		S	O	0.43	1.87	11.69
4k[e]	Cl		S	O	0.15	16.30	31.81
4l	F		S	O	0.34	3.00	27.95
4m	F		S	O	1.22	18.81	20.39
4n	F		S	O	0.22	12.72	25.13
4o	F		S	O	0.60	9.95	25.79
4p	Br		S	O	2.18	>50	37.31
4q	Br		S	O	1.06	9.59	28.37
4r	Br		S	O	0.75	7.46	37.45

4s	Br		S	O	1.48	7.49	>50
4t	Br		S	O	0.76	18.67	>50
4u	NO_2		S	O	NA[b]	NT[c]	NT[c]
4v	NO_2		S	O	NA[b]	15.03	25.87
4w	NO_2		S	O	NA[b]	10.70	42.57
4x	NO_2		S	O	NA[b]	NT[c]	NT[c]
4y	NO_2		S	O	NA[b]	16.72	>50
5[e]	Cl		S	O	0.54	6.79	21.24
6[e]	Cl		S	O	0.81	8.85	15.14
7a	Cl		S	S	0.086	8.28	22.05
7b	Cl		S	S	0.17	22.05	29.04
7c	Cl		S	S	0.32	7.41	27.74
7d	Cl		S	S	0.58	>50	34.02
10a	Cl	OCH_2CH_3	NH	O	1.24	2.53	16.14
10b	Cl		NH	O	0.53	4.62	19.33
Nutlin-3	—	—	—	—	0.055	4.62	20.04

[a] Values are means of two experiments.

[b] NA, no activity.

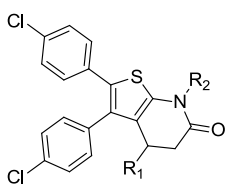
[c] NT, not tested.

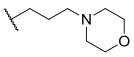
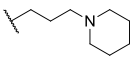
[d] These compounds were reported in our previous studies[11].

[e] These compounds were reported in our another previous studies[12].

Table 2

P53–MDM2 binding inhibitory activities and i*n vitro* tumor cell anti-proliferation activities of 2,3,4-trisubstituted dihydrothieno[2,3-b]pyridone derivatives (**12a-c**, **13a**, **b**).



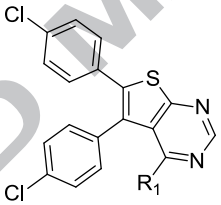
Compd.	R_1	R_2	Inhibition at 10μM (%)	SRB IC_{50} (μM) A549[a]	PC3[a]
12a		H	6.07	17.22	>50
12b		H	0	20.21	>50
12c		H	0	25.44	>50
13a			23.75	NT[b]	NT[b]
13b			0	NT[b]	NT[b]
Nutlin-3	—	—	100	4.62	20.04

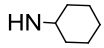
[a] Values are means of two experiments.

[b] NT, not tested.

Table 3

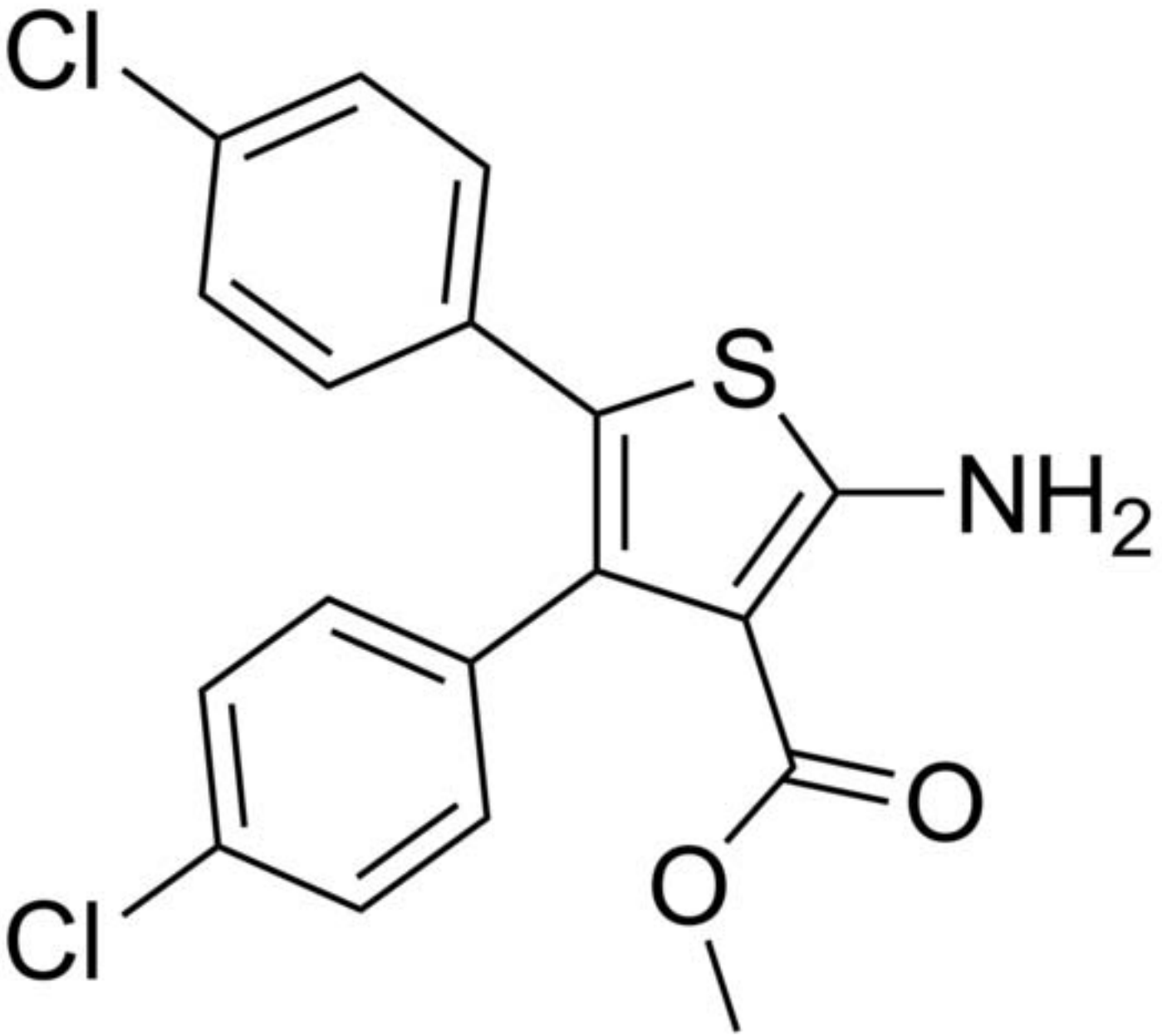
P53–MDM2 binding inhibitory activities and i*n vitro* tumor cell anti-proliferation activities of 4,5,6-trisubstituted thieno[2,3-d]pyrimidines (**17a-d**).



Compd.	R_1	Inhibition at 10μM (%)	SRB IC_{50} (μM) A549[a]	PC3[a]
17a		30.5	42.75	>50
17b		12.5	>50	>50
17c		16.8	>50	>50
17d		18.9	>50	>50
Nutlin-3	—	100	4.62	20.04

[a] Values are means of two experiments.

Figure 1



MCL0527

Figure 2

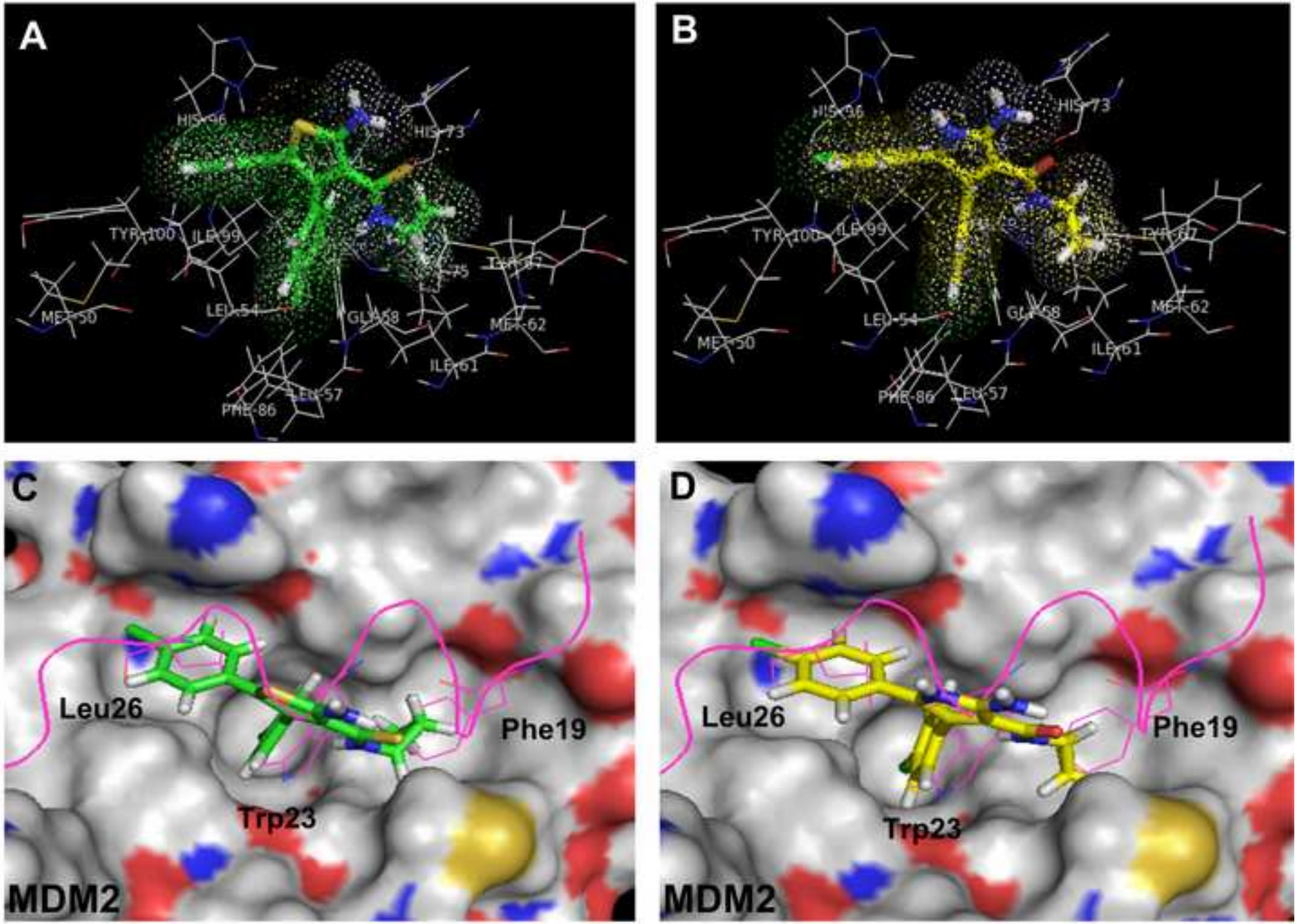
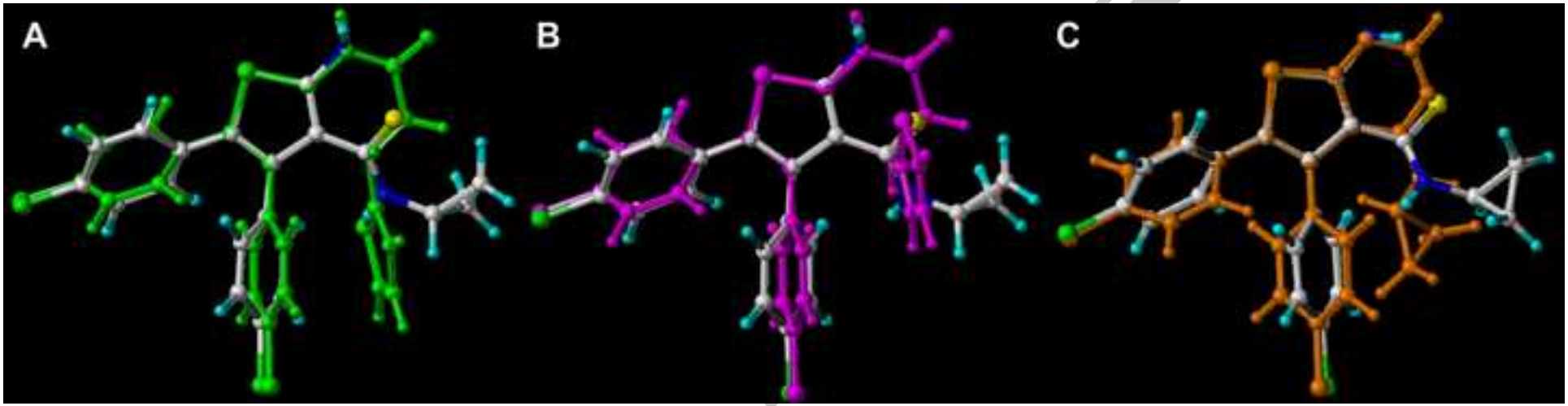
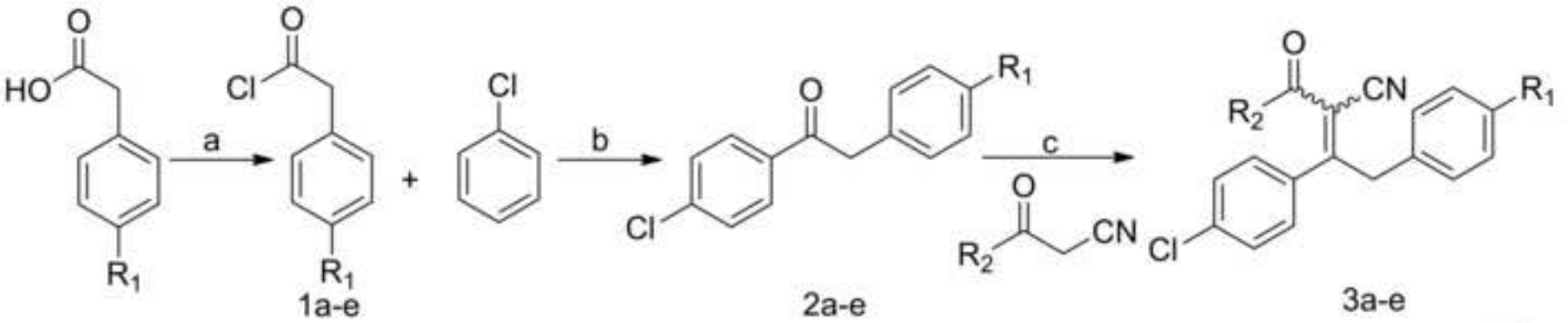
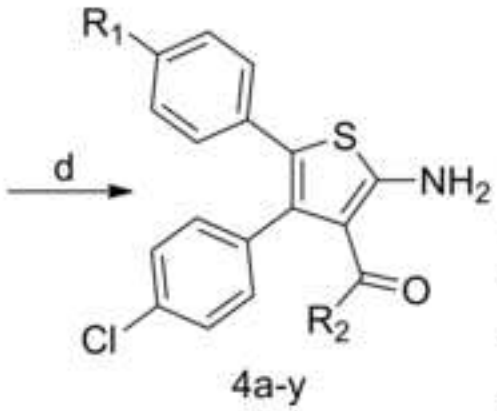


Figure 3



Scheme 1



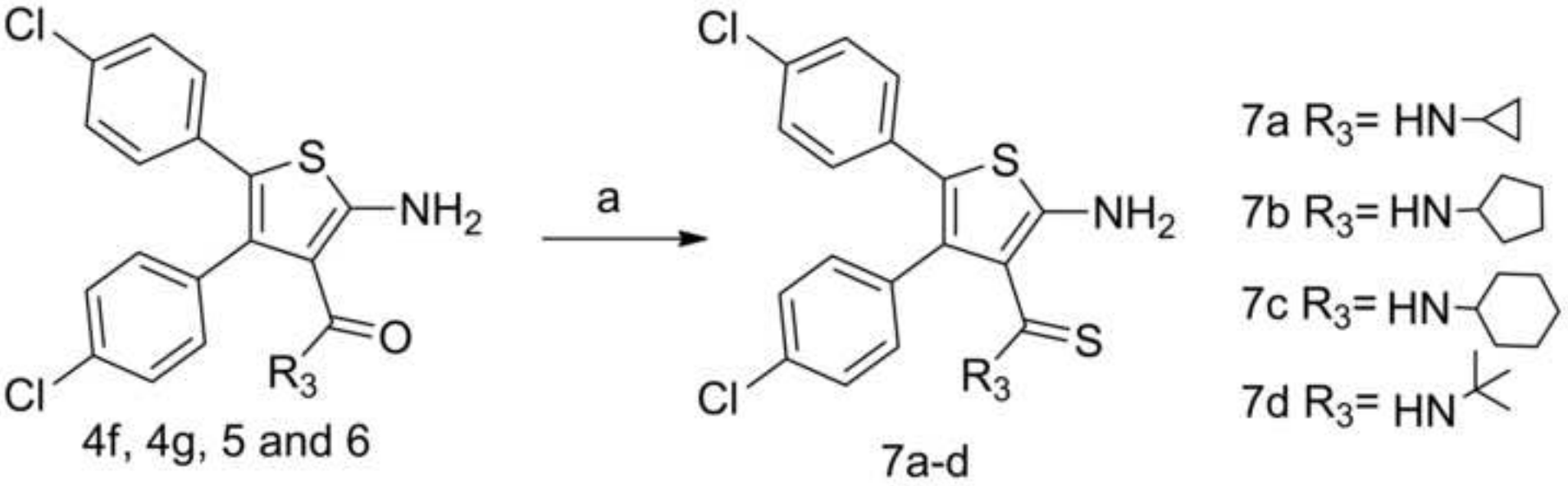


4a R_1= H, R_2= HN
4b R_1= H, R_2= N
4c R_1= H, R_2= N O
4d R_1= H, R_2= HN
4e R_1= H, R_2= OCH_3
4f R_1= Cl, R_2= HN
4g R_1= Cl, R_2= HN
4h R_1= Cl, R_2= N

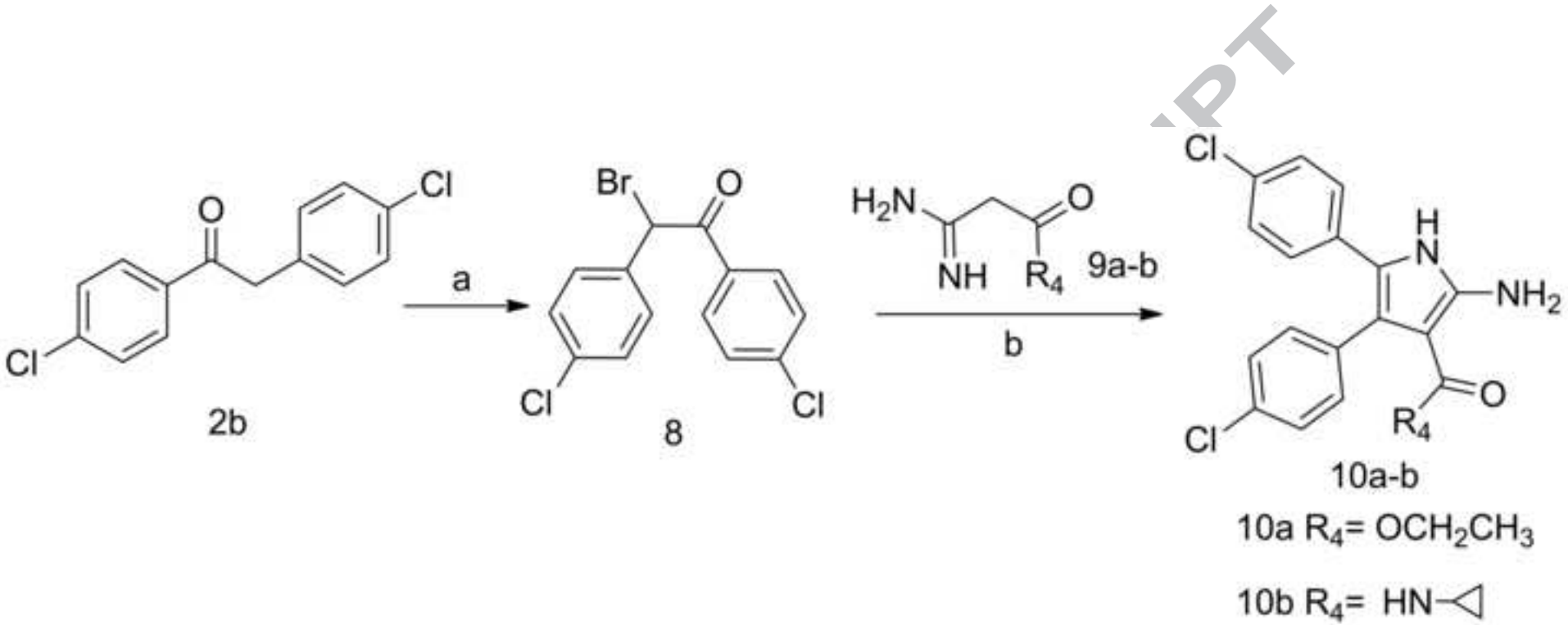
4i R_1= Cl, R_2= N
4j R_1= Cl, R_2= N O
4k R_1= Cl, R_2= HN
4l R_1= F, R_2= HN
4m R_1= F, R_2= N
4n R_1= F, R_2= N O
4o R_1= F, R_2= HN
4p R_1= Br, R_2= HN

4q R_1= Br, R_2= HN
4r R_1= Br, R_2= N
4s R_1= Br, R_2= N O
4t R_1= Br, R_2= HN
4u R_1= NO_2, R_2= HN
4v R_1= NO_2, R_2= HN
4w R_1= NO_2, R_2= N
4x R_1= NO_2, R_2= N
4y R_1= NO_2, R_2= N O

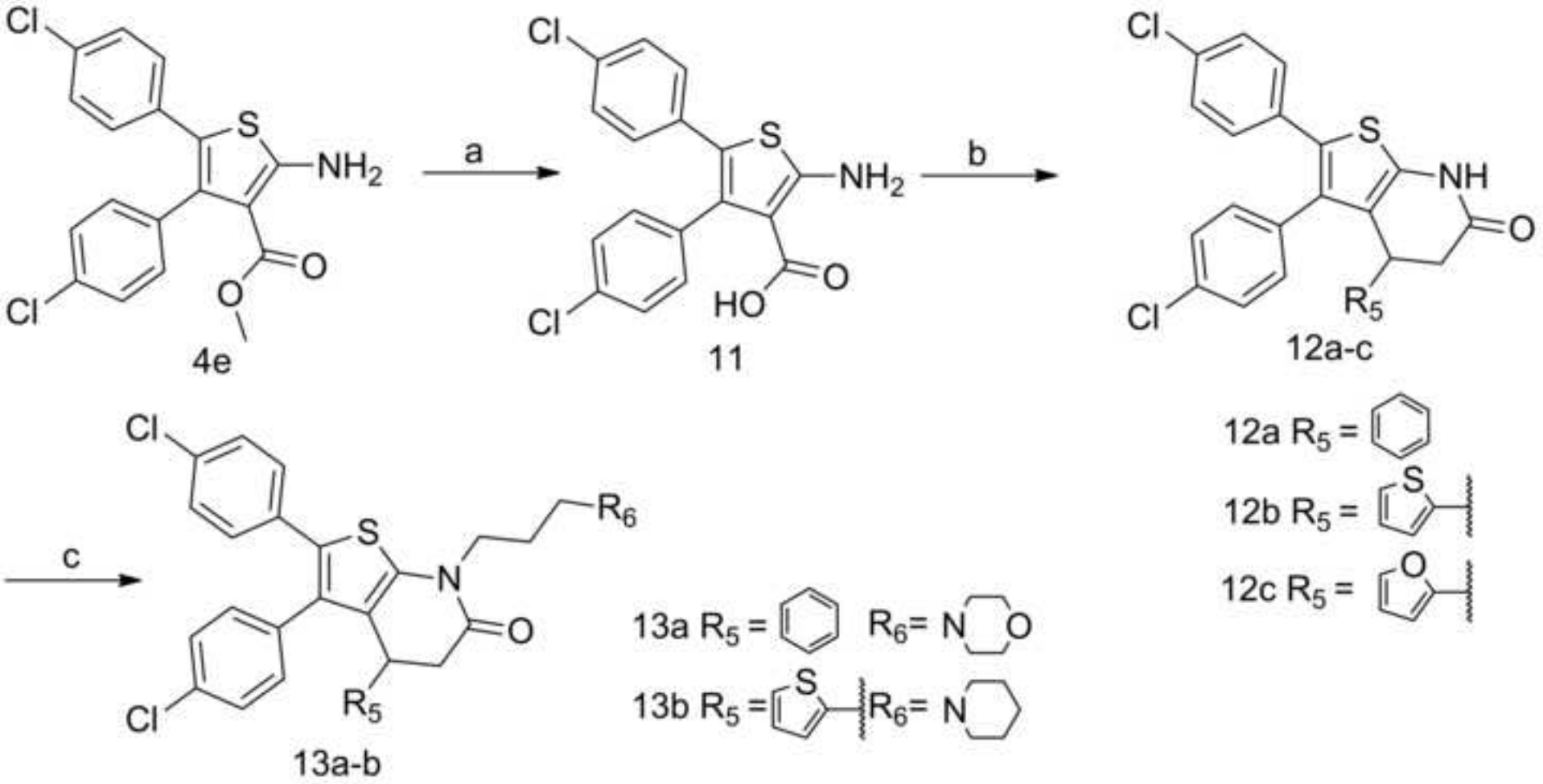
Scheme 2



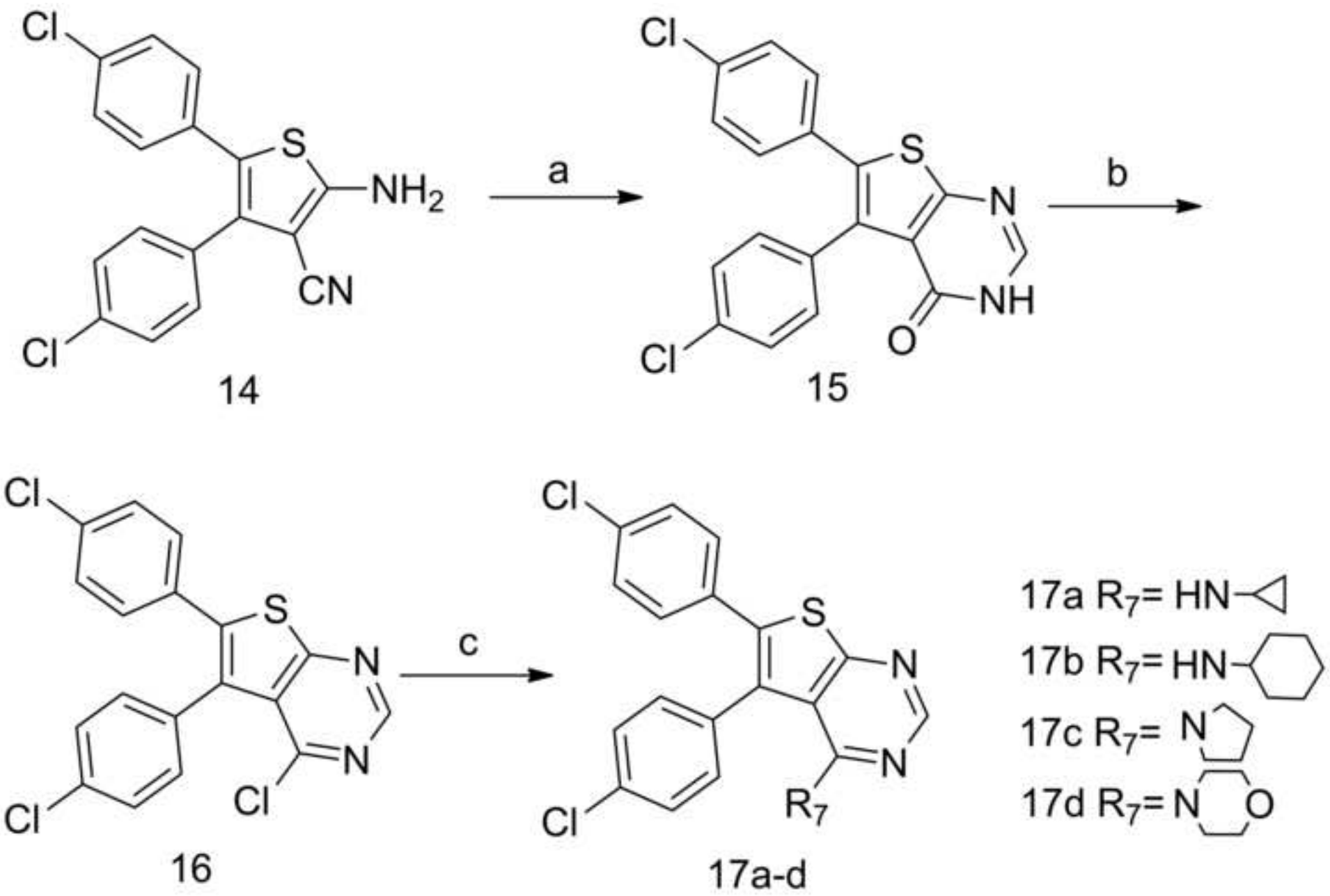
Scheme 3



Scheme 4



Scheme 5



Graphical abstract

Five series of novel 3,4,5-trisubstituted aminothiophene derivatives were designed and synthesized as p53–MDM2 binding inhibitors.