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Synthesis and Thrombin, Factor Xa and U46619 Inhibitory Effects of Non-amidino and Amidino N^2-Thiophenecarbonyl- and N^2-Tosylanthranilamides

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Abstract: Thrombin (factor IIa) and factor Xa (FXa) are key enzymes at the junction of the intrinsic and extrinsic coagulation pathways and are the most attractive pharmacological targets for the development of novel anticoagulants. Twenty non-amidino N^2-thiophencarbonyl- and N^2-tosyl anthranilamides **1**-**20** and six amidino N^2-thiophencarbonyl- and N^2-tosylanthranilamides **21**-**26** were synthesized and evaluated prothrombin time (PT) and activated partial thromboplastin time (aPTT) using human plasma at concentration 30 μg/mL *in vitro*. From these results, compounds **5**, **9**, and **21**-**23** were selected to study the further antithrombotic activity. The anticoagulant properties of **5**, **9**, and **21-23** significantly exhibited a concentration-dependent prolongation of *in vitro* PT and aPTT, *in vivo* bleeding time, and *ex vivo* clotting time. These compounds concentration-dependently inhibited the activities of thrombin and FXa and inhibited the generation of thrombin and FXa in human endothelial cells. In addition, data showed that **5**, **9**, and **21-23** significantly inhibited thrombin catalyzed fibrin polymerization and mouse platelet aggregation and inhibited platelet aggregation induced U46619 *in vitro* and *ex vivo*. *N*-(3'-Amidinophenyl)-2-((thiophen-2''-yl)carbonyl amino)benzamide (**21**) was most active.

Keywords: N^2-Arylcarbonyl/sulfonylanthranilamides; Prothrombin time; Activated partial thromboplastin time; Thrombin; Factor Xa; U46619

1. Introduction

Thromboembolic disorders such as deep vein thrombosis, pulmonary embolism, myocardial infarction, unstable angina, and thromboembolic stroke are one of the major disorders that cause morbidities and mortalities worldwise [1]. Several anticoagulants, such as heparins and vitamin K antagonists (e.g., warfarin), have proved to be effective in the prevention and treatment of thromboembolic diseases [2]. But considerable shortcomings, such as inconvenient drug administration, monitoring, unneglectable side effects for heparins, and extensive drug and food interactions for vitamin K antagonists, restrict their clinical use [3].

Thrombin (factor IIa, FIIa) and factor Xa (FXa) occupy central positions in the blood coagulation cascade and play prominent role in various thromboembolic complications. Thrombin promotes blood clot formation by the conversion of fibrinogen to insoluble fibrin and activating platelets [4]. Inhibition of thrombin attenuates formation of fibrin, reduces thrombin generation, and may limit platelet aggregation. FXa is a trypsin like serine protease which plays a pivotal role in the sequence of blood coagulation events. By directly binding to FXa active site, FXa inhibitors lead to interruption of the intrinsic and extrinsic coagulation cascade pathways and thus to inhibition of thrombin formation and thrombus development.

Heparin inhibits both thrombin and factor Xa indirectly via complex formation with modulation of the activity of the serine protease inhibitor antithrombin III [5]. Fondaparinux sodium (Arixtra®), heparin pentasaccharide, is the first of a new class of synthetic antithrombin III–mediated selective inhibition of FXa, distinct from low molecular weight heparin and unfractional heparin. Its pharmacokinetic properties allow for a simple, fixed-dose, once-daily regimen of subcutaneous injection, without the need for monitoring [6,7]. Unexpectedly, fondaparinux sodium are inconvenient for long-term use and require dose adjustment according to the therapeutic range either using the activated partial thromboplastin time (aPTT) or chromogenic assays, particularly in renal impairment, and they may also require the control of platelet contents. [8].

The first synthetic direct thrombin inhibitor was ximelagatran, but it was not approved for medicinal use because of its hepatotoxicity. The direct thrombin inhibitor, dabigatran etexilate memesyla (Pradaxa®), argatroban, and synthetic 20-amino acid peptide, bivalirudin are approved for use on the market [9]. Apixaban (Eliquis®) and rivaroxaban (Xarelto®) are oral direct FXa inhibitors. But, while these anticoagulants carry similar side effects to warfarin, such as risk for gastrointestinal bleeding and intracranial hemorrhage, INR and PT monitoring are not required.

The structures of thrombin and FXa show similarities in their active sites and for this reason, attempts have been made to develop synthetic agents containing in a single molecular inhibitory activity against two of the enzymes of the blood coagulation cascade. A combination of thrombin and FXa inhibitory activity in a single, synthetic, orally bioavailable, small molecular weight compound as a novel approach to antithrombotic therapy should therefore result in potent anticoagulant with potentially superior features over currently available therapies [10].

The first compounds to demonstrate dual inhibitor properties were BIBM 1015 and tanogitran (BIBT986) which belong to the methylbenzimidazole series [5]. Hexadecasaccharide (e.g. SR123781) [11,12] is a synthetic heparin mimetic that inhibits both FXa and thrombin via antithrombin III that was advanced to phase I clinical trials. This dual inhibitor demonstrated superior antithrombotic properties in three rat models of thrombosis following intravenous administration compared to selective factor Xa inhibitor, synthetic pentasaccharide fondaparinux (Arixtra®) [13]. In addition,

novel anti-FXa pentasaccharides coupled to active site thrombin inhibitors to obtain dual inhibitory effects have also been reported [14,15]. However, problems with thrombin-rebound remain with these mimetics, which appear to result from the inability to inhibit clot-bound thrombin.

EP 217609 is a dual-action parenteral anticoagulant that combines an indirect factor Xa inhibitor (fondaparinux analog) and a direct thrombin inhibitor (α-NAPAP analog) in a single molecule without the compilcations of thrombin rebound [16]. In recent years several groups [17-20] have reported the development of dual FXa and thrombin inhibitors and proposed a stronger anticoagulant activity for such compounds compared to monoselective inhibitors.

Furthermore, some patients with cardiovascular disease have indications for anticoagulant and dual antiplatelet therapy. It is estimated that between 5 and 10 percent of patients scheduled for coronary artery stenting, and who thus require dual antiplatelet therapy, are receiving oral anticoagulant, most often for atrial fibrillation [21]. The concomitant use of dual antiplatelet therapy and oral anticoagulant is referred to as triple oral antithrombotic therapy (triple therapy).

We previously reported the synthesis and anticoagulant and antiplatelet activities of N^3-aroylbenzamide derivatives. Two amidine compounds, *N*-(3'-amidino-, and *N*-(4'-amidinophenyl)-3-(thiophen-2''-ylcarbonylamino)benzamide exhibited weak thrombin, FXa, and U46619 inhibitory activities [22]. Anthranilamide derivatives have been reported as FXa inhibitors [23-26], therefore anthranilic acid was considered as central ring of diamide to improve the activity. We now report the synthesis and evaluation of the thrombin, FXa, and U46619 inhibitory activities of non-amidino N^2-thiophenecarbonyl- and N^2-tosylanthranilamides and amidino N^2-thiophenecarbonyl- and N^2-tosylanthranilamides.

2. Results and Discussion

2.1. Chemistry

All synthetic methods used in this study are shown in Scheme 1, 2, 3, and 4. Scheme 1 and 2 showed the synthesis of non-amidine derivatives and scheme 3 and 4 showed the synthesis of amidine derivatives. Scheme 1 illustrated the synthesis of N^2-(thiophenecarbonyl)anthranilamide derivatives **1**-**10**, which were prepared according to synthetic procedures for N^3-(thiophenecarbonyl)benzamide derivatives [22]. The 2-nitrobenzoyl chloride was coupled with 4-substituted anilines (4-chloro, 4-bromo, 4-fluoro, 4-methoxy-, and 4-morpholinoaniline) to give **1a**-**5a** and these nitro amides reduced with Fe and NH_4Cl to provide the corresponding amino amides **1b**-**5b**. Intermediates **1b**-**5b** were then coupled with thiophene-2-carbonyl chloride and 5-chlorothiophene-2-carbonyl chloride to furnish N^2-(thiophene-2-carbonyl)anthranilamides **1**-**5** and N^2-(5-chlorothiophene-2-carbonyl)anthranilamides **6**-**10**, respectively.
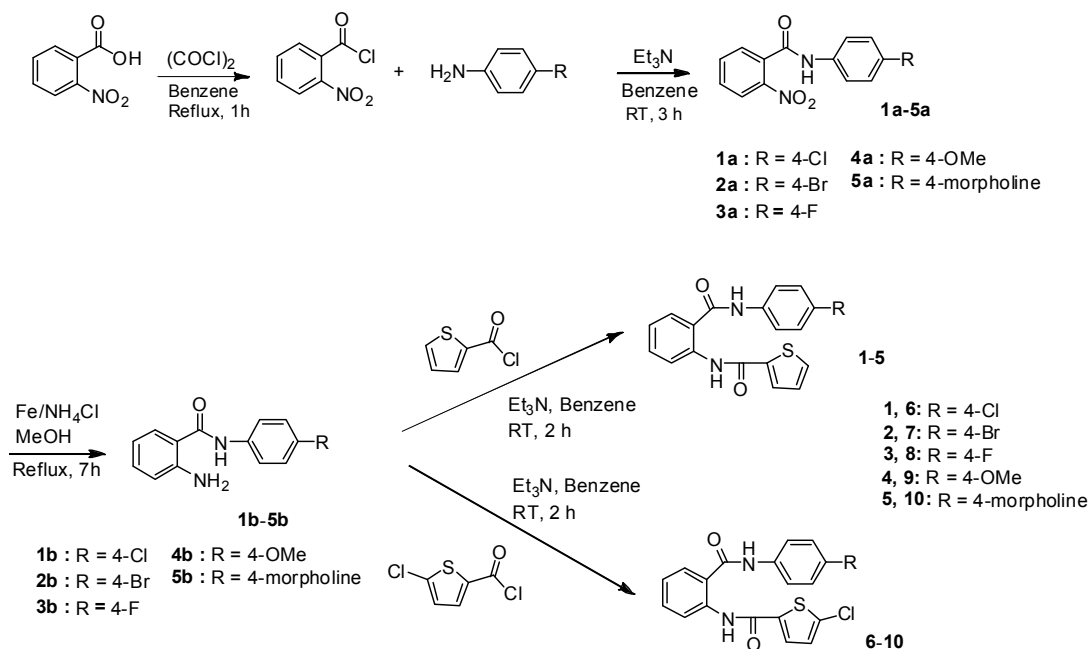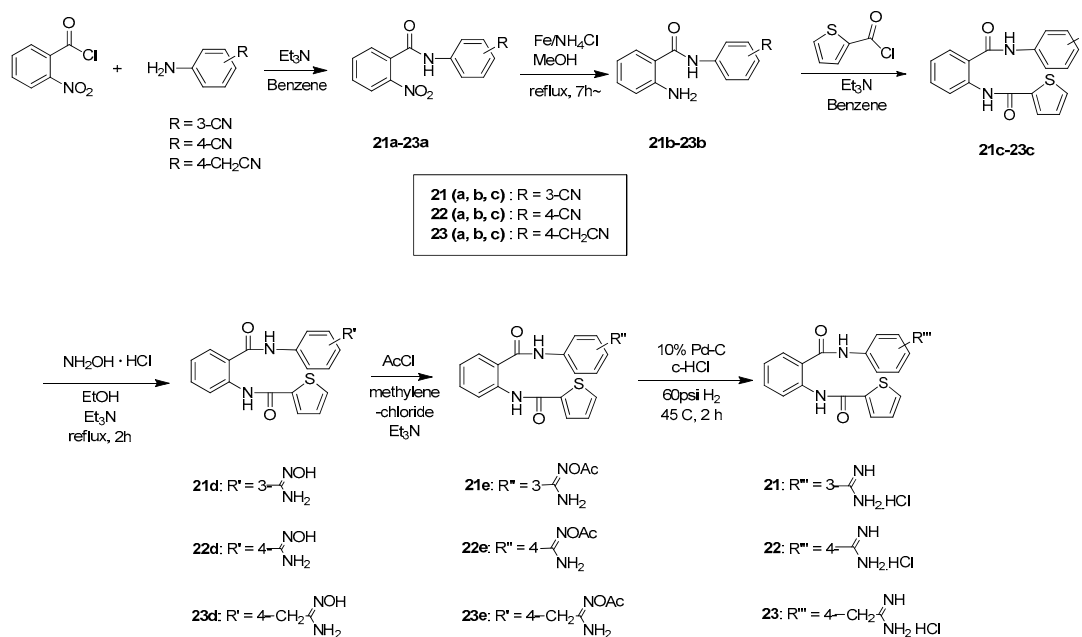
Scheme 1. Synthesis of N^2-(thiophenecarbonyl)anthranilamides derivatives **1**-**10**.

The introduction of sulfonamide instead of one carboxamide in the linker of diamide may give rise to differences in the possible hydrogen-binding interaction between the ligand and the residues in the thrombin/FXa active site. The N^2-arylsulfonamide series **11**-**20** were synthesized to compare with the activity of N^2-arylcarboxamide series **1**-**10**. N^2-tosylanthranilamides were synthesized as beginning compounds for development of N^2-arylsulfonamide derivatives.

The preparative route to N^2-tosylanthranilamide derivatives **11**-**20** are shown in Scheme 2. Anthranilic acid was reacted with *p*-tosyl chloride to give *N*-tosylbenzoic acid, followed by chlorination with oxalyl chloride to give *N*-tosylbenzoyl chloride. This acid chloride was coupled with 3-cyano-, 4-cyano-, 4-cyanomethyl-, 4-chloro-, 4-bromo-, 4-methoxy-, 4-carboxy-, 4-(2-(2-thienyl)ethyl)-, 4-morpholino-, and 4-(2-oxomorpholino)aniline to give N^2-tosylanthranilamide derivatives **11a**-**13a** and **14**-**20**, respectively. To consider the pharmacokinetic problems we replaced the amidine group with less basic residues, aminoalkyl groups. The cyano group containing compounds **11a**-**13a** were reduced by catalytic hydrogenation in the presence of 10% Pd/C and c-HCl at 45 ℃ to give aminoalkyl amides **11**-**13**.
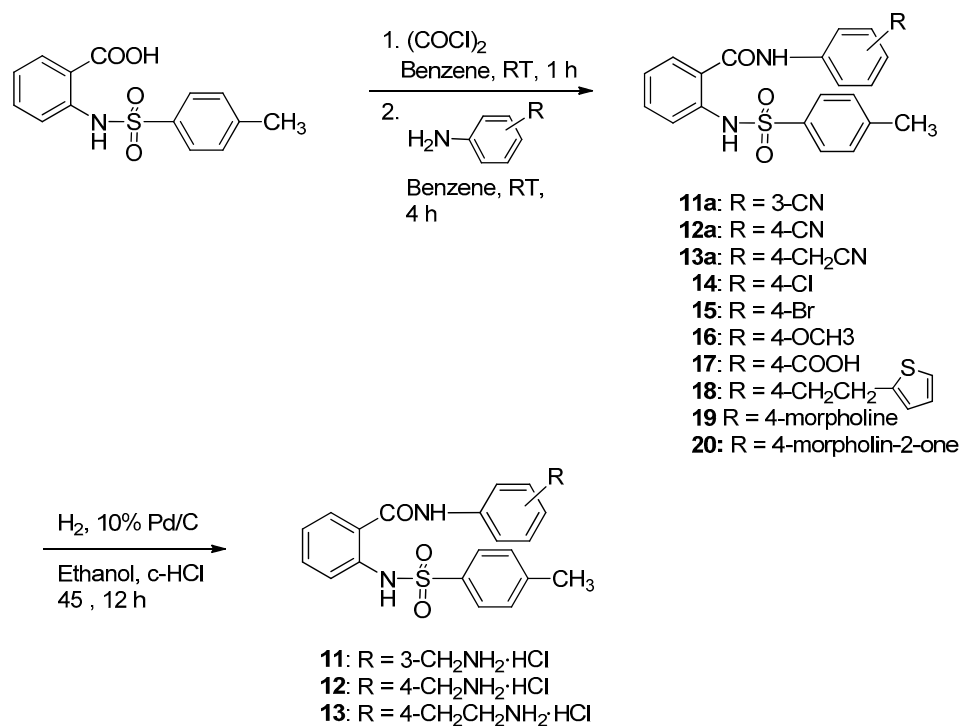
Scheme 2. Synthetic procedures of N^2-tosylanthranilamide derivatives **11**-**20**.

Scheme 3 illustrated the synthetic procedures of amidino N^2-thiophenecarbonyl anthranilamide derivatives **21**-**23**. The first side chain was introduced through acylation of 2-nitrobenzoyl chloride with 3-cyano-, 4-cyano-, and 4-cyanomethyl-aniline to give **21a**–**23a**. The nitro group of these 2-nitrobenzamides was reduced by Fe and NH_4Cl to give 2-aminobenzamides **21b**–**23b**. An acylation with thiophene-2-carbonyl chloride provided the N^2-(thiophene-2-carbonyl)-N-(3-cyano- or -4-cyanophenyl)benzamides **21c** and **22c**, and the N^2-(thiophene-2-carbonyl)-N-(4-cyanobenzyl)benzamide **23c**, which were converted to amidoximes **21d**–**23d** by treatment with hydroxylamine·HCl and triethylamine. The N-O bond of amidoximes **21d**–**23d** was converted to amidinium chloride **21**–**23** via the *O*-acetyl amidoximes **21e**-**23e** and catalytically hydrogenated over 10% Pd/C in the presence of c-HCl at 60 psi, 45 °C for 2 h. In the ^{1}H-NMR spectra, the four proton peaks of amidinium chloride in **21** were identified as one broad singlet at 9.45 ppm, and otherwise the four proton peaks of amidinium chloride in **22** and **23** were identified, respectively, as two broad singlets at 9.17 and 9.36 ppm and at 8.83 and 9.28 ppm. Two NH proton peaks of compound **22** were confirmed by observing the correlation of NH (10.77 ppm) and CO (167.5 ppm) of the -NHCO group and the correlation of NH (11.53 ppm) and CO (164.9 ppm) of the -CONH group in the HMBC spectrum, respectively.

Scheme 3. Synthetic procedures of amidino N^2-(thiophenecarbonyl)anthranilamide derivatives (**21**-**23**).

Scheme 4 illustrated the synthetic procedures of amidino N^2-tosylanthranilamide derivatives **24**-**26**. Compounds **11a**-**13a** were refluxed with hydroxylamine hydrochloride and triethylamine in ethanol to provide the amidoxime derivatives **24b-26b** to form amidinium chloride derivatives **24-26** via the *O*-acetyl amidoximes **24c–26c** and catalytic hydrogenated over 10% Pd/C in the presence of c-HCl at 60 psi, 45 °C for 12 h.
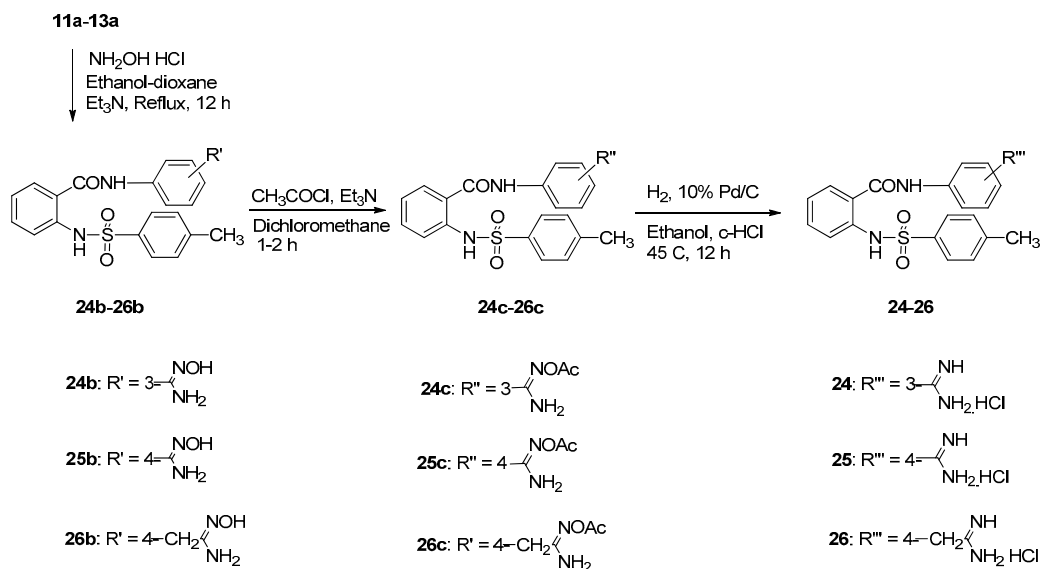
Scheme 4. Synthetic procedures of amidino N^2-tosylanthranilamide derivatives (**24**-**26**).

2.2. Biology

2.2.1. Effects of non-amidino N^2-aroylanthranilamides on *in vitro* aPTT, PT, *in vivo* tail bleeding time, and ex vivo clotting time

Most of the amidine-type compounds reported as FXa and thrombin inhibitor were exhibited to be insufficiently absorbed when administered orally, because of strongly basic amidine groups [27]. Therefore, the trend in synthetic studies seems to be shifted to non-amidine type compounds containing weak basic groups. Rivaroxaban and apixaban are non-amidine direct FXa inhibitors, and argatroban and dabigatran eterxilate are non-amidine direct thrombin inhibitors.

The anticoagulant effects of twenty non-amidino N^2-thiophenecarbonyl anthranilamides **1-10** and N^2-tosylanthranilamides **11-20** were screened in aPTT and PT assays using human plasma at concentration 30 μg/mL *in vitro* and were demonstrated in Table 1. As shown in Table 1, *N*-(4-morpholinophenyl)-2-(thiophen-2-ylcarbonyl)benzamide (**5**), *N*-(4-fluorophenyl)-2-(5-chloro thiophen-2-ylcarbonyl)benzamide (**8**), and *N*-(4-methoxyphenyl)-2-(5-chlorothiophen-2-ylcarbonyl) benzamide (**9**) displayed high aPTT values (60.6, 59.3, and 65.1 s), respectively. Any N^2-tosylanthranilamides **11**-**20** did not exhibit the prolongation of PT and aPTT. This result demostrate that both carboxamide linker and thiophene ring are necessary for anticoagulant activity. For the further anticoagulant and antiplatelet experiments, thiophene compound **5** and 5-chlorothiophene compound **9** were investigated the effects of **5** and **9** on anticoagulant activities (*in vitro* and *ex vivo*) and on tail bleeding time (*in vivo*) on mouse.

Table 1. Anticoagulant activities of non-amidine compounds **1-20** at 30 μg/mL.

Compound No.	aPPT (s)	PT (s)	Compound No.	aPTT (s)	PT (s)
DMSO[1]	39.0	11.1	**11**	40.2	11.8
1	44.5	14.1	**12**	41.2	11.7
2	37.4	13.2	**13**	42.3	10.9
3	41.6	11.8	**14**	38.6	13.6
4	41.7	11.6	**15**	35.8	11.3
5	60.6	12.8	**16**	36.2	11.0
6	39.9	12.2	**17**	41.5	11.7
7	44.9	12.2	**18**	38.4	11.0
8	59.3	12.7	**19**	35.8	10.7
9	65.1	12.5	**20**	33.6	10.8
10	53.3	14.7	Heparin[2]	34 (10 mg/ml)	85 (0.5 mg/ml)

[1] DMSO was used as the negative control. [2] Heparin was used as the positive control. PT: prothrombin time, aPTT: activated partial thromboplastin time.

As shown Table 2, aPTT in the vehicle-treated group was 23.6±0.6 s (mean±SEM, n=5) and in compounds **5**, **9** and heparin, aPTT was increased as 33.2±0.5 s, 37.6±0.3 s and 53.3±0.5 s at dose 20

µM, respectively. Although the *in vitro* anticoagulant activities of compound **5** and **9** were weaker than those of heparin, aPTT was significantly prolonged by **5** and **9** at concentrations 20 µM and above as compared to the vesicle group. Unlike methoxy compound **9**, morpholine compound **5** showed PT prolongation at concentrations 20 µM and above as compared to the vesicle group.

Noting that a prolongation of aPTT suggests the inhibition of intrinsic and/or common coagulation pathway, and a PT prolongation suggests inhibition of the extrinsic and/or the common pathway, obtained results in this study showing prolongation of aPTT and PT of compound **5** suggests inhibition of the extrinsic, and intrinsic and/or common pathway. The prolongation of aPTT of **9** suggests inhibition of intrinsic pathway and/or common pathway.

Table 2. *In vitro* anticoagulant activities of **5** and **9**.

Compound No.	Dose	aPTT (s)	PT (s)	PT (INR)
Control	saline	23.6 ± 0.6	12.4 ± 0.4	1.00
5	5 µM	23.7 ± 0.2	12.2 ± 0.4	0.96
	10 µM	24.2 ± 0.8	12.6 ± 0.6	1.04
	20 µM	33.2 ± 0.5*	14.0 ± 0.3*	1.34*
	30 µM	40.4 ± 0.6*	15.2 ± 0.5*	1.63*
	40 µM	58.5 ± 0.5*	18.5 ± 0.4*	2.61*
	50 µM	60.5 ± 0.7*	19.1 ± 0.6	2.78*
9	5 µM	23.2 ± 0.4	12.0 ± 0.2	0.92
	10 µM	23.4 ± 0.5	12.2 ± 0.5	0.96
	20 µM	37.6 ± 0.3*	12.0 ± 0.2	0.92
	30 µM	44.1 ± 0.4*	11.6 ± 0.6	0.85
	40 µM	65.6 ± 0.6*	12.7 ± 0.5	1.06
	50 µM	63.2 ± 0.8*	13.0 ± 0.8	1.12
Heparin	20 µM	53.3 ± 0.5*	21.6 ± 0.4*	3.79*
	30 µM	62.5 ± 0.8*	27.2 ± 0.6*	6.59*

Each value represents the means ± SEM (n=5). * $p < 0.05$ as compared to control.

To confirm these *in vitro* results, tail bleeding times were determined. The average circulating blood volume for mice is 72 mL/kg [28]. Because the average weight of used mouse is 27 g, the molecular weight of **5** and **9** are 407.49 and 386.85, respectively and the average blood volume is 2 mL, the amount of synthesized compounds (24.4, 32.6, 40.8 µg/mouse for **5** and 25.6, 34.2, 42.7 µg/mouse for **9**) injected yielded a maximum concentration of 30, 40, or 50 µM in the peripheral blood. As shown in Table 3, tail bleeding times were significantly prolonged by compound **5** and **9** in at concentrations 24.4 and 25.6 µg/mouse and above as compared to the control, respectively.

Table 3. *In vivo* bleeding time of **5** and **9**.

Compound No.	Dose	Tail bleeding time (s)
Control	saline	30.8 ± 0.8
5	24.4 µg/mouse	37.4 ± 1.0*
	32.6 µg/mouse	53.7 ± 1.2*
	40.8 µg/mouse	61.8 ± 1.2*
9	25.6 µg/mouse	48.2 ± 1.6*
	34.2 µg/mouse	62.2 ± 1.4*
	42.7 µg/mouse	65.4 ± 1.6*
Heparin	140.0 µg/mouse	51.2 ± 1.2*
	210.0 µg/mouse	68.4 ± 1.2*

Each value represents the means ± SEM (n=5). * $p < 0.05$ as compared to control.

aPTT values were significantly prolonged by both **5** and **9** at concentration 24.4 and 25.6 µg/mouse and above *ex vivo* clotting times, while prolongation in PT was found in compound **5**. (Table 4)

Collectively, aPTT (*in vitro* and *ex vivo*) of **9** was longer than those of **5** suggesting that methoxy group of **9** is more effective for anticoagulant activity than morpholine group of **5**, while 5-chloro group of 5-chlorothiophene was seemed not to influence on the anticoagulant activity.

Table 4. *Ex vivo* clotting time of **5** and **9**.

Compound No.	Dose	aPTT (s)	PT (s)	PT (INR)
Control	saline	32.2 ± 0.8	12.8 ± 0.4	1.00
5	24.4 µg /mouse	38.4 ± 0.8*	14.8 ± 0.4*	1.42*
	32.6 µg/mouse	51.2 ± 1.4*	17.4 ± 0.8*	2.09*
	40.8 µg/mouse	60.8 ± 1.2*	17.2 ± 0.7*	2.03*
9	25.6 µg/mouse	42.2 ± 0.6*	12.6 ± 0.8	0.96
	34.2 µg/mouse	57.4 ± 1.2*	13.0 ± 0.6	1.04
	42.7 µg/mouse	61.2 ± 1.0*	12.4 ± 0.8	0.93
Heparin	140.0 µg/mouse	63.4 ± 0.9*	25.4 ± 0.4*	5.18*
	210.0 µg/mouse	75.4 ± 1.2*	31.4 ± 0.6*	8.62*

Each value represents the means ± SEM (n=5). * $p < 0.05$ as compared to control.

To obtain more active compound, six amidino N^2-aroylantranilamide derivatives (**21-26**) derivatives were synthesized and also screened in aPTT and PT assays using human plasma at

concentration 30 µg/mL *in vitro* and are demonstrated in Table 5. In case of N^2-thiophene compounds, both aPTT and PT of 3-amidino- and 4-amidinomethyl- compounds **21** and **23** were longer than 4-amidino compound **22**. In reverse, among N^2-tosyl derivatives **24**-**26**, 4-amidino derivative **25** showed longer aPTT than 3-amidino- and 4-amidinomethyl- compounds **24** and **26**. From these aPTT and PT results, **21-23** were further investigated the effect of **21-23** on anticoagulant activities (*in vitro* and *ex vivo*) and on tail bleeding time (*in vivo*) on mouse.

Table 5. Anticoagulant activities of amidine compounds **21-26** at 30 µg/mL.

Compound No.	PT (s)	aPTT (s)	Compound No.	PT (s)	aPTT (s)
DMSO[1]	11.1	39.0	**24**	11.5	41.7
21	22.0	74.3	**25**	12.8	63.1
22	19.9	50.7	**26**	11.7	40.5
23	20.9	65.8	Heparin[2]	34 (10 mg/ml)	85 (0.5 mg/ml)

[1]   DMSO was used as the negative control.[2] Heparin was used as the positive control. PT: prothrombin time, aPTT: activated partial thromboplastin time.

As shown Table 6, aPTT in the vehicle-treated group was 23.6±0.6 s (mean±SD, n=5) and in compounds **21-23** and heparin, aPTT showed 38.5±0.4 s, 30.2±0.3 s, 38.5±0.7 s, and 53.3±0.5 s at dose 20 µM, respectively. aPTT was significantly prolonged by **21** and **23** at concentrations 10 µM and above, and **22** at concentration 20 µM and above as compared to the vesicle group, respectively. Unlike amidino N^2-tosylanthranilamides **24**-**26**, amidino thiophene compounds **21-23** significantly showed PT prolongation (16.7±0.5 s, 13.9±0.3 s, 14.2±0.5 s) and INR (2.04, 1.52, and 1.38) at concentrations 20 µM and above as compared to the vesicle group (12.4±0.4 s). The obtained results in this study showing prolongation of aPTT and PT of N^2-thiophenecarbonyl anthranilamides **21-23** suggest inhibition of both extrinsic and intrinsic, and/or common pathway.

Table 6. *In vitro* anticoagulant activity of amidino N^2-thiophene compounds **21-23**.

Compound No.	Dose	aPTT (s)	PT (s)	PT (INR)
Control	saline	23.6 ± 0.6	12.4 ± 0.4	1.00
	5 µM	24.1 ± 0.6	12.8 ± 0.7	1.08
	10 µM	30.8 ± 0.5*	14.5 ± 0.5*	1.46*
	20 µM	38.5 ± 0.4*	16.7 ± 0.5*	2.04*
21	30 µM	52.7 ± 0.7*	19.3 ± 0.6*	2.89*
	40 µM	71.4 ± 0.6*	22.7 ± 0.7*	4.27*
	50 µM	75.2 ± 0.5*	23.5 ± 0.5*	4.64

	5 µM	22.9 ± 0.5	12.2 ± 0.4	0.96
	10 µM	23.7 ± 0.6	12.7 ± 0.4	1.06
22	20 µM	30.2 ± 0.3*	13.9 ± 0.3*	1.32*
	30 µM	32.8 ± 0.5*	16.1 ± 0.7*	1.87*
	40 µM	48.6 ± 0.4*	20.8 ± 0.5*	3.46*
	50 µM	51.4 ± 0.7*	21.4 ± 0.6*	3.70*
	5 µM	24.2 ± 0.2	12.1 ± 0.6	0.94
	10 µM	30.2 ± 0.5*	12.5 ± 0.4	1.02
23	20 µM	38.5 ± 0.7*	14.2 ± 0.5*	1.38*
	30 µM	43.2 ± 0.4*	17.4 ± 0.4*	2.25*
	40 µM	63.8 ± 0.6*	20.1 ± 0.6*	3.19*
	50 µM	64.1 ± 0.4*	20.2 ± 0.5*	3.23*
Heparin	20 µM	53.3 ± 0.5*	21.6 ± 0.4*	3.79*
	30 µM	62.5 ± 0.8*	27.2 ± 0.6*	6.59*

Each value represents the means ± SEM (n=5). * $p < 0.05$ as compared to control.

To confirm these *in vitro* results, tail bleeding times were determined. As shown in Table 7, tail bleeding times were significantly prolonged by compounds **21** and **22** in at concentrations 24.1 µg/mouse and above, and by compound **23** in at concentrations 24.9 µg/mouse and above as compared to the controls, respectively.

Table 7. *In vivo* bleeding time of amidino N^2-thiophene compounds **21-23**.

Compound No.	Dose	Tail bleeding time (s)
Control	saline	30.8 ± 0.8
21	24.1 µg/mouse	49.2 ± 1.2*
	32.1 µg/mouse	68.4 ± 1.0*
	40.1 µg/mouse	70.4 ± 1.6*
22	24.1 µg/mouse	33.1 ± 1.0*
	32.1 µg/mouse	46.2 ± 0.6*
	40.1 µg/mouse	48.3 ± 1.2*
23	24.9 µg/mouse	42.3 ± 0.6*
	33.2 µg/mouse	56.4 ± 1.6*
	41.5 µg/mouse	58.2 ± 1.4*
Heparin	140.0 µg/mouse	51.2 ± 0.8*
	210.0 µg/mouse	68.4 ± 1.2*

Each value represents the means ± SEM (n=5). * $p < 0.05$ as compared to control.

As shown Table 8, both aPTT and PT values were significantly prolonged by both **21** and **22** at concentration 24.1 µg/mouse and above, and by **23** at concentration 25.6 µg/mouse and above *ex vivo* clotting times.

Table 8. *Ex vivo* clotting time of amidino N^2-thiophene compounds **21-23**.

Compound No.	Dose	aPTT (s)	PT (s)	PT (INR)
Control	Saline	32.2 ± 0.8	12.8 ± 0.4	1.00
21	24.1 µg/mouse	48.8 ± 1.0*	19.4 ± 0.8*	2.71*
	32.1 µg/mouse	65.8 ± 0.8*	21.2 ± 0.6*	3.36*
	40.1 µg/mouse	69.4 ± 1.0*	23.7 ± 1.2*	4.39*
22	24.1 µg/mouse	37.2 ± 1.2*	15.7 ± 0.6*	1.63*
	32.1 µg/mouse	47.5 ± 0.6*	19.8 ± 0.8*	2.85*
	40.1 µg/mouse	49.6 ± 0.8*	20.1 ± 0.8*	2.95*
23	24.9 µg/mouse	39.8 ± 0.8*	16.1 ± 0.5*	1.73*
	33.2 µg/mouse	54.2 ± 1.2*	18.2 ± 0.7*	2.33*
	41.5 µg/mouse	56.4 ± 1.8*	19.4 ± 0.6	2.71*
Heparin	140.0 µg/mouse	63.4 ± 0.9*	25.4 ± 0.4*	5.18*
	210.0 µg/mouse	75.4 ± 1.2*	31.4 ± 0.6*	8.62*

Each value represents the means ± SEM (n=5). * $p < 0.05$ as compared to control.

Collectively, aPTT (*in vitro* and *ex vivo*), PT, and tail bleeding time (*in vivo*) on mouse of **21** were longer than those of **5**, **9**, **22** and **23**. The prolongation in PT of amidine N^2-thiophene compounds **21-23** exhibited more longer than non-amidine N^2-thiophene compounds **5** and **9**.
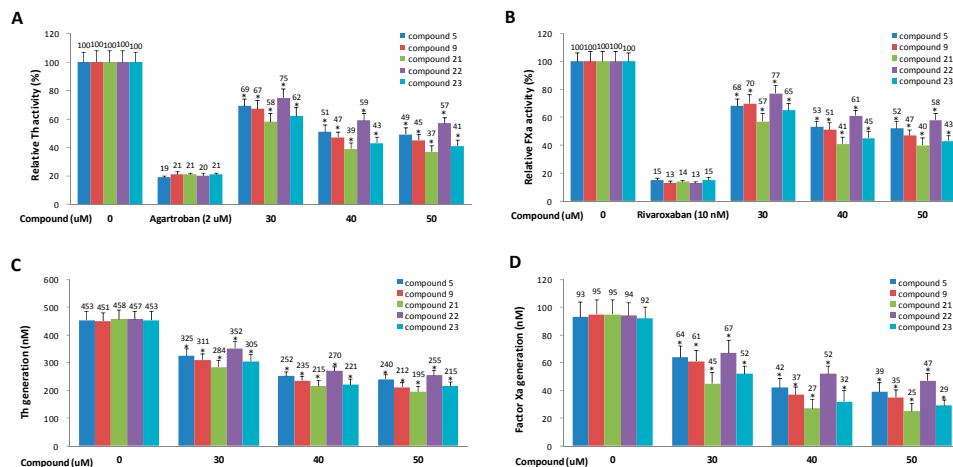
2.2.2. Thrombin and Factor Xa (FXa) activity

In order to determine the underlying mechanism of **5**, **9** and **21-23**, mediated inhibiting of coagulation, the effects of **5**, **9** and **21-23** on the activities of thrombin and FXa were measured. As shown in Fig 1A, compound **5**, **9**, and **21-23** dose-dependently inhibited the activity of thrombin. The direct thrombin inhibitor, argatroban was used as positive control. In addition, treatment with **5**, **9**, and **21-23** resulted in a dose-dependent inhibition of amidolytic activity of FXa, indicating direct inhibition of FXa activity. Rivaroxaban, direct FXa inhibitor, was used as a positive control (Fig. 1B).

2.2.3. Thrombin and Factor Xa (FXa) generation

In previous study, Sugo et al. [29] reported that endothelial cells are able to support prothrombin activation by FXa. In the current study, pre-incubation of HUVECs with FVa and FXa in the presence of CaCl$_2$ prior to addition of prothrombin resulted in production of thrombin (Fig. 1C). According to finding reported by Rao et al. [30], the endothelium provides the functional equivalent of pro-coagulant phospholipids and supports activation of FXa, and, in TNF-α stimulated HUVECs,

activated of FX by FVIIa occurred in a tissue factor (TF) expression-dependent manner. Thus, we investigated the effects of **5**, **9**, and **21-23** on activation of FX by FVIIa. Pre-incubation with **5**, **9**, and **21-23** resulted in dose-dependent inhibition of FX activation by FVIIa (Fig.1D). Therefore, these results suggested that **5**, **9**, and **21-23** can inhibit production of thrombin and FXa.

Figure 1. Effects of 5, 9, and 21-23 on inactivation and production of thrombin and factor Xa. (A) Inhibition of thrombin (Th) by **5** or **9** or **21-23** was measured using a chromogenic assay, as described in the "Materials and Methods". (B) Inhibition of factor Xa (FXa) by **5** or **9** or **21-23** was monitored using a chromogenic assay, as described in the "Materials and Methods". Argatroban (A) or rivaroxaban (B) was used as positive control. (C) HUVEC monolayers were pre-incubated with FVa (100 pM) and FXa (1 nM) for 10 min with the indicated concentrations of **5** or **9** or **21-23**. Prothrombin was added to a final concentration of 1 M and prothrombin activation was determined 30 min later, as described in the "Materials and Methods". (D) HUVECs were pre-incubated with the indicated concentrations (μM) of **5** or **9** or **21-23** for 10 min. After TNF-(10 ng/mL for 6 h) stimulated HUVECs were incubated with FVIIa (10 nM) and FX (175 nM), FXa production was determined as described in the "Materials and Methods". Data represent the mean±SEM of three independent experiments performed in triplicate. *p < 0.05
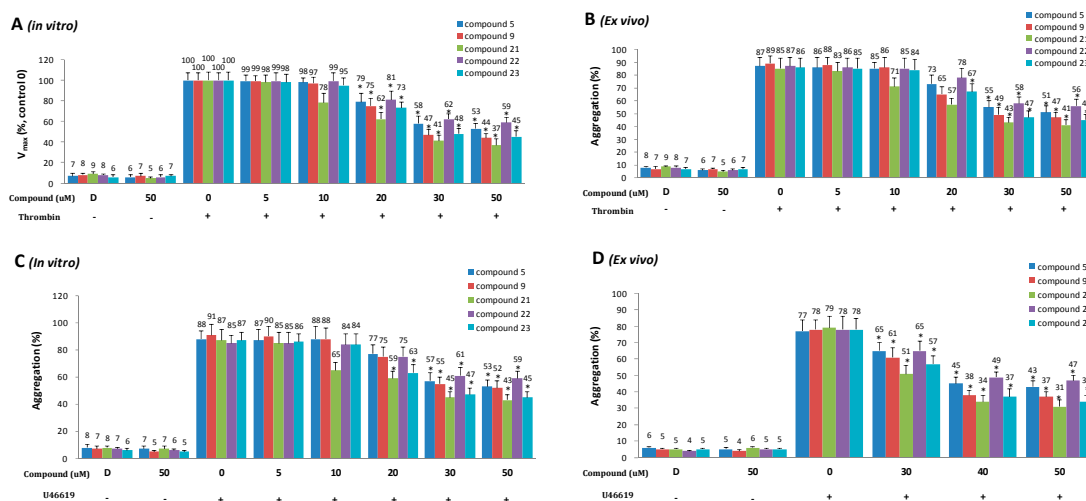
2.2.4.　　Effects of **5**, **9**, and **21-23** on thrombin-catalyzed fibrin polymerization and platelet aggregation

The effects of **5** or **9** or **21-23** on thrombin-catalyzed fibrin polymerization in human plasma were monitored as changes in absorbance at 360 nm, as described in the Experimental section. The results, shown in Fig. 2A, demonstrate that incubation of human plasma with **5** or **9** or **21-23** resulted in a significant decrease in the maximal rate of fibrin polymerization (Fig 2A). To eliminate the effect of sample pH, all dilutions were performed using 50 mM TBS (pH 7.4). We also evaluated the effect of the same volume of DMSO on human plasma; however, coagulation properties were unaffected. To confirm the antiplatelet activities of compound **5** and **9**, thrombin-catalyzed platelet aggregation assay was performed. As shown in Fig. 2B, treatment with compounds **5** or **9** or **21-23** resulted in significantly inhibited mouse platelet aggregation induced by thrombin (final concentration: 3 U/mL) in a concentration dependent manner. In order to exclude the possibility that the decrease of

polymerization could be due to a direct effect on thrombin leading to a decrease in fibrin generation rather than polymerization of fibrin formed, reptilase-catalyzed polymerization assay was performed. Results showed that **5**, **9**, and **21-23** induced a significant decrease in reptilase-catalyzed polymerization (data not shown).

### 2.2.5.   Effects of **5**, **9**, and **21-23** on U46619 catalyzed platelet aggregation

To confirm the antiplatelet activities of compounds **5** or **9** or **21-23**, a U46619-(a stable thromboxane A2 analog/aggregation agonist) catalyzed platelet aggregation assay was performed. As shown in figure 2C, treatment with compounds **5** or **9** or **21-23** significantly inhibited human platelet aggregation induced by U46619 (final concentration: 2 µM) in a concentration-dependent manner. These *in vitro* results were confirmed in an *ex vivo* platelet aggregation assay (i.v. injection, figure 2D). As shown in Fig. 2D, treatment with **5** or **9** or **21-23** resulted in significantly inhibited mouse platelet aggregation induced U46619 (final concentration: 2 µM) in a concentration dependent manner. So far, most of amidine-type compounds have been reported as FXa inhibitor, these non-amidines **5** and **9**, and amidines **21-23** exhibited the potential as platelet aggregation inhibitor.

Figure 2. Effects of 5, 9, and 21-23 on fibrin polymerization in human plasma. (A) Thrombin-catalyzed fibrin polymerization at the indicated concentrations of **5** or **9** or **21-23** was monitored using a catalytic assay, as described in the "Materials and Methods". The results are Vmax values expressed as percentages versus controls. (B) Effect of **5** or **9** or **21-23** on mouse platelet aggregation induced by 3U/mL thrombin. (C) The effect of each compound on human platelet aggregation induced by 2 mM U46619. (D) The indicated each compound concentration in DMSO was injected intravenously. The effects of each compound on mouse platelet aggregation induced by 2 µM U46619 (U) were monitored *ex vivo*. D: 0.2% DMSO is the vehicle control. Data represent the mean±SEM of three independent experiments performed in triplicate. * *p* < 0.05 *vs.* Thrombin or U46619 alone.

2.2.6.　Cellular viability

To determine the cellular viability of compounds **5**, **9**, and **21-23**, cellular viability assay (MTT assay) was performed in HUVECs treated with **5**, **9**, and **21-23** for 24 h. Compounds **5**, **9**, and **21-23** did not affect cell viability at concentration up to 100 µM (Fig. 3).
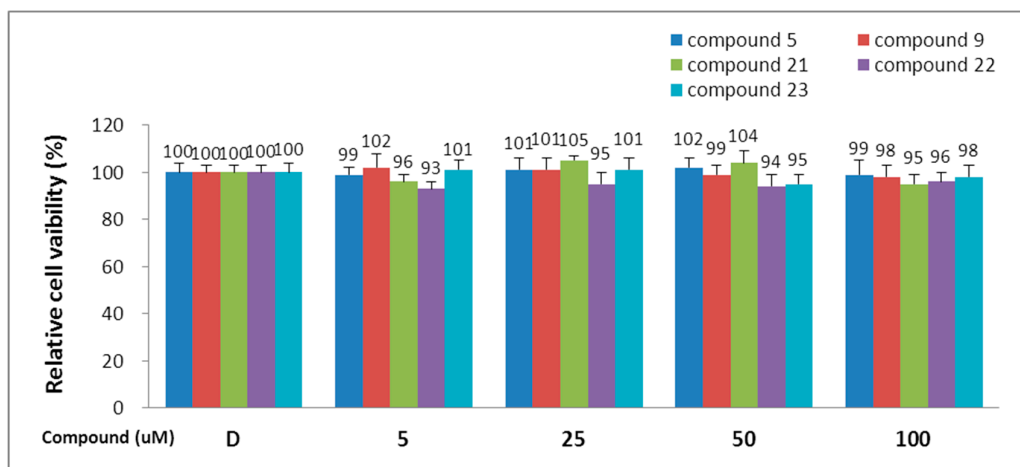
The data represent the means ± SEM of three independent experiments performed in triplicate. . D : 0.2% DMSO is the vehicle control.

Figure 3. Effects of **5**, **9,** and **21-23** on cellular viability were measured by MTT assay.

3.　Materials and Methods

3.1.　Chemistry

3.1.1.　Reagents and instruments

The commercial reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or TCI (Tokyo, Japan). Solvents were purified and dried prior to use. All non-aqueous reactions were performed under a dry atmosphere of nitrogen. Melting points were measured on Thomas-Hoover melting point apparatus (Thomas Scientific, Swedesboro, NJ, USA) and not corrected. ^{1}H, ^{13}C-NMR and HMBC spectra were taken on a Varian 400 MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA) in DMSO-d_6, CDCl$_3$, or (CD$_3$)$_2$CO. Chemical shifts (δ) are in parts per million (ppm) relative to tetramethylsilane, and coupling constants (*J*) are in Hertz. DIP-MS (EI) was obtained on an Agilent 7890A-5975C GC/MSD (Agilent Technologies, Santa Clara, CA, USA). GC/MS (EI) was obtained on a SHIMADZU QP 2010 model (Shimadzu, Kyoto, Japan) and FAB-MS was obtained on a JEOL JMS-700 Mstation (JEOL, Tokyo, Japan). Fraction collection was performed on an EYELA fraction collector DC-1500 (Tokyo Rikakikai, Tokyo, Japan). An analytical TLC was performed on pre-coated silica gel 60 F$_{254}$ plates (Merck, Kenilworth, NJ, USA). Solvent systems for TLC were ethyl acetate/*n*-hexane mixtures and 10% methanol in dichloromethane. Column chromatography was carried out on Merck silica gel 9385 (Merck, Kenilworth, NJ, USA) (230–400 mesh).

3.1.2.   General synthetic procedures for **1a-5a**

To a stirred solution of 2-nitrobenzoic acid (29.94 mmol) in anhydrous dichloromethane (50 mL) was dropwise added oxalyl chloride (38.92 mmol) and then triethylamine (32.93 mmol) at room temperature. The reaction mixture was refluxed for 1 h and the solvent and unreacted oxalyl chloride were evaporated off under reduced pressure and the acid chloride was used to next reaction without purification. To a solution of 3-aminobenzonitrile, 4-aminobenzonitrile, and 4-aminobenzyl cyanide (7.19 mmol) in anhydrous dichloromethane (30 mL) was added acyl chloride (8.99 mmol) and triethylamine (7.19 mmol) and stirred for 3 h at room temperature. To the reaction mixture, water was added and extracted with dichloromethane (30 mL x 3), dried with anhydrous magnesium sulfate and filtrated. The filtrate was evaporated under reduced pressure to give the crude compound, which was purified by column chromatography to give pure white or pale yellow compound, respectively

N-(4'-Chlorophenyl)-2-nitrobenzamide (**1a**). Yield : 85%; m.p. : 184-186 ℃ (186-187 ℃ [31]).

N-(4'-Bromophenyl)-2-nitrobenzamide (**2a**) [32,34]. Yield : 86%; m.p. : 199-201 ℃ (202-205℃ [33]).

N-(4'-Fluorophenyl)-2-nitrobenzamide (**3a**). Yield : 78%; m.p. : 165-169 ℃ (167-171℃ [35]).

N-(4'-Methoxyphenyl)-2-nitrobenzamide (**4a**) [36]. Yield : 82%; m.p. : 153-154 ℃.

N-(4'-Morpholinophenyl)-2-nitrobenzamide (**5a**). Yield : 76%; m.p. : 220-221 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.03 (4H, t, *J* = 4.8 Hz, H-3''×2, H-5''×2), 3.71 (4H, t, *J* = 4.8 Hz, H-2''×2, H-6''×2), 6.91 (2H, d, *J* = 9.0 Hz, H-3',5'), 7.49 (2H, d, *J* = 9.0 Hz, H-2',6'), 7.68-7.71 (2H, m, H-4,6), 7.82 (1H, dt, *J* = 8.0, 1.1 Hz, H-5), 8.09 (1H, d, *J* = 7.6 Hz, H-3), 10.42 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 48.9 (C-3'',5''), 66.0 (C-2'',6''), 115.4 (C-2',6'), 120.8 (C-3',5'), 124.2 (C-1), 129.3 (C-3), 130.8 (C-6), 131.0 (C-1'), 132.8 (C-4), 134.0 (C-5), 146.6 (C-2), 147.8 (C-4'), 163.4 (C=O); GC-MS (EI) *m/z* : 327 [M]$^+$.

3.1.3. General synthetic procedures for **1b-5b**

To a stirred solution of **1a – 5a** (3.37 mmol) in methanol (30 mL) was added ammonium chloride (33.7 mmol) and iron powder (6.03 mmol) and the reaction mixture was reflux for 7 h. The reaction mixture was added with water and extracted with dichloromethane (30 mL x 3), dried with anhydrous magnesium sulfate and filtrated. The filtrate was evaporated under reduced pressure to give crude compound, which was recrystallized with ethyl acetate ad *n*-hexane to give pure white or pale yellow compounds **1b-5b**, respectively

2-Amino-*N*-(4'-chlorophenyl)benzamide (**1b**). Yield : 63%; m.p. : 148-150 ℃ (147 ℃ [37]).

2-Amino-*N*-(4'-bromophenyl)benzamide (**2b**). Yield : 70%; m.p. : 143-145 ℃ (139-144 ℃ [38]).

2-Amino-*N*-(4'-fluorophenyl)benzamide (**3b**). Yield : 62%; m.p. : 118-120 ℃ (122 ℃ [37]).

2-Amino-*N*-(4'-methoxyphenyl)benzamide [38] (**4b**). Yield : 80%; m.p. : 118-119 ℃ (121 ℃ [37]).

2-Amino-*N*-(4''-morpholinophenyl)benzamide (**5b**). Yield : 97%; m.p. : 167-168 ℃; ¹H NMR (400 MHz, DMSO-*d₆*) δ: 3.06 (4H, t, *J* = 4.8 Hz, H-3''×2, H-5''×2), 3.74 (4H, t, *J* = 4.8 Hz, H-2''×2, H-6''×2), 6.29 (2H, s, NH₂), 6.57 (1H, dt, *J* = 7.7, 1.1 Hz, H-5), 6.73 (1H, dd, *J* = 8.2, 1.0 Hz, H-3), 6.91 (2H, d, *J* = 9.0 Hz, H-3',5'), 7.17 (1H, dt, *J* = 8.4, 1.5 Hz, H-4), 7.56 (2H, d, *J* = 9.0 Hz, H-2',6'), 7.59 (1H, dd, *J* = 7.8, 1.8 Hz, H-6), 9.80 (1H, s, CONH); ¹³C NMR (100 MHz, DMSO-*d₆*) δ: 39.9 (C-3'',5''), 65.1 (C-2'',6''), 114.5 (C-1), 115.2 (C-2',6'), 115.6 (C-3), 116.3 (C-5), 121.7 (C-3',5'), 129.5 (C-6), 131.4 (C-1'), 131.8 (C-4), 147.4 (C-4'), 149.6 (C-2), 167.4 (C=O); GC-MS (EI) *m/z* : 297 [M]⁺.

3.1.4. General synthetic procedures for **1-10**

Thiophene-2-carbonioride and 5-chlorothiophenecarbonyl chloride were prepared according to the synthetic procedures for **1a-5a**. To a solution of **1b-5b** (0.41 mmol) in anhydrous benzene (20 mL) was slowly added triethylamine (0.41 mmol) and thiophene-2-carbonyl chloride (0.51 mmol) or 5-chlorothiophene-2-carbonyl chloride (0.51 mmol) at room temperature and the reaction mixture was stirred for 2 h. The following procedures were same as procedures for **1a-5a**. Compounds 1-10 were recrystallized with ethyl acetate and *n*-hexane to give a pure white or pale yellow compound.

N-(4′-Chlorophenyl)-2-(thiophen-2′′-ylcarbonylamino)benzamide (**1**).Yield : 79%; m.p. : 197-199 ℃; ¹H NMR (400 MHz, DMSO-*d₆*) δ: 7.25 (1H, dd, *J* = 4.9, 3.8 Hz, H-4''), 7.29 (1H, dt, *J* = 7.7, 1.1 Hz, H-5), 7.43 (2H, d, *J* = 8.8 Hz, H-3',5'), 7.61 (1H, dt, *J* = 8.0, 1.2 Hz, H-4), 7.74-7.78 (3H, m, H-2',6',5''), 7.88-8.02 (2H, m, H-3,6), 8.32 (1H, d, *J* = 8.0 Hz, H-3''), 10.63 (1H, s, NHCO), 11.54 (1H, s, CONH); ¹³C NMR (100 MHz, DMSO-*d₆*) δ: 121.6 (C-3), 122.5 (C-2',6'), 123.0 (C-1), 123.4 (C-5), 127.9 (C-4'), 128.4 (C-3',5'), 128.6 (C-6), 128.8 (C-4''), 129.0 (C-5''), 132.2 (C-4), 132.3 (C-3''), 137.5 (C-2''), 138.1 (C-1'), 139.5 (C-2), 159.4 (CONH), 167.3 (NHCO); DIP-MS (EI): m/z 356 [M]⁺; HRMS (FAB): m/z calcd for C₁₈H₁₄ClN₂O₂S [M+H]⁺ 357.0465, found 357.0478.

N-(4′-Bromophenyl)-2-((thiophen-2′′-yl)carbonylamino)benzamide (**2**). Yield : 75%; m.p. : 210-212 ℃; ¹H NMR (400 MHz, DMSO-*d₆*) δ: 7.25 (1H, dd, *J* = 4.9, 3.8 Hz, H-4''), 7.30 (1H, dt, *J* = 7.8, 1.1 Hz, H-5), 7.56 (2H, d, *J* = 8.9 Hz, H-3',5'), 7.61 (1H, dt, *J* = 8.0, 1.2 Hz, H-4), 7.71 (2H, d, *J* = 8.9 Hz, H-2',6'), 7.75 (1H, dd, *J* = 3.7, 1.0 Hz, H-5''), 7.88-8.03 (2H, m, H-3,6), 8.30 (1H, d, *J* = 8.2 Hz, H-3''), 10.64 (1H, s, NHCO), 11.51 (1H, s, CONH); ¹³C NMR (100 MHz, DMSO-*d₆*) δ: 116.0 (C-4'), 121.6 (C-3), 122.9 (C-2',6'), 123.1 (C-1), 123.4 (C-5), 128.4 (C-6), 128.8 (C-4''), 129.0 (C-5''), 131.5 (C-3',5'), 132.2 (C-4), 132.3 (C-3''), 138.0 (C-2''), 138.1 (C-1'), 139.5 (C-2), 159.4 (CONH), 167.3 (NHCO); DIP-MS (EI): m/z 400 [M-1]⁺, 402 [M+1]⁺; HRMS (FAB): m/z calcd for C₁₈H₁₄BrN₂O₂S [M+H]⁺ 400.9959, found 400.9973.

N-(4′-Fluorophenyl)-2-((thiophen-2′′-yl)carbonylamino)benzamide (**3**). Yield : 73%; m.p. : 215-216 ℃; ¹H NMR (400 MHz, DMSO-*d₆*) δ: 7.19-7.26 (3H, m, H-3',5',4''), 7.29 (1H, dt, *J* = 7.7, 1.1 Hz, H-5), 7.61 (1H, dt, *J* = 8.0, 1.0 Hz, H-4), 7.73-7.75 (3H, m, H-2',6', 5''), 7.89 (1H, dd, *J* = 5.0, 1.1 Hz, H-6), 7.92 (1H, dd, *J* = 7.8, 1.2 Hz, H-3), 8.36 (1H, d, *J* = 7.6 Hz, H-3''), 10.58 (1H, s, NHCO), 11.68 (1H, s, CONH); ¹³C NMR (100 MHz, DMSO-*d₆*) δ: 115.9 (*J* = 22.3 Hz, C-3',5'), 122.0 (C-3), 123.3 (C-1), 123.7 (*J* = 8.2 Hz, C-2',6'), 124.1 (C-5), 129.1 (C-6), 129.4 (C-4''), 129.6 (C-5''), 133.0 (C-4,3''), 135.5 (*J* = 3.0 Hz, C-1'), 139.0 (C-2''), 140.2 (C-2), 159.4 (*J* = 239.4 Hz, C-4'), 160.0 (CONH), 167.9 (NHCO); DIP-MS (EI): m/z 340 [M]⁺; HRMS (FAB): m/z calcd for C₁₈H₁₄FN₂O₂S [M+H]⁺ 341.0760, found 341.0781.

N-(4´-Methoxyphenyl)-2-(thiophen-2´´-ylcarbonylamino)benzamide (**4**). Yield : 77%; m.p. : 170-172 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.73 (3H, s, CH$_3$), 6.96 (2H, d, J = 9.0 Hz, H-3',5'), 7.31-7.23 (2H, m, H-5,4''), 7.59-7.64 (3H, m, H-4,2',6'), 7.73 (1H, dd, J = 3.7, 0.6 Hz, H-5''), 7.89 (1H, dd, J = 5.0, 0.6 Hz, H-6), 7.94 (1H, d, J = 6.9 Hz, H-3), 8.43 (1H, d, J = 8.2 Hz, H-3''), 10.45 (1H, s, NHCO), 11.92 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.2 (CH$_3$), 113.8 (C-3',5'), 120.9 (C-3), 122.1 (C-1), 122.9 (C-2',6'), 123.2 (C-5), 128.4 (C-6), 128.6 (C-4''), 128.8 (C-5''), 131.3 (C-1'), 132.2 (C-4), 132.3 (C-3''), 138.5 (C-2''), 139.6 (C-2), 156.1 (C-4'), 159.3 (CONH), 167.0 (NHCO); DIP-MS (EI): m/z 352 [M]$^+$; HRMS (FAB): m/z calcd for C$_{19}$H$_{17}$N$_2$O$_3$S [M+H]$^+$ 353.0960, found 353.0979.

N-(4´-Morpholinophenyl)-2-(thiophen-2´´´-ylcarbonylamino)benzamide (**5**). Yield : 76%; m.p. : 246-247 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.09 (4H, t, J = 4.8 Hz, H-3''×2, H-5''×2), 3.74 (4H, t, J = 4.8 Hz, H-2''×, H-6''×2), 6.96 (2H, d, J = 9.0 Hz, H-3',5'), 7.22-7.30 (2H, m, H-5,4'''), 7.57-7.61 (3H, m, H-4,2',6'), 7.72 (1H, dd, J = 3.7, 0.6 Hz, H-5'''), 7.90 (1H, dd, J = 4.9, 0.5 Hz, H-6), 7.93 (1H, d, J = 7.3 Hz, H-3), 8.43 (1H, d, J = 8.1 Hz, H-3'''), 10.39 (1H, s, NHCO), 11.97 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 48.7 (C-3'',5''), 66.1 (C-2'',6''), 115.2 (C-3',5'), 120.8 (C-3), 122.0 (C-1), 122.4 (C-2',6'), 123.1 (C-5), 128.4 (C-6), 128.6 (C-4''), 128.8 (C-5''), 130.3 (C-1'), 132.2 (C-4), 132.3 (C-3''), 138.6 (C-2''), 139.6 (C-2), 148.1 (C-4'), 159.3 (CONH), 166.9 (NHCO); DIP-MS (EI): m/z 407 [M]$^+$; HRMS (FAB): m/z calcd for C$_{22}$H$_{22}$N$_3$O$_3$S [M+H]$^+$ 408.1382, found 408.1368.

N-(4´-Chlorophenyl)-2-((5-chlorothiophen-2´´-yl)carbonylamino)benzamide (**6**). Yield : 60%; m.p. : 207-209 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.29 (1H, d, J = 4.0 Hz, H-4''), 7.32 (1H, dt, J = 7.7, 1.1 Hz, H-5), 7.43 (2H, d, J = 8.9 Hz, H-3',5'), 7.60 (1H, dt, J = 8.0, 1.2 Hz, H-4), 7.64 (1H, d, J = 4.1 Hz, H-6), 7.75 (2H, d, J = 8.9 Hz, H-2',6'), 7.88 (1H, dd, J = 7.8, 1.0 Hz, H-3), 8.17 (1H, d, J = 8.2 Hz, H-3''), 10.63 (1H, s, NHCO), 11.44 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 122.2 (C-3), 122.4 (C-2',6'), 123.9 (C-1), 124.2 (C-5), 127.8 (C-4'), 128.6 (C-3',5'), 128.7 (C-6), 129.1 (C-4''), 132.2 (C-4,3''), 134.2 (C-2''), 137.4 (C-5''), 137.7 (C-1'), 138.7 (C-2), 158.4 (CONH), 167.1 (NHCO); DIP-MS (EI): m/z 390 [M-1]$^+$; HRMS (FAB): m/z calcd for C$_{18}$H$_{13}$Cl$_2$N$_2$O$_2$S [M+H]$^+$ 391.0075, found 391.0089.

N-(4´-Bromophenyl)-2-((5-chlorothiophen-2´´-yl)carbonylamino)benzamide (**7**). Yield : 41%; m.p. : 198-199 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (1H, d, J = 4.1 Hz, H-4''), 7.32 (1H, dt, J = 7.7, 1.1 Hz, H-5), 7.55 (2H, d, J = 8.9 Hz, H-3',5'), 7.60 (1H, dt, J = 8.0, 1.2 Hz, H-4), 7.64 (1H, d, J = 4.1 Hz, H-6), 7.70 (2H, d, J = 8.9 Hz, H-2',6'), 7.88 (1H, dd, J = 7.8, 1.3 Hz, H-3), 8.18 (1H, dd, J = 8.2, 0.8 Hz, H-3''), 10.60 (1H, s, NHCO), 11.42 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 115.9 (C-4'), 122.1 (C-3), 122.8 (C-2',6'), 123.9 (C-1), 124.2 (C-5), 128.7 (C-6), 129.0 (C-4''), 131.5 (C-3',5'), 132.1 (C-4,3''), 134.1 (C-2''), 137.4 (C-5''), 138.0 (C-1'), 138.6 (C-2), 158.4 (CONH), 167.1 (NHCO); DIP-MS (EI): m/z 434 [M-1]$^+$, 436 [M+1]$^+$; HRMS (FAB): m/z calcd for C$_{18}$H$_{13}$BrClN$_2$O$_2$S [M+H]$^+$ 434.9570, found 434.9549.

N-(4´-Fluorophenyl)-2-((5-chlorothiophen-2´´-yl)carbonylamino)benzamide (**8**). Yield : 73%; m.p. : 215-216 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.22 (2H, t, J = 8.9 Hz, H-3',5'), 7.27-7.34 (2H, m, H-5,4''), 7.64-7.57 (2H, m, H-4,6), 7.73 (2H, dd, J = 9.1, 5.1 Hz, H-2',6'), 7.91 (1H, dd, J = 7.9, 1.1 Hz, H-3), 8.24 (1H, d, J = 8.1 Hz, H-3''), 10.57 (1H, s, NHCO), 11.60 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 115.2 (J = 22.3 Hz, C-2',6'), 121.8 (C-3), 123.0 (J = 8.1 Hz, C-3',5'), 123.5 (C-1), 123.8 (C-5), 128.6 (C-6), 128.9 (C-4''), 132.2 (C-4), 134.1 (C-2''), 134.8 (J = 2.2 Hz, C-1'), 137.7 (C-5''), 138.6 (C-2), 158.3 (CONH), 158.6

(J = 239.5 Hz, C-4'), 167.0 (NHCO); DIP-MS (EI): m/z 374 [M]$^+$; HRMS (FAB): m/z calcd for C$_{18}$H$_{13}$ClFN$_2$O$_2$S [M+H]$^+$ 375.0370, found 375.0388.

N-(4′-Methoxyphenyl)-2-((5-chlorothiophen-2′′-yl)carbonylamino)benzamide (**9**). Yield : 77%; m.p. : 190-191 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.75 (3H, s, CH$_3$), 6.95 (2H, d, J = 8.8 Hz, H-3',5'), 7.26-7.32 (2H, m, H-5,4''), 7.56-7.65 (4H, m, H-4,6,2',6'), 7.94 (1H, d, J = 7.7 Hz, H-3), 8.31 (1H, d, J = 8.3 Hz, H-3''), 10.45 (1H, s, NHCO), 11.87 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.4 (CH$_3$), 114.0 (C-2',6'), 121.6 (C-3), 123.0 (C-3',5'), 123.1 (C-1), 123.8 (C-5), 128.7 (C-6), 128.8 (C-4''), 129.1 (C-1'), 131.5 (C-3''), 132.3 (C-4), 134.4 (C-2''), 138.2 (C-5''), 139.9 (C-2), 156.3 (C-4'), 158.5 (CONH), 167.1 (NHCO); DIP-MS (EI): m/z 386 [M]$^+$; HRMS (FAB): m/z calcd for C$_{19}$H$_{16}$ClN$_2$O$_3$S [M+H]$^+$ 387.0457, found 387.0470.

N-(4′-Morpholinophenyl)-2-((5-chlorothiophen-2′′′-yl)carbonylamino)benzamide (**10**). Yield : 80%; m.p. : 230-231 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.08 (4H, t, J = 4.8 Hz, H-3''×2, H-5''×2), 3.74 (4H, t, J = 4.8 Hz, H-2''×2, H-6''×2), 6.96 (2H, d, J = 9.1 Hz, H-3',5'), 7.23-7.32 (2H, m, H-5,4'''), 7.51-7.64 (4H, m, H-4,6,2',6'), 7.92 (1H, dd, J = 7.9, 1.1 Hz, H-3), 8.32 (1H, d, J = 8.3 Hz, H-3'''), 10.39 (1H, s, NHCO); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 51.6 (C-3'',5''), 68.9 (C-2'',6''), 118.0 (C-3',5'), 124.1 (C-3), 125.2 (C-2',6'), 125.6 (C-1), 126.4 (C-5), 131.2 (C-6), 131.5 (C-4''), 131.7 (C-3''), 133.2 (C-4), 135.0 (C-2''), 137.0 (C-1'), 140.9 (C-5''), 141.6 (C-2), 150.9 (C-4'), 161.1 (CONH), 169.6 (NHCO); DIP-MS (EI): m/z 441[M]$^+$; HRMS (FAB): m/z calcd for C$_{22}$H$_{21}$ClN$_3$O$_3$S [M+H]$^+$ 442.0992, found 442.0977.

3.1.6. 3.1.7. General synthetic procedures for **11a-13a**

To a solution of 4-(methylphenylsulfonamido)benzoyl chloride **2** (2.06 mmol) was dissolved in anhydrous benzene (30 mL) and added with 3- or 4-aminobenzonitrile (220 mg, 1.87 mmol), 4-aminobenzylcyanide (265 mg, 1.87 mmol) and triethylamine (0.1 mL). The following procedures were same as procedures for **1a-5a**. **11a-13a** were recrystallized with dichloromethane and *n*-hexane mixture to afford a white powder.

N-(3′-Cyanophenyl)-2-(4′′-methylphenylsulfonamido)benzamide (**11a**). Yield : 75%; m.p. : 216-218 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ : 2.23 (3H, s, CH$_3$), 7.21 (2H, d, J = 8.4 Hz, H-3'',5''), 7.23 (1H, t, J = 7.6 Hz, H-5), 7.36 (1H, d, J = 8.0 Hz, H-3), 7.48 (1H, t, J = 7.6 Hz, H-4), 7.54-7.65 (4H, m, H-6, 5',2'',6''), 7.69 (1H, d, J = 7.2 Hz, H-4'), 7.88 (1H, d, J = 7.6 Hz, H-6'), 8.08 (1H, s, H-2'), 10.26 (1H, s, NHSO$_2$), 10.53 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 21.6 (CH$_3$), 112.1 (C-3'), 119.3 (CN), 122.9 (C-3), 124.1 (C-1), 125.9 (C-5), 127.6 (C-6), 127.8 (C-6'), 128.3 (C-2'',6''), 129.8 (C-5'), 130.4 (C-3'',5''), 130.8 (C-2'), 133.1 (C-4), 136.6 (C-4'), 137.1 (C-1''), 137.4 (C-4''), 140.0 (C-1'), 144.3 (C-2), 167.5 (C=O); DIP-MS (EI): m/z 391 [M]$^+$.

N-(4′-Cyanophenyl)-2-(4′′-methylphenylsulfonamido)benzamide (**12a**). Yield : 70%; m.p. : 253-255 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ : 2.22 (3H, s, CH$_3$), 7.12-7.26 (3H, m, H-5,3'',5''), 7.33 (1H, d, J = 7.6 Hz, H-3), 7.47 (1H, t, J = 7.6 Hz, H-4), 7.56 (2H, d, J = 7.6 Hz, H-2'',6''), 7.67 (1H, d, J = 7.2 Hz, H-6), 7.77-7.87 (4H, m, H-2',3',5',6'), 10.16 (1H, s, NHSO$_2$), 10.59 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 21.6 (CH$_3$), 106.4 (C-4'), 119.7 (CN), 121.2 (C-2',6'), 123.1 (C-3), 125.2 (C-1), 126.4 (C-5), 127.5 (C-6), 128.0 (C-2'',6''), 130.4 (C-3'',5''), 133.1 (C-3',5'), 133.8 (C-4), 137.2 (C-1'',4''), 143.6 (C-1'), 144.3 (C-2), 167.6 (C=O); DIP-MS (EI): m/z 391 [M]$^+$.

N-(4′-Cyanomethylphenyl)-2-(4″-methylphenylsulfonamido)benzamide (**13a**). Yield: 68 %; m.p. : 142-146 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ : 2.27 (3H, s, CH$_3$), 4.02 (2H, s, CH$_2$), 7.20-7.29 (3H, m, H-5,3″,5″), 7.36 (2H, d, *J* = 8.8 Hz, H-3',5'), 7.45-7.53 (2H, m, H-3,4), 7.67 (2H, d, *J* = 8.4 Hz, H-2″,6″), 7.76 (1H, dd, *J* = 8.0, 1.2 Hz, H-6), 10.39 (1H, s, NHSO$_2$), 10.59 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 21.0 (CH$_3$), 21.9 (CH$_2$), 119.3 (CN), 121.4 (C-2',6'), 124.0 (C-3), 124.1 (C-1), 126.8 (C-5), 127.0 (C-6), 128.4 (C-2″,6″), 129.1 (C-3',5'), 129.8 (C-3″,5″), 132.4 (C-4), 135.9 (C-1'), 137.2 (C-1″), 137.7 (C-4″), 143.7 (C-2), 166.7 (C=O); GC-MS (EI) *m/z* : 405 [M]$^+$.

3.1.7. General synthetic procedures for **11**-**13**

A suspension of 10% Pd-C (200 mg) in ethanol (50 mL) was added with 1*N*-HCl (1 mL) and **11a-13a** (1.28 mmol) and the reaction mixture was catalytically hydrogenated in *parr* hydrogenation apparatus (50 psi of hydrogen) at room temperature for 6 h. When the starting material could be no longer detected by TLC, the reaction mixture was cooled down to room temperature and filtrated on celite pad. The filtrate was evaporated to give the oily product which was dissolved in ethanol and then crystallized with diethyl ether to form the brown precipitate which was recrystallized ethanol and diethyl ether mixture to afford **11**-**13** as a white powder.

N-(3′-Aminomethyl)phenyl-2-(4″-methylphenylsulfonamido)benzamide (**11**). Yield : 72 %; m.p : 163-166 ℃; ^{1}H NMR (400 MHz, CD$_3$OD) δ: 2.29 (3H, s, CH$_3$), 4.03 (2H, s, CH$_2$), 7.23 (1H, t, *J* = 7.2 Hz, H-4), 7.27 (2H, d, *J* = 8.4 Hz, H-3″,5″), 7.32 (1H, d, *J* = 7.6 Hz, H-4'), 7.40-7.45 (2H, m, H-5',6'), 7.42 (1H, t, *J* = 7.6 Hz, H-5), 7.55 (1H, d, *J* = 8.4 Hz, H-3), 7.63 (2H, d, *J* = 8.4 Hz, H-2″,6″), 7.83 (1H, d, *J* = 7.6 Hz, H-6), 7.90 (1H, s, H-2'), 8.50 (3H, br s, aminium chloride), 10.52 (1H, s, NHSO$_2$), 10.69 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 20.9 (CH$_3$), 42.3 (CH$_2$), 121.0 (C-2'), 121.2 (C-6'), 121.5 (C-3), 123.6 (C-1), 124.8 (C-5), 126.8 (C-2″,6″), 128.9 (C-6), 129.2 (C-4'), 129.8 (C-3″,5″), 132.5 (C-4,1'), 134.5 (C-3'), 137.4 (C-1″), 138.5 (C-4″), 143.7 (C-2), 166.8 (C=O); DIP-MS (EI): m/z 395 [M-HCl]$^+$; HRMS (FAB): calcd for C$_{21}$H$_{22}$N$_3$O$_3$S [M+H]$^+$ 396.1382, found 396.1396.

N-(4′-Aminomethyl)phenyl-2-(4″-methylphenylsulfonamido)benzamide (**12**). Yield : 81%; m.p : 92-95 ℃; ^{1}H NMR (400 MHz, CD$_3$OD) δ: 2.29 (3H, s, CH$_3$), 4.03 (2H, s, CH$_2$), 7.18-7.24 (3H, m, H-4,3″,5″), 7.42 (1H, t, *J* = 8.0 Hz, H-5), 7.45-7.53 (3H, m, H-3,3',5'), 7.61 (2H, d, *J* = 8.4 Hz, H-2″,6″), 7.69 (2H, d, *J* = 8.4 Hz, H-2',6'), 7.80 (1H, d, *J* = 8.0 Hz, H-6), 8.36 (3H, br s, aminium chloride), 10.44 (1H, s, NHSO$_2$), 10.61 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 20.9 (CH$_3$), 41.8 (CH$_2$), 120.9 (C-2',6'), 121.4 (C-3), 124.0 (C-1), 124.1 (C-5), 126.8 (C-2″,6″), 129.1 (C-6), 129.3 (C-3',5'), 129.8 (C-3″,5″), 132.4 (C-4,1'), 135.9 (C-4'), 137.2 (C-1″), 138.4 (C-4″), 143.7 (C-2), 166.7 (C=O); DIP-MS (EI): m/z 395 [M-HCl]$^+$; HRMS (FAB): calcd for C$_{21}$H$_{22}$N$_3$O$_3$S [M]$^+$ 396.1382, found 396.1399.

N-(4′-Aminoethyl)phenyl-2-(4″-methylphenylsulfonamido)benzamide (**13**). Yield : 77 %; m.p : 253-255 ℃; ^{1}H NMR (400 MHz, CD$_3$OD) δ: 2.27 (3H, s, CH$_3$), 2.98 (2H, t, *J* = 7.8 Hz, CH$_2$), 3.21 (2H, t, *J* = 7.8 Hz, CH$_2$), 7.14 (2H, m, H-3',5'), 7.23 (1H, t, *J* = 7.6 Hz, H-4), 7.30 (2H, d, *J* = 8.0 Hz, H-3″,5″), 7.46-7.61 (6H, m, H-3,5,2',6',2″,6″), 7.67 (1H, d, *J* = 7.6 Hz, H-6), 8.33 (3H, br s, aminium chloride), 10.41 (1H, s, NHSO$_2$), 10.63 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 20.2 (CH$_3$), 32.9 (CH$_2$), 40.7 (CH$_2$), 121.8 (C-2',6'), 123.5 (C-3), 124.8 (C-1), 125.3 (C-5), 127.1 (C-2″,6″), 128.2 (C-6), 129.0 (C-3',5'), 129.5 (C-3″,5″), 132.2 (C-4), 133.2 (C-4'), 136.1 (C-1'), 137.3 (C-1″), 137.7 (C-4″), 144.1 (C-2), 167.7 (C=O); DIP-MS (EI): m/z 409 [M-HCl]$^+$; HRMS (FAB): calcd for C$_{22}$H$_{24}$N$_3$O$_3$S [M+H]$^+$ 410.1538, found 410.1520.

3.1.8. General synthetic procedures for **14**-**20**

To a solution of 4-(methylphenylsulfonamido)benzoyl chloride **2** (1.50 mmol) was dissolved in anhydrous benzene (30 mL) and added with 4-chloroaniline, 4-bromoaniline, 4-methoxyaniline, 4-carboxyaniline, 2-(thiophen-2-yl)ethylamine, 4-morpholinoaniline, and 4-(2-oxomorpholino)aniline (1.36 mmol) and triethylamine (0.1 mL). The following procedures were same as procedures for **1a-5a**. Compounds **14-20** were recrystallized with dichloromethane and *n*-hexane mixture to afford a white powder, respectively.

N-(4′-Chlorophenyl)-2-(4″-methylphenylsulfonamido)benzamide (**14**). Yield : 82%; m.p : 220-223 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ : 2.29 (3H, s, CH_3), 7.20-7.30 (3H, m, H-4,3″,5″), 7.58 (2H, d, *J* = 7.6 Hz, H-3′,5′), 7.69 (2H, d, *J* = 8.0 Hz, H-2″,6″), 7.71 (2H, d, *J* = 7.6 Hz, H-2′,6′), 7.98 (1H, t, *J* = 7.6 Hz, H-5), 8.03 (1H, d, *J* = 8.0 Hz, H-3), 8.18 (1H, d, *J* = 8.0 Hz, H-6) 11.95 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 21.6 (CH_3), 121.2 (C-2′,6′), 123.1 (C-3), 125.2 (C-1), 126.4 (C-5), 127.5 (C-6), 128.0 (C-2″,6″), 129.9 (C-4′), 130.4 (C-3″,5″), 133.1 (C-4,3′,5′), 136.8 (C-1″), 137.2 (C-4″), 144.3 (C-2), 167.6 (C=O); DIP-MS (EI) *m/z* : 400 [M]$^+$; HRMS (FAB): calcd for $C_{20}H_{18}ClN_2O_3S$ [M+H]$^+$ 401.0727, found 401.0741.

N-(4′-Bromophenyl)-2-(4″-methylphenylsulfonamido)benzamide (**15**). Yield : 80%; m.p : 211-213 ℃; ^{1}H NMR (400 MHz, CDCl$_3$) δ : 2.26 (3H, s, CH_3), 7.12-7.21 (3H, m, H-4,3″,5″), 7.38 (2H, d, *J* = 8.8 Hz, H-3′,5′), 7.43-7.51 (4H, m, H-3,5,2′,6′), 7.62 (2H, d, *J* = 8.4 Hz, H-2″,6″), 7.69 (1H, d, *J* = 7.8 Hz, H-6) 10.1 (1H, s, NHSO$_2$), 10.45 (1H, s, CONH); ^{13}C NMR (100 MHz, CDCl$_3$) δ : 21.7 (CH_3), 118.2 (C-4′), 122.2 (C-2′,6′), 122.7 (C-3), 122.9 (C-1), 124.3 (C-5), 126.9 (C-6), 127.5 (C-2″,6″), 129.8 (C-3″,5″), 132.3 (C-3′,5′), 133.3 (C-4), 136.4 (C-1′), 136.5 (C-1″), 138.8 (C-4″), 144.0 (C-2), 166.7 (C=O); DIP-MS (EI) *m/z* : 444 [M]$^+$; HRMS (FAB): calcd for $C_{20}H_{18}BrN_2O_3S$ [M+H]$^+$ 445.0222, found 445.0239.

N-(4′-Methoxyphenyl)-2-(4″-methylphenylsulfonamido)benzamide (**16**). Yield : 87%; m.p : 157-159 ℃; ^{1}H NMR (400 MHz, CDCl$_3$) δ : 2.28 (3H, s, CH_3), 3.76 (3H, s, OCH_3), 6.95 (2H, d, *J* = 8.8 Hz, H-3′,5′), 7.21 (1H, t, *J* = 6.8 Hz, H-4), 7.26 (2H, d, *J* = 8.0 Hz, H-3″,5″), 7.44-7.50 (2H, m, H-3,5), 7.54 (2H, d, *J* = 8.8 Hz, H-2′,6′), 7.61 (2H, d, *J* = 8.4 Hz, H-2″,6″), 7.77 (1H, d, *J* = 7.6 Hz, H-6), 10.23 (1H, s, NHSO$_2$), 10.81 (1H, s, CONH); ^{13}C NMR (100 MHz, CDCl$_3$) δ : 21.6 (CH_3), 55.9 (OCH_3), 114.4 (C-3′,5′), 121.6 (C-3), 123.4 (C-2′,6′), 124.0 (C-1), 124.6 (C-5), 127.5 (C-2″,6″), 129.6 (C-6), 130.5 (C-3″,5″), 131.8 (C-1′), 133.0 (C-4), 136.5 (C-1″), 138.2 (C-4″), 144.4 (C-2), 156.8 (C-4′), 167.0 (C=O) DIP-MS (EI) *m/z* : 396 [M]$^+$; HRMS (FAB): calcd for $C_{21}H_{21}N_2O_4S$ [M+H]$^+$ 397.1222, found 397.1241.

N-(4′-Carboxyphenyl)-2-(4″-methylphenylsulfonamido)benzamide (**17**). Yield : 85%; m.p :   ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ : 2.23 (3H, s, CH_3), 7.18-7.25 (3H, m, H-4,3″,5″), 7.36 (1H, d, *J* = 8.4 Hz, H-3), 7.47 (1H, t, *J* = 7.6 Hz, H-5), 7.57 (2H, d, *J* = 8.0 Hz, H-2″,6″), 7.61 (2H, d, *J* = 8.8 Hz, H-2′,6′), 7.86 (2H, d, *J* = 8.8 Hz, H-3′,5′), 7.71 (1H, d, *J* = 8.0 Hz, H-6), 10.31 (1H, s, NHSO$_2$), 10.52 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 21.3 (CH_3), 117.5 (C-2′,6′), 122.0 (C-3), 124.3 (C-1), 124.3 (C-5), 126.8 (C-4′), 127.1 (C-2″,6″), 129.0 (C-6), 128.9 (C-3″,5″), 130.8 (C-3′,5′), 132.8 (C-4), 136.1 (C-1″), 138.5 (C-4″), 144.1 (C-2), 167.3 (C=O) DIP-MS (EI) *m/z* : 410 [M]$^+$; HRMS (FAB): calcd for $C_{21}H_{19}N_2O_5S$ [M+H]$^+$ 411.1015, found 411.1036.

N-(2-(thiophen-2'-yl)ethyl)-2-(4″-methylphenylsulfonamido)benzamide (**18**). Yield : 60%; m.p : 119-121 ℃; ^{1}H NMR (400 MHz, CDCl$_3$) δ : 2.31 (3H, s, CH_3), 3.02 (2H, t, *J* = 6.8 Hz, CH_2), 3.45 (2H, q, *J* = 6.8 Hz,

CH$_2$), 6.91 (1H, d, J = 2.4 Hz, H-3'), 6.96 (1H, d, J = 5.2, 3.6 Hz, H-4'), 7.13 (1H, t, J = 7.2 Hz, H-4), 7.31 (2H, d, J = 8.0 Hz, H-3",5"), 7.35 (1H, dd, J = 5.2, 1,2 Hz, H-5'), 7.42-7.53 (2H, m, H-3,5), 7.61 (2H, d, J = 8.4 Hz, H-2",6"), 7.66 (1H, d, J = 8.0 Hz, H-6), 8.91 (1H, s, NHSO$_2$), 11.57 (1H, s, CONH); ^{13}C NMR (100 MHz, CDCl$_3$) δ : 21.0 (CH$_3$), 28.7 (CH$_2$), 40.9 (CH$_2$), 119.7 (C-3), 120.6 (C-1), 123.4 (C-5), 124.2 (C-5′), 125.3 (C-4′), 126.8 (C-2",6"), 127.0 (C-3′), 128.3 (C-6), 129.9 (C-3",5"), 132.4 (C-4), 135.9 (C-1"), 138.4 (C-4"), 141.2 (C-2′), 143.8 (C-2), 168.0 (C=O); DIP-MS (EI) *m/z* : 400 [M]$^+$; HRMS (FAB): calcd for C$_{20}$H$_{21}$N$_2$O$_3$S$_2$ [M+H]$^+$ 401.0994, found 401.0982.

N-(4′-morpholinophenyl)-2-(4″-methylphenylsulfonamido)benzamide (**19**). Yield : 55%; m.p : 206-209 ℃; ^{1}H NMR (400 MHz, CDCl$_3$) δ : 2.29 (3H, s, CH$_3$), 3.09 (4H, t, J = 4.8 Hz, CH$_2$), 3.75 (4H, t, J = 4.4 Hz, CH$_2$), 6.95 (2H, d, J = 8.8 Hz, H-3′,5′), 7.20 (1H, t, J = 6.0 Hz, H-4), 7.26 (2H, d, J = 8.4 Hz, H-3",5"), 7.45-7.53 (4H, m H-3,5,2′,6′), 7.61 (2H, d, J = 8.4 Hz, H-2",6"), 7.77 (1H, d, J = 7.6 Hz, H-6), 10.18 (1H, s, NHSO$_2$), 10.89 (1H, s, CONH); ^{13}C NMR (100 MHz, CDCl$_3$) δ : 21.7 (CH$_3$), 49.4 (2 x CH$_2$), 66.8 (2 x CH$_2$), 115.8 (C-3′,5′), 122.9 (C-3), 123.9 (C-1,2′,6′), 124.6 (C-5), 127.6 (C-2",6"), 129.6 (C-6), 130.5 (C-3",5"), 130.7 (C-1′), 133.0 (C-4), 136.5 (C-1"), 138.2 (C-4"), 144.4 (C-2), 148.8 (C-4′), 166.9 (C=O); DIP-MS (EI) *m/z* : 451 [M]$^+$; HRMS (FAB): calcd for C$_{24}$H$_{26}$N$_3$O$_4$S [M+H]$^+$ 452.1644, found 452.1627.

N-(4′-(2-oxomorpholino)phenyl)-2-(4″-methylphenylsulfonamido)benzamide (**20**). Yield : 44 %; m.p : 270-273 ℃; ^{1}H NMR (400 MHz, CDCl$_3$) δ : 2.28 (3H, s, CH$_3$), 3.74 (2H, t, J = 4.8 Hz, CH$_2$), 3.99 (2H, t, J = 4.8 Hz, CH$_2$), 4.21 (2H, s, CH$_2$), 7.20-7.30 (3H, m, H-4,3′,5′), 7.39 (2H, d, J = 8.8 Hz, H-3",5"), 7.40-7.52 (2H, m H-3,5), 7.61 (2H, d, J = 8.4 Hz, H-2',6'), 7.67 (2H, d, J = 8.8 Hz, H-2",6"), 7.77 (1H, d, J = 7.6 Hz, H-6), 10.40 (1H, s, NHSO$_2$), 10.61 (1H, s, CONH); ^{13}C NMR (100 MHz, CDCl$_3$) δ : 21.0 (CH$_3$), 40.1 (CH$_2$), 63.5 (CH$_2$), 67.8 (CH$_2$), 117.8 (C-3′,5′), 121.2 (C-3), 124.0 (C-1), 124.1 (C-5), 125.7 (C-2′,6′), 126.8 (C-2",6"), 129.1 (C-6), 129.8 (C-3",5"), 132.4 (C-4), 135.9 (C-1′), 136.4 (C-4′), 137.2 (C-1″), 137.7 (C-4"), 143.8 (C-2), 166.0 (C=O), 166.6 (C=O); DIP-MS (EI) *m/z* : 465 [M]$^+$; HRMS (FAB): calcd for C$_{24}$H$_{24}$N$_3$O$_5$S [M+H]$^+$ 466.1437, found 466.1454.

3.1.9.   General synthetic procedures for **21a-23a**

To a stirred solution of 2-nitrobenzoic acid (29.94 mmol) in anhydrous dichloromethane (50 mL) was dropwise added oxalyl chloride (38.92 mmol) and triethylamine (32.93 mmol) at room temperature. The reaction mixture was refluxed for 1 h and both solvent and unreacted oxalyl chloride were evaporated off under reduced pressure and the acid chloride was used without purification. To a solution of 3-aminobenzonitrile, 4-aminobenzonitrile, and 4-aminobenzyl cyanide (7.19 mmol) in anhydrous dichloromethane (30 mL) was added acyl chloride (8.99 mmol) and triethylamine (7.19 mmol) at room temperature and stirred for 3 h. The reaction mixture was added with water and extracted with dichloromethane (30 mL x 3), dried with anhydrous magnesium sulfate and filtrated. The filtrate was evaporated under reduced pressure to give crude compound, which was recrystallized with ethyl acetate ad *n*-hexane to give pure white or pale yellow compound, respectively.

N-(3′-Cyanophenyl)-2-nitrobenzamide (**21a**). Yield : 80%; m.p. : 170-171 ℃; ^{1}H NMR (400 MHz, DMSO-*d$_6$*) δ: 7.59-7.92 (6H, m, H-4,5,6,4',5',6'), 8.13 (1H, s, H-2'), 8.18 (1H, d, J = 8.0 Hz, H-3), 11.02 (1H, s,

CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 111.7 (C-3'), 118.6 (C≡N), 122.2 (C-6'), 124.2 (C-5'), 124.4 (C-1), 127.5 (C-2'), 129.3 (C-3), 130.4 (C-4'), 131.3 (C-6), 132.0 (C-4), 134.3 (C-5), 139.6 (C-1'), 146.3 (C-2), 164.6 (C=O); GC-MS (EI) *m/z* : 267 [M]$^+$.

N-(4′-Cyanophenyl)-2-nitrobenzamide (**22a**). Yield : 84%; m.p. : 217-218 ℃; ; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.77-7.84 (6H, m, H-4,6,2',3',5',6'), 7.90 (1H, td, *J* = 7.6 Hz, 1.2 Hz, H-5), 8.19 (1H, dd, *J* = 8.0, 4.0 Hz, H-3), 11.08 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 105.7 (C-4'), 118.9 (C≡N), 119.6 (C-2',6'), 124.8 (C-1), 129.3 (C-3), 131.3 (C-6), 132.1 (C-4), 133.4 (C-3',5'), 134.3 (C-5), 142.9 (C-1'), 146.2 (C-2), 164.7 (C=O); GC-MS (EI) *m/z* : 267 [M]$^+$.

N-(4′-Cyanomethylphenyl)-2-nitrobenzamide (**23a**). Yield : 76%; m.p. : 174-175 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.01 (2H, s, CH$_2$), 7.35 (2H, d, *J* = 8.0 Hz, H-3',5'), 7.68 (2H, d, *J* = 8.0 Hz, H-2',6'), 7.75-7.79 (2H, m, H-4,6), 7.88 (1H, t, *J* = 8.0 Hz, H-5), 8.16 (1H, d, *J* = 8.0 Hz, H-3), 10.71 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 21.9 (CH$_2$), 119.3 (C≡N), 120.0 (C-2',6'), 124.3 (C-1), 126.6 (C-4'), 128.6 (C-3',5'), 129.3 (C-3), 131.0 (C-6), 132.5 (C-4), 134.1 (C-5), 138.2 (C-1'), 146.4 (C-2), 164.1 (C=O); GC-MS (EI) *m/z* : 281 [M]$^+$.

3.1.10. General synthetic procedures for **21b**-**23b**

To a solution of **21a–23a** (1.79 mmol) in methanol (20 mL) was added ammonium chloride (17.9 mmol) and iron powder (3.58 mmol), the reaction mixture was refluxed for 7 h. The following procedures were same as synthetic procedures for **1b**-**5b**. Compounds **21b**-**23b** were recrystallized with ethyl acetate and *n*-hexane to give a pure white or pale-yellow solid.

2-Amino-N-(3′-cyanophenyl)benzamide (**21b**). Yield : 82%; m.p. : 158-160 ℃, (161-163 ℃ [39]).

2-Amino-N-(4′-cyanophenyl)benzamide (**22b**). Yield : 64%; m.p. : 233-234 ℃, (231 ℃ [39]).

2-Amino-N-(4′-cyanomethylphenyl)benzamide (**23b**). Yield : 61%; m.p. : 144-145 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.98 (2H, s, CH$_2$), 6.32 (2H, s, NH$_2$), 6.59 (1H, t, *J* = 6.8 Hz, H-5), 6.75 (1H, d, *J* = 7.6 Hz, H-3), 7.18-7.22 (2H, m, H-4,6), 7.30 (2H, d, *J* = 8.0 Hz, H-3',5'), 7.73 (2H, d, *J* = 8.0 Hz, H-2',6'), 10.03 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 21.8 (CH$_2$), 114.6 (C-1), 115.0 (C-3), 116.3 (C-5), 119.3 (C≡N), 120.8 (C-2',6'), 128.2 (C-3',5'), 128.6 (C-4'), 129.3 (C-6), 132.1 (C-4), 138.6 (C-1'), 149.7 (C-2), 167.8 (C=O); DIP-MS (EI) *m/z* : 251 [M]$^+$.

3.1.11. General synthetic procedures of **21c – 23c**

To a solution of **21b–23b** (1.48 mmol) in anhydrous benzene (20 mL) was added triethylamine (1.48 mmol) and thiophene-2-carbonyl chloride (1.85 mmol) at room temperature and the reaction mixture was refluxed for 1 h. The following procedures were same as synthetic procedures for **1-10**. Compounds **21c-23c** were recrystallized with ethyl acetate and *n*-hexane to give a pure white or pale yellow compound, respectively.

N-(3′-Cyanophenyl)-2-((thiophen-2′′-yl)carbonylamino)benzamide (**21c**). Yield : 98%; m.p. : 208-209 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.25 (1H, dd, *J* = 5.0, 4.0 Hz, H-4''), 7.32 (1H, td, *J* = 7.6, 1.2 Hz, H-5), 7.56-7.65 (3H, m, H-4,4',6'), 7.80 (1H, dd, *J* = 3.8, 0.8 Hz, H-5''), 7.88-7.91 (2H, m, H-3,6), 7.99-8.02 (1H,

m, H-5'), 8.19 (1H, s, H-2'), 8.24 (1H, dd, J = 8.2, 0.8 Hz, H-3''), 10.80 (1H, s, NHCO), 11.36 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 111.2 (C-3'), 118.5 (C≡N), 121.8 (C-3), 123.3 (C-1), 124.5 (C-5), 125.2 (C-6'), 127.3 (C-2'), 128.2 (C-6), 128.7 (C-4''), 128.8 (C-5''), 129.9 (C-4'), 132.0 (C-4), 132.1 (C-3''), 137.6 (C-2''), 139.2 (C-1'), 139.3 (C-2), 159.5 (CONH), 167.5 (NHCO); DIP-MS (EI) *m/z* : 347 [M]+.

N-(4′-Cyanophenyl)-2-((thiophen-2′′-yl)carbonylamino)benzamide (**22c**). Yield : 69%; m.p. : 266-267 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.24 (1H, t, J = 4.0 Hz, H-4''), 7.32 (1H, t, J = 7.6 Hz, H-5), 7.62 (1H, t, J = 8.0 Hz, H-4), 7.79 (1H, d, J = 3.2 Hz, H-5''), 7.83 (2H, d, J = 8.0 Hz, H-3',5'), 7.87-7.89 (2H, m, H-3,6), 7.94 (2H, d, J = 8.0 Hz, H-2',6'), 8.20 (1H, d, J = 8.0 Hz, H-3''), 10.86 (1H, s, NHCO), 11.23 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 105.6 (C-4'), 119.0 (C≡N), 120.6 (C-3',5'), 122.2 (C-3), 123.7 (C-1), 124.2 (C-5), 128.3 (C-6), 128.9 (C-4''), 129.1 (C-5''), 132.2 (C-4), 132.3 (C-3''), 133.1 (C-2',6'), 137.7 (C-2''), 139.4 (C-2), 143.1 (C-1'), 159.5 (CONH), 167.6 (NHCO); DIP-MS (EI) *m/z* : 347 [M]+.

N-(4′-Cyanomethylphenyl)-2-((thiophen-2′′-yl)carbonylamino)benzamide (**23c**). Yield : 88%; m.p. : 221-222 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.02 (2H, s, CH$_2$), 7.25 (1H, dd, J = 5.0, 4.0 Hz, H-4''), 7.29 (1H, dt, J = 7.7, 1.1 Hz, H-5), 7.36 (2H, d, J = 8.0 Hz, H-3',5'), 7.61 (1H, dt, J = 8.0, 1.2 Hz, H-4), 7.74 (2H, d, J = 8.0 Hz, H-2',6'), 7.75 (1H, d, J = 7.6 Hz, H-6), 7.89 (1H, d, J = 5.0 Hz, H-5''), 7.93 (1H, dd, J = 7.8, 1.2 Hz, H-3), 8.35 (1H, dd, J = 8.2, 1.2 Hz, H-3''), 10.60 (1H, s, NHCO), 11.66 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 21.9 (CH$_2$), 119.3 (C≡N), 121.4 (C-3), 121.5 (C-2',6'), 122.8 (C-1), 123.4 (C-5), 126.9 (C-4'), 128.4 (C-3',5'), 128.8 (C-6), 129.0 (C-4''), 129.0 (C-5''), 132.2 (C-4), 132.3 (C-3''), 137.9 (C-2''), 138.3 (C-1'), 139.5 (C-2), 159.4 (CONH), 167.3 (NHCO); DIP-MS (EI) *m/z* : 361 [M]+.

3.1.12.   General synthetic procedures for **21d – 23d**

To a suspension of intermediate **21c–23c** (0.86 mmol) in absolute ethanol (25 mL) and 1,4-dioxane (5 mL) was added triethylamine (2.59 mmol) and refluxed to dissolve. To the reaction mixture hydroxylamine·HCl (3.45 mmol) was added and refluxed for 4–6 h. The mixture was evaporated to remove the ethanol and ice water was poured into the residue. The resulting precipitate was filtrated, washed with water, and dried under reduced pressure to give a pure white or pale yellow solid.

N-(3′-Amidoximephenyl)-2-((thiophen-2′′-yl)carbonylamino)benzamide (**21d**). Yield : 94%; m.p. : 230-232 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 5.78 (2H, s, NH$_2$), 7.25 (1H, dd, J = 5.0, 4.0 Hz, H-4''), 7.29 (1H, dt, J = 7.7, 1.1 Hz, H-5), 7.38 (1H, t, J = 8.0 Hz, H-5'), 7.43 (1H, dt, J = 7.7, 1.4 Hz, H-4'), 7.61 (1H, dt, J = 7.6, 2.4 Hz, H-4), 7.68-7.71 (2H, m, H-6,6'), 7.89 (1H, dd, J = 5.0, 1.1 Hz, H-5''), 7.95 (1H, dd, J = 7.6, 1.2 Hz, H-3), 8.04 (1H, t, J = 1.7 Hz, H-2'), 8.38 (1H, dd, J = 4.0, 0.8 Hz, H-3''), 9.65 (1H, s, NOH), 10.59 (1H, s, NHCO), 11.71 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 116.6 (C-2'), 122.8 (C-3), 123.4 (C-1), 124.6 (C-5), 124.9 (C-3'), 129.2 (C-4''), 129.3 (C-5''), 129.8 (C-6), 132.1 (C-4), 132.8 (C-3''), 134.8 (C-5'), 138.1 (C-2''), 138.7 (C-1'), 139.3 (C-2), 159.1 (CONH), 167.7 (NHCO); FAB-MS *m/z* : 363 [M+1-H$_2$O]+, 380 [M]+, 381 [M+1]+, 403 [M+Na]+.

N-(4′-Amidoximephenyl)-2-(thiophen-2′′-ylcarbonylamino)benzamide (**22d**). Yield : 52%; m.p. : 241-243 ℃; ^{1}H NMR (400MHz, DMSO-d_6) δ: 5.40 (2H, s, NH$_2$), 7.20-7.29 (4H, m, H-5,2',6',4''), 7.56-7.64 (3H, m, H-4,3',5'), 7.80 (1H, d, J = 7.6 Hz, H-6), 7.88 (1H, d, J = 4.0 Hz, H-5''), 7.96 (1H, d, J = 7.6 Hz, H-3), 8.30 (1H, d, J = 8.4 Hz, H-3''), 8.80 (1H, s, NOH), 10.20 (1H, s, NHCO), 11.12 (1H, s, CONH); ^{13}C NMR (100 MHz,

DMSO-d_6) δ: 120.6 (C-2',6'), 122.5 (C-3), 123.2 (C-1), 124.3 (C-5), 128.8 (C-3',5'), 128.7 (C-6), 129.0 (C-4'',5''), 133.1 (C-4), 134.2 (C-3''), 136.6 (C-1'), 138.0 (C-2''), 138.7 (C-2), 150.2 (C=NOH), 154.3 (CONH), 165.3 (NHCO); FAB-MS m/z : 363 [M+1-H$_2$O]$^+$, 380 [M]$^+$, 381 [M+1]$^+$.

N-(4′-Amidoximemethylphenyl)-2-(thiophen-2′′-ylcarbonylamino)benzamide **(23d)**
Yield : 89%; m.p. : 209-211 ℃; ^{1}H NMR (400MHz, DMSO-d_6) δ: 3.26 (2H, s, CH$_2$), 5.43 (2H, s, NH$_2$), 7.19-7.27 (4H, m, H-5,3',5',4''), 7.56-7.62 (3H, m, H-4,2',6'), 7.73 (1H, d, *J* = 2.8 Hz, H-6), 7.89 (1H, d, *J* = 4.0 Hz, H-5''), 7.93 (1H, d, *J* = 7.6 Hz, H-3), 8.39 (1H, d, *J* = 8.4 Hz, H-3''), 8.92 (1H, s, NOH), 10.50 (1H, s, NHCO), 11.79 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 36.7 (CH$_2$), 121.1 (C-2',6'), 122.5 (C-3), 123.2 (C-1), 128.4 (C-4'), 128.8 (C-3',5'), 128.9 (C-6), 128.9 (C-4'',5'') 132.3 (C-4), 133.9 (C-3''), 136.6 (C-1'), 138.4 (C-2''), 139.7 (C-2), 152.2 (C=NOH), 159.3 (CONH), 167.3 (NHCO); FAB-MS m/z : 377 [M+1-H$_2$O]$^+$, 394 [M]$^+$, 395 [M+1]$^+$, 417 [M+Na]$^+$.

3.1.13.   General synthetic procedures for **21 - 23**

To a solution of amidoximes **21d–23d** (0.53 mmol) in anhydrous dichloromethane (10 mL) was added triethylamine (1.58 mmol) and acetyl chloride (0.6 mmol) at 0 °C. The reaction mixture was stirred for 1–2 h at room temperature and ice water was added. The aqueous layer was extracted with dichloromethane (30 mL × 3). The organic phase was washed with water, saturated NaHCO$_3$, and water. The organic layer was dried with MgSO$_4$, filtrated, and evaporated in reduced pressure to give the acetylated amidoximes (**21e–23e**), which were used next reaction without purification. To a solution of 10% Pd-C in absolute ethanol (10 mL) was added acetylated amidoximes (1.0 eq.) and c-HCl (1.0 eq.) and hydrogenated for 2 h at 60 psi, 45 °C. The reaction mixture was filtrated and concentrated under reduced pressure to give a crude oily compound, which was purified to yield a white or pale yellow solid by column chromatography.

N-(3′-Amidinophenyl)-2-((thiophen-2′′-yl)carbonylamino)benzamide (**21**). Yield : 32%; m.p. : 262-264 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.24 (1H, dd, *J* = 5.0, 4.0 Hz, H-4''), 7.33 (1H, t, *J* = 8.0 Hz, H-5), 7.53 (1H, d, *J* = 8.0 Hz, H-4'), 7.60-7.65 (2H, m, H-4,5'), 7.79 (1H, dd, *J* = 3.7, 0.8 Hz, H-6), 7.89 (1H, dd, *J* = 5.0, 0.8 Hz, H-5''), 7.92 (1H, d, *J* = 8.0 Hz, H-6'), 8.00 (1H, d, *J* = 8.3 Hz, H-3), 8.21 (1H, s, H-2'), 8.26 (1H, dd, *J* = 8.0, 0.8 Hz, H-3''), 9.08 (2H, br s, hydrogens of amidine·HCl), 9.36 (2H, br s, hydrogens of amidine·HCl), 10.86 (1H, s, NHCO), 11.45 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 120.0 (C-2'), 122.0 (C-3), 123.5 (C-1), 123.7 (C-5), 125.7 (C-6'), 128.3 (C-6), 128.9 (C-4''), 129.0 (C-5''), 129.5 (C-4'), 132.3 (C-4), 132.4 (C-3''), 137.9 (C-2''), 139.2 (C-1'), 139.5 (C-2), 159.4 (carbon of amidine), 166.0 (CONH), 167.6 (NHCO); FAB-MS m/z : 388 [M-Cl+Na]$^+$, 365 [M-Cl]$^+$; HRMS (FAB): calcd for C$_{19}$H$_{17}$N$_4$O$_2$S [M+H]$^+$ 365.1072, found 365.1084.

N-(4′-Amidinophenyl)-2-((thiophen-2′′-yl)carbonylamino)benzamide (**22**). Yield : 51%; m.p. : 211-213 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.24 (1H, dd, *J* = 5.0, 4.0 Hz, H-4''), 7.30 (1H, t, *J* = 8.0 Hz, H-5), 7.61 (1H, dt, *J* = 8.0, 1.2 Hz, H-4), 7.82 (1H, dd, *J* = 4.0, 1.2 Hz, H-6), 7.88 (2H, d, *J* = 8.0 Hz, H-3',5'), 7.88 (1H, d, *J* = 4.0, 1.2 Hz, H-5''), 7.94 (1H, dd, *J* = 8.0, 1.2 Hz, H-3), 7.97 (2H, d, *J* = 8.0 Hz, H-2', 6'), 8.18 (1H, dd, *J* = 8.0, 0.8 Hz, H-3''), 9.36 (4H, br s, hydrogens of amidine·HCl), 10.77 (1H, s, NHCO), 11.53 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 120.2 (C-2',6'), 122.4 (C-3), 122.5 (C-4'), 123.6 (C-1), 124.4 (C-5), 128.3 (C-6), 128.9 (C-3',5',4''), 129.2 (C-5''), 132.1 (C-4), 132.2 (C-3''), 137.9 (C-2''), 139.7 (C-2), 143.9

(C-1'), 159.6 (carbon of amidine), 164.9 (CONH), 167.5 (NHCO); FAB-MS *m/z* : 365 [M-Cl]⁺; HRMS (FAB): calcd for $C_{19}H_{17}N_4O_2S$ [M+H]⁺ 365.1072, found 365.1089.

N-(4′-Amidinomethylphenyl)-2-((thiophen-2′′-yl)carbonylamino)benzamide (**23**). Yield : 42%; m.p. : 172-174 ℃; ¹H NMR (400 MHz, DMSO-d_6) δ: 3.69 (2H, s, CH₂), 7.24 (1H, dd, *J* = 5.0, 4.0 Hz, H-4''), 7.30 (1H, dt, *J* = 7.7, 1.1 Hz, H-5), 7.42 (2H, d, *J* = 8.0 Hz, H-3',5'), 7.61 (1H, dt, *J* = 8.0, 1.2 Hz, H-4), 7.72 (2H, d, *J* = 8.0 Hz, H-2',6'), 7.75 (1H, dd, *J* = 4.0, 1.2 Hz, H-6), 7.89 (1H, dd, *J* = 4.0, 1.2 Hz, H-5''), 7.92 (1H, dd, *J* = 8.0, 1.2 Hz, H-6), 8.33 (1H, d, *J* = 8.0 Hz, H-3''), 8.92 (4H, br s, hydrogens of amidine·HCl), 10.61 (1H, s, NHCO), 11.64 (1H, s, CONH); ¹³C NMR (100 MHz, DMSO-d_6) δ: 121.4 (C-2', 6'), 121.5 (C-3), 123.0 (C-1), 123.4 (C-5), 128.4 (C-6), 128.8 (C-4''), 129.0 (C-5''), 129.1 (C-3',5'), 130.7 (C-4'), 132.2 (C-4), 132.3 (C-3''), 138.0 (C-2''), 138.2 (C-1'), 139.6 (C-2), 159.4 (carbon of amidine), 167.3 (CONH), 169.1 (NHCO); FAB-MS *m/z* : 379 [M-Cl]⁺; HRMS (FAB): calcd for $C_{20}H_{19}N_4O_2S$ [M+H]⁺ 379.1229, found 379.1243.

3.1.14. General synthetic procedures for **24b-26b**

To a solution of **11a-13a** (1.54 mmol) in ethanol (40 mL) was added with hydroxylamine hydrochloride (300 mg, 4.60 mmol) and triethylamine (0.5 mL) at room temperature. The reaction mixture was refluxed for 12 h and the following procedures were same as synthetic procedure for **21b-23b**. Compounds **24b-26b** were recrystallized with dichloromethane and *n*-hexane mixture to afford as a white powder, respectively.

N-(3′-Amidoximino)-2-(4″-methylphenylsulfonamido)benzamide (**24b**). Yield : 85%; m.p : 182-184 ℃; ¹H NMR (400 MHz, CDCl₃) δ : 2.23 (3H, s, CH₃), 5.05 (2H, s, NH₂), 6.61 (1H, s, =NOH), 7.02-7.12 (4H, m, H-3,4,5,5′), 7.37 (2H, d, *J* = 8.0 Hz, H-3'',5''), 7.50-7.60 (3H, m, H-6,4',6'), 7.61 (2H, d, *J* = 8.0 Hz, H-2'',6''), 7.85 (1H, s, H-2'), 7.91 (1H, s, NHSO₂), 8.26 (1H, s, CONH); ¹³C NMR (100 MHz, CDCl₃) δ : 21.4 (CH₃), 118.3 (C-2'), 121.9 (C-6'), 122.2 (C-3), 122.5 (C-1), 124.0 (C-5), 124.1 (C-3'), 127.2 (C-2'',6''), 129.4 (C-3'',5''), 129.7 (C-6), 132.9 (C-4), 133.1 (C-5'), 136.2 (C-1''), 137.5 (C-4''), 138.6 (C-1'), 143.9 (C-2), 158.8 (C=NOH), 166.8 (C=O); GC-MS (EI) *m/z* : 409 [M+1-NH₂]⁺, 392 [M+1-NH₂OH]⁺.

N-(4′-Amidoximino)-2-(4″-methylphenylsulfonamido)benzamide (**25b**). Yield : 74%; m.p : 213-215 ℃; ¹H NMR (400 MHz, (CD₃)₂CO) δ : 2.26 (3H, s, CH₃), 6.60 (1H, s, =NOH), 7.19 (3H, m, H-4,3'',5''), 7.54 (1H, t, *J* = 7.2 Hz, H-5), 7.62 (2H, d, *J* = 7.6 Hz, H-2'',6''), 7.69 (1H, d, *J* = 8.4 Hz, H-3), 7.75-7.88 (3H, m, H-6,2',6'), 7.98 (2H, d, *J* = 8.4 Hz, H-3',5'), 9.72 (1H, s, NHSO₂), 10.50 (1H, s, CONH); ¹³C NMR (100 MHz, (CD₃)₂CO) δ :21.4 (CH₃), 120.9 (C-3), 122.9 (C-1), 124.2 (C-5), 125.0 (C-4'), 128.0 (C-2',6'), 129.2 (C-3',5',2'',6''), 130.6 (C-3'',5''), 131.2 (C-6), 133.6 (C-4), 137.3 (C-1''), 139.5 (C-4''), 142.0 (C-1'), 144.8 (C-2), 168.2 (C=NOH), 168.3 (C=O); GC-MS (EI) *m/z* : 409 [M+1-NH₂]⁺, 392 [M+1-NH₂OH]⁺.

N-(4′-Amidoximinobenzyl)-2-(4″-methylphenylsulfonamido)benzamide (**26b**). Yield : 63%; m.p : 70-74 ℃; ¹H NMR (400 MHz, DMSO-d_6) δ : 2.29 (3H, s, CH₃), 3.52 (2H, s, CH₂), 7.22 (1H, t, *J* = 7.2 Hz, H-4), 7.25 (2H, d, *J* = 7.6 Hz, H-3',5'), 7.26 (2H, d, *J* = 8.4 Hz, H-3'',5''), 7.42-7.50 (2H, m, H-3,5), 7.52 (2H, d, *J* = 7.6 Hz, H-2',6'), 7.61 (2H, d, *J* = 8.4 Hz, H-2'',6''), 7.78 (1H, d, *J* = 8.0 Hz, H-6), 8.49 (2H, br s, NH₂), 9.50 (1H, br s, =NOH), 10.35 (1H, s, NHSO₂), 10.75 (1H, s, CONH); ¹³C NMR (100 MHz, DMSO-d_6) δ :21.4 (CH₃), 120.9 (C-3), 122.9 (C-1), 124.2 (C-5), 125.0 (C-4'), 128.0 (C-2',6'), 129.2 (C-3',5',2'',6''), 130.6 (C-3'',5''), 131.2

(C-6), 133.6 (C-4), 137.3 (C-1"), 139.5 (C-4"), 142.0 (C-1'), 144.8 (C-2), 168.2 (C=NOH), 168.3 (C=O); GC-MS (EI) m/z : 423 [M+1-NH2]+, 406 [M+1-NH2OH]+.

3.1.15. General experimental procedures for **24 – 26**

To a solution of amidoximes **24b–26b** (1.18 mmol) in anhydrous dichloromethane (10 mL) was added triethylamine (1.18 mmol) and acetyl chloride (1.20 mmol) at 0 °C. The following procedures were same as synthetic procedures for acetylated amidoximes **21c-23c**. To a solution of 10% Pd-C in absolute ethanol (50 mL) was added acetylated amidoximes (1.12 mmol.) and c-HCl (1.12 mmol.) and hydrogenated for 12 h at 50 psi, 45 °C. The reaction mixture was filtrated on celite pad and the filtrate was concentrated under reduced pressure to give a crude oily compound, which was purified to yield a white or pale yellow solid by column chromatography.

N-(3′-Amidinophenyl)-2-(4″-methylphenylsulfonamido)benzamide·HCl (**24**). Yield : 37% m.p : 216-218 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ : 2.29 (3H, s, CH3), 7.20-7.29 (3H, m, H-4,3",5"), 7.39 (1H, d, J = 6.8 Hz, H-3), 7.55 (1H, t, J = 8.0Hz, H-5), 7.58-7.68 (4H, m, H-,2",6"), 7.86 (1H, d, J = 7.6 Hz, H-6), 7.92 (1H, d, J = 6.8 Hz, H-6'), 8.24 (1H, s, H-2'), 9.28 (2H, br s, hydrogens of amidine·HCl), 9.46 (2H, s, hydrogens of amidine·HCl), 10.57 (1H, s, NHSO2), 10.80 (1H, s, CONH); ^{13}C NMR (100 MHz, CD3OD) δ : 20.2 (CH3), 119.4 (C-2'), 121.3 (C-3), 121.8 (C-1), 122.4 (C-5), 123.0 (C-6'), 124.2 (C-4'), 125.8 (C-3'), 126.8 (C-2",6"), 126.9 (C-3",5"), 129.1 (C-4), 129.2 (C-6), 129.8 (C-5'), 130.6 (C-1'), 132.3 (C-1"), 139.9 (C-4"), 142.9 (C-2), 167.3 (C=NH), 167.6 (C=O); DIP-MS (EI) m/z : 392 [M-HCl-NH2]+; HRMS (FAB): calcd for C21H21N4O3S [M+H]+ 409.1334, found 409.1347.

N-(4'-Amidinophenyl)-2-(4"-methylphenylsulfonamido)benzamide·HCl (**25**). Yield : 43%; m.p : 250-252 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ : 2.27 (3H, s, CH3), 7.23-7.32 (3H, m, H-4,3",5"), 7.35 (1H, d, J = 8.0 Hz, H-3), 7.50 (1H, t, J = 8.4 Hz, H-5), 7.60 (2H, d, J = 8.0 Hz, H-2",6"), 7.78 (1H, dd, J = 8.0, 1.2 Hz, H-6), 9.10 (2H, br s, hydrogens of amidine·HCl), 9.35 (2H, br s, hydrogens of amidine·HCl), 10.34 (1H, s, NHSO2), 10.73 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 20.9 (CH3), 120.1 (C-2',6'), 122.2 (C-3), 122.6 (C-1), 124.5 (C-5), 125.4 (C-4'), 126.8 (C-3',5'), 128.3 (C-2",6"), 129.3 (C-6), 129.5 (C-3",5"), 132.4 (C-4), 136.0 (C-1"), 136.7 (C-4"), 143.5 (C-1'), 143.6 (C-2), 164.8 (C=NH), 166.9 (C=O); DIP-MS (EI) m/z : 392 [M-HCl-NH2]+; HRMS (FAB): calcd for C21H21N4O3S [M+H]+ 409.1334, found 409.1348.

N-(4'-Amidinomethylphenyl)-2-(4"-methylphenylsulfonamido)benzamide·HCl (**26**). Yield : 33%; m.p. : 172-173 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.28 (3H, s, CH3), 3.67 (2H, s, CH2), 7.23 (1H, t, J = 8.0 Hz, H-4), 7.26 (2H, d, J = 8.0 Hz, H-3",5"), 7.41-7.53 (4H, m, H-3,5, 3',5'), 7.60 (2H, d, J = 8.4 Hz, H-2',6'), 7.66 (2H, d, J = 8.8 Hz, H-2",6"), 7.81 (1H, dd, J = 8.0, 1,2 Hz, H-6), 8.79 (2H, br s, hydrogens of amidine·HCl), 9.28 (2H, br s, hydrogens of amidine·HCl), 10.44 (1H, s, NHCO), 10.65 (1H, s, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 20.9 (CH3), 37.1 (CH2), 121.2 (C-2',6'), 123.8 (C-3), 124.1 (C-1), 126.8 (C-3',5'), 128.3 (C-6), 129.2 (C-2",6"), 129.8 (C-3",5"), 129.9 (C-4), 132.4 (C-4'), 135.9 (C-1'), 137.3 (C-1"), 137.7 (C-4"), 143.7 (C-2), 166.7 (C=NH), 169.2 (NHCO); DIP-MS (EI) m/z : 406 [M-HCl-NH2]+; HRMS (FAB): calcd for C22H23N4O3S [M+H]+ 423.1491, found 423.1478.

3.2. Biology

3.2.1. Reagents and Instruments

Heparin was purchased from Sigma (St. Louis, MO, USA) and TNF-α was purchased from Abnova (Taipei, Taiwan). Anti-tissue factor (TF) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, GA, USA) and U46619, thromboxane A_2 (TXA_2) analog, was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). Factor VIIa, X, Xa, prothrombin, thrombin were purchased from Haematologic Technologies (Essex Junction, VT, USA) and aPTT (APTT-XL) and PT (Thromboplastin-D) assay reagent was purchased from Fisher Diagnostics (Middletown, VA, USA). S-2222 (for Factor Xa) and S-2238 (for thrombin) were purchased from ChromogenixAB (Mölndal, Sweden). Rivaroxaban (direct factor Xa inhibitor) and argatroban (direct Factor IIa inhibitor) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Microplate reader (Tecan Austria GmbH, Grödig, Austria), thrombotimer (Behnk Elektronik, Norderstedt, Germany), aggregometer (Chronlog, Havertown, PA, USA), spectrophotometer (TECAN, Männedorf, Switzerland) were used.

3.2.2. Preparation of plasma

Blood samples were taken in the morning from 10 healthy volunteers in fasting status (aged between 24 and 28 years, four males and six females) without cardiovascular disorders, allergy and lipid or carbohydrate metabolism disorders, untreated with drugs. All subjects gave written informed consent before participation. Healthy subjects did not use additive substances or antioxidant supplementation, and their diet was balanced (meat and vegetables). Human blood was collected into sodium citrate (0.32% final concentration) and immediately centrifuged ($2,000 \times 15$ min) in order to obtain plasma. The study protocol (KNUH 2012-01-010) was approved by the Institutional Review Board of Kyungpook National University Hospitals (Daegu, Republic of Korea).

3.2.3. *In vitro* anticoagulant assay

aPTT and PT were determined using a Thrombotimer (Behnk Elektronik, Norderstedt, Germany), according to the manufacturer's instructions, as described previously [40]. In brief, citrated normal human plasma (90 μL) was mixed with 10 μL of target compounds incubated for 1 min at 37 ℃, aPTT assay reagent (100 μL) was added and incubated for 1 min at 37 ℃, followed by addition of 20 mM $CaCl_2$ (100 μL). Clotting times were recorded. For PT assays, citrated normal human plasma (90 μL) was mixed with 10 μL of synthesized compounds stock and incubated for 1 min at 37 ℃. PT assay reagent (200 μL), which had been pre-incubated for 10 min at 37 ℃, was then added and clotting time was recorded. PT results are expressed in seconds and as International Normalized Ratios (INR), and aPTT results are expressed in seconds. INR = (PT sample/PT control)[ISI], ISI = international sensitivity index.

3.2.4. *In vivo* bleeding time

Tail bleeding times were measured using the method described by Dejana et al [41]. Briefly, ICR mice were fasted overnight before experiments. One hour after intravenous administration of synthesized compounds (**5**, **9**, and **21**-**23**), tails of mice were transected at 2 mm from their tips.

Bleeding time was defined as the time elapsed until bleeding stopped. When the bleeding time exceeded 15 min, bleeding time was recorded as 15 min for the analysis. All animals were treated in accordance with the guidelines for the Care and Use of Laboratory Animals issued by Kyungpook National University.

3.2.5. Thrombin activity assay

Compounds **5**, **9**, and **21-23** in 50 nM Tris-HCl buffer (pH 7.4) containing 7.5 mM EDTA were mixed with 150 mM NaCl. Following incubation at 37 ℃ for 2 min, throminb solution (150 μL; 10 U/ mL) was added, followed by incubation at 37 ℃ for 1 min. S-2238 as substrate (150 μL; 1.5 mM) solution was then added and absorbance at 405 nm was monitored for 120 s using a spectrophotometer (TECAN, Männedorf, Switzerland).

3.2.6. Factor Xa activity assay

These assays were performed in the saline manner as the thrombin activity assay, but using FXa (150 μL; 1 U/mL) and S-2222 (a factor Xa substrate) as substrate instead of thrombin and S-2238.

3.2.7. Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and were maintained using a previously described method [42,43]. Briefly, cells were cultured until confluent at 37 ℃ at 5% CO_2 in EBM-2 basal media supplemented with growth supplements from Cambrex Bio Science (Charles City, IA).

3.2.8. Thrombin generation on the surfaces of HUVECs

Measurement of thrombin generation of HUVECs was quantitated as previously described [40]. Briefly, HUVECs were pre-incubated in 300 μL containing synthesized compounds in 50 mM Tris-HCl buffer, 100 pM FVa, and 1 nM FXa for 10 min, followed by addition of prothrombin to a final concentration of 1 μM. After 10 min, duplicate samples (10 μL each) were transferred to a 96-well plate containing 40 μL of 0.5 M EDTA in Tris-buffered saline per well in order to determinate prothrombin activation. Activated prothrombin was determined by measuring the rate of hydrolysis of S2238 at 405 nm. Standard curves were prepared using amounts of purified thrombin.

3.2.9. Factor Xa generation on the surfaces of HUVECs

TNF-α (10 ng/mL for 6 h in serum-free medium) stimulated confluent monolayers of HUVECs (preincubated with the indicated concentrations of **5**, **9**, and **21-23** for 10 min) in a 96-well culture plates were incubated with FVIIa (10 nM) in buffer B (buffer A supplemented with 5 mg/mL bovine serum albumin (BSA) and 5 mM $CaCl_2$) for 5 min at 37 ℃. FXa (175 nM) was added to the cells (final reaction mixture volume, 100 μL) and incubated for 15 min. The reaction was stopped by addition of buffer A (10 mM HEPES, pH 7.45, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) containing 10 mM EDTA and the amounts of FXa generated were measured at 405 nm over 2 min were monitored using a microplate reader. Initial rates of color development were converted into FXa concentrations using a standard curve prepared with known dilutions of purified human FXa.

3.2.10. Thrombin-catalyzed fibrin polymerization

Thrombin-catalyzed polymerization was determined every 6 s for 20 min by monitoring turbidity at 360 nm using a spectrophotometer (TECAN, Männedorf, Switzerland) at ambient temperature. Control plasma and plasma incubated with synthesized compounds (**5**, **9**, and **21-23**) were diluted three times in TBS (50 mM Tris-buffered physiological saline solution pH 7.4) and clotted with thrombin (final concentration-0.5 U/ml). The maximum polymerization rate (Vmax, ΔmOD/min) of each absorbance curve was recorded. All experiments were performed three times.

3.2.11. Platelet aggregation assay

Mouse platelets from platelet-rich plasma (PRP) were washed once with HEPES buffer (5 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 2 mM $MgCl_2$, 5.6 mM glucose, 1% BSA (w/v), pH to 7.45). The platelet aggregation study was performed according to a previously reported method [44]. Washed plasma was incubated with the indicated concentration of synthesized compounds for 3 min, and followed by stimulation with thrombin (0.1 U/mL, Sigma) and TxA$_2$ analog, U46619 (2 μM) in 0.9% saline at for 5 min. Platelet aggregations were recorded using a aggregometer (Chronolog, Havertown, PA, USA).

3.2.12. Cell viability assay

MTT was used as an indicator of cell viability. Cells were grown in 96-sell plates at density of 5 × 10^3/well. After 24 h, cells were washed with fresh medium, followed by treatment with compounds **5**, **9**, and **21-23**. After a 48 h incubation period, cells were washed and 100 μL of 1 mg/mL MTT was added, followed by incubation for 4 h. Finally, 150 μL DMSO was added in order to solubilize the formazan salt formed, the amount of which was determined by measuring the absorbance at 540 nm using a microplate reader (Tecan Austria GmbH, Austria). Data were expressed as mean ± SD at least three independent experiments.

3.2.13. Statistical Analysis

Data are expressed as mean SEM (standard error of the mean) of at least three independent experiments. Statistical significance between two groups was determined using the Student's *t*-test. Statistical significance was accepted for *p* values < 0.05.

4. Conclusions

Non-amidino N^2-thiophenecarbonyl- and N^2-tosylanthranilamides **1**-**20**, and amidino N^2-thiophenecarbonyl- and N^2-tosylanthranilamides **21**-**26** synthesized and evaluated against PT and aPTT *in vitro*. Non-amidine thiophenecarbonyl compounds **5**, **9**, and amidine thiophenecarbonyl compounds **21**-**23** showed prolongation in aPTT *in vitro* and *ex vivo*, and *in vivo* bleeding time, respectively. Compounds **5** and **21**-**23** exhibited prolongation in PT *in vitro* and *ex vivo*. The activities of FXa and thrombin as well as the generation of thrombin and FXa in HUVECs were dose-dependently inhibited but weak. These compounds inhibited thrombin-catalyzed fibrin polymerization and platelet aggregation induced by U46619. Any sulfonamide group containing

compounds **11**-**20**, **24**, and **26** did not exhibit prolongation of PT and aPTT. This study demonstrated that the amidine group in the *meta* or *para* position of the B-ring and thiophenecarbonyl linker of **21**-**23** were essential for anticoagulant activity.

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