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ORIGINAL PAPER

Design, synthesis, biological evaluation and molecular docking studies of dabigatran analogs as potential thrombin inhibitors

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Abstract A series of fluorinated dabigatran analogs were designed and synthesized. All the target compounds were characterized by ^{1}H NMR, ^{13}C NMR, and FT-ICR-MS. The thrombin inhibitory activities of the new synthesized compounds were also evaluated in vitro. The results show that compound **12a** has the highest IC_{50} of thrombin inhibition (IC_{50} = 5.41 nM). Moreover, molecular docking simulation was carried out to elucidate the conformations of the compounds and key amino acid residues at the active site of thrombin protein. The results show there is an appropriate relationship between IC_{50} and the docking scores for compounds **12a–e**. We suggest that the hydrogen bond interaction between Asp189, Gly219 of thrombin and the compounds appear to play major role in thrombin inhibition.

Keywords Dabigatran analogs · Thrombin inhibition · Molecular docking

Introduction

Thromboembolic disease, which occurs in the arterial or venous circulation, is a major cause of mortality and morbidity in the developed world and is caused by excessive stimulation of coagulation [1]. The most widely used drugs for antithrombotic therapy are indirect thrombin inhibitors (e.g., heparins) and vitamin K antagonists such as warfarin [2]. Recently, the introduction of new oral anticoagulants on the market has enabled the availability of new options. The first oral anticoagulant drug dabigatran etexilate is a novel, potent, nonpeptidic direct thrombin inhibitor [3]. The orally administered double prodrug is hydrolyzed in vivo by esterases into the active form dabigatran [4] (Fig. 1b). Dabigatran reversibly binds to the active site of both free and clot-bound thrombin, thereby effectively preventing the formation of fibrin clots [5]. Dabigatran is approved in over 70 countries for stroke prevention in patients with atrial fibrillation and for thrombosis prevention after orthopedic hip and knee surgery [6]. However, this new anticoagulant has several disadvantages, including low oral bioavailability and probability of hemorrhage when used in large dose [7, 8].

To date, fluorinated drugs account for a considerable proportion of drugs used in clinical treatment [9–13]. With the introduction of fluorine atoms or fluoride groups, physical and chemical properties of drug molecules could be changed [14], which consequently alters the pharmacokinetic properties of drug molecules. Changes in the structural conformation of compounds may enhance their capacity to bind with ligands and target proteins, as well as their selectivity for target proteins. Fluorination could block metabolic pathways of drugs in a particular site, change the rate of their metabolism, prolong their effects in the body, and improve their metabolic stability [10]. Furthermore, the introduction of fluorine into drug molecules may enhance membrane permeability [15] and specific loci formed with target proteins during hydrophobic interaction [16]. Since the introduction of 5-fluorouracil, the addition of fluorine atoms or fluoride groups into drug molecules has gradually become an important research strategy in drug design and structural transformation [17].

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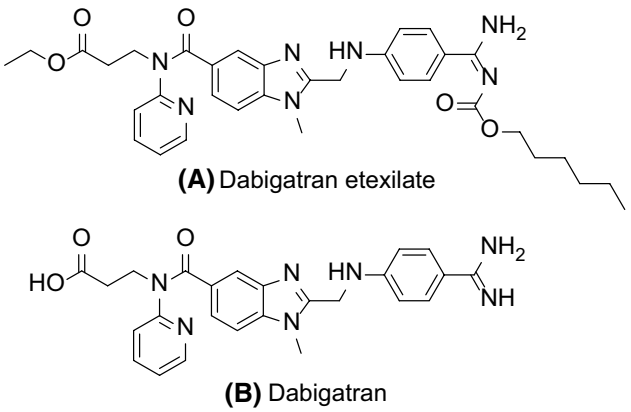


Fig. 1 Structures of dabigatran etexilate and dabigatran

According to the literature, derivatization by an electron-donating group at the N–H position of benzimidazole resulted in good biological activity [18, 19]. Therefore, to combine the benefits of dabigatran etexilate and fluorine atoms during treatment, fluoride groups were introduced into dabigatran molecule to modify its structure. This modification was expected to generate a series of fluoride-modified dabigatran analogs that could maximize the selected characteristics of fluorine atoms and improve the shortcomings of dabigatran etexilate. Consequently, more efficient and safer novel anticlotting drug candidate compounds are provided.

Researches on novel compounds and their development have become more efficient with advancements in computer-aided simulation and design. In this study, we designed and synthesized a novel series of fluoride dabigatran analogs using dabigatran as the reference molecule, studied their antithrombin activities, and validated their activity using molecular modeling approaches. Initially, docking experiments with designed structures at the active site (PDB entry code: 1KTS) [3] were performed in silico to assess their binding (docking scores) with respect to that of dabigatran. Dabigatran etexilate has a basic benzimidazole ring and a benzamidine group that are interlinked using amino linker. According to the literature [20], 3-(pyridyl-2-amino) propionic acid ethyl ester and its analogs account for its bioactivity. Therefore, we replaced the pyridine ring with a benzene ring and introduced various fluoride groups at different sites (Fig. 2). The effects of introducing fluorine atoms on anticoagulant activity were determined. Throughout this research, both strategies of preferential selectivity toward thrombin and the addition of a fluorine group release moiety were combined. Then, novel analogs of fluorinated dabigatran were synthesized and evaluated for their antithrombin activity. Docking studies were performed for validation of the observed pharmacological properties of the investigated antithrombin

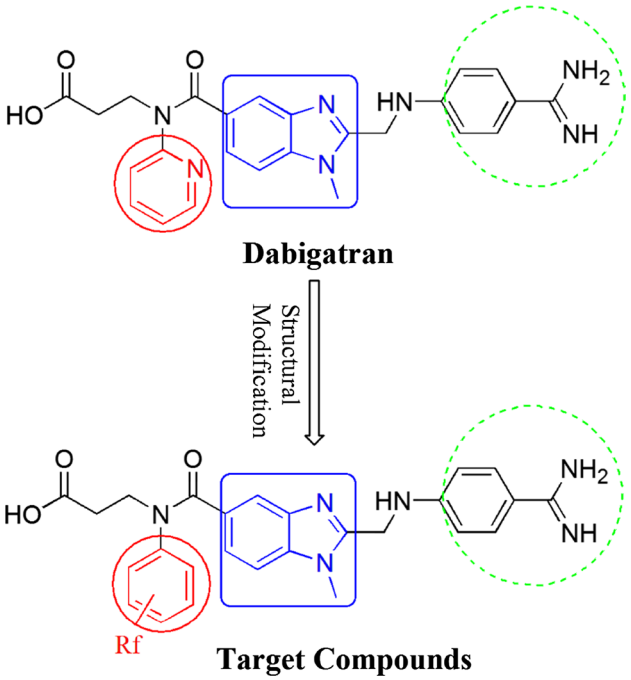


Fig. 2 Design strategy of fluorinated thrombin inhibitor derivatives

compounds and for the determination of the most important parameters controlling these properties.

Experimental

General

All chemicals and solvents were purchased from Darui and Titan Corporation and used without further purification. Melting points were measured on WRS-1B and were uncorrected. NMR spectra were carried out using a Brucker Avance 500 MHz NMR spectrometer using TMS as internal reference. Chemical shifts are reported in δ scale (ppm). Mass spectra of the synthesized compounds were recorded on a Brucker FT-ICR-MS. Elemental analysis was recorded on Germany Vario EL III Elementar.

*General procedure for the synthesis of ethyl 3-((fluorinated-phenyl)-amino) propanoate (**3a–e**)*

To a solution of commercial fluorinated benzenamine (**1a–e**) (0.05 mol) and ethyl acrylate (0.09 mol) without solvent, trifluoromethanesulfonic acid (0.005 mol) was added. The resulting mixture was heated to 100 °C for 24 h. The progress of the reaction was monitored by thin layer chromatography using petroleum ether/dichloromethane (2:1) as the solvent system. The crude mixture was purified by silica gel column chromatography eluting 5:1 petroleum

ether: dichloromethane to give the intermediate compounds, **3a**–**e**.

*Synthetic procedure for 4-(ethylamino)-3-nitrobenzoic acid (**6**)* A 250-mL round-bottom flask was charged with 4-chloro-3-nitrobenzoic acid (20 g, 0.1 mol) and a solution of ethylamine in water (60 %, 86 mL, 0.8 mol).The mixture was refluxed at 80 °C for 5 h. The solution was adjusted to pH 4–5 with acetic acid. The precipitations of yellow solids were filtered to get products 5 (19.6 g, 100 %).

*Synthetic procedure for 4-(ethylamino)-3-nitrobenzoyl chloride (**7**)* A 250-mL round-bottom flask was charged with 4-(ethylamino)-3-nitrobenzoic acid (9.0 g, 0.046 mol). 30 mL of dichloromethane and 5 drops of *N*,*N*-dimethylformamide were added in one portion. The mixture was stirred at room temperature (30 °C). Thionyl chloride (20 mL, 0.23 mol) was added dropwise via addition funnel. The reaction solution was stirred at room temperature for 5–6 h. The progress of the reaction was monitored by thin layer chromatography using ethyl acetate: petroleum ether (1:1) as solvent system. The reaction solvent was evaporated in vacuo to dryness to give the product 6 without further operation.

*General procedure for the synthesis of ethyl 3-(N-(flourinated-phenyl)-4-(ethylamino)-3-nitrobenzamido) propanoate (**8a**–**e**)*

To a vigorously stirred mixture of 3 (0.03 mol) and triethylamine (0.03 mol) in anhydrous dichloromethane (50 mL) at room temperature, 4-(ethylamine)-3-nitrobenzoyl-chloride (0.03 mol) in anhydrous dichloromethane was added dropwise for 30 min. The reaction mixture was further stirred for 5 h at room temperature. The progress of the reaction was monitored by thin layer chromatography using ethyl acetate: petroleum ether (1:1) as the solvent system. The reaction mixture was washed with aqueous sodium chloride solution three times and dried over anhydrous sodium sulfate. The crude product was purified by silica gel column chromatography eluting 2:1 petroleum ether: ethyl acetate to give the products **8a**–**e**.

*Ethyl 3-(N-(3-fluorophenyl)-4-(methylamino)-3-nitrobenzamido)propanoate (**8a**)* Red–orange solid; yield: 68 %; m.p.: 79.5–80.4 °C; ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.17 (d, $J = 2.0$ Hz, 1H), 7.45–7.38 (m, 1H), 7.30–7.21 (m, 1H), 6.91 (td, $J = 7.9$, 2.0 Hz, 2H), 6.85 (dt, $J = 9.5$, 2.0 Hz, 1H), 6.62 (d, $J = 9.0$ Hz, 1H), 4.18 (t, $J = 7.2$ Hz, 2H), 4.07 (q, $J = 7.1$ Hz, 2H), 2.97 (d, $J = 5.1$ Hz, 3H), 2.69 (t, $J = 7.2$ Hz, 2H), 1.20 (t, $J = 7.1$ Hz, 3H). FT-MS *m*/*z* Calcd. for $C_{19}H_{20}FN_3O_5$ ([M+H] +): 390.14598, Found: 390.14826.

*Ethyl 3-(N-(3, 5-difluorophenyl)-4-(methylamino)-3-nitrobenzamido)propanoate (**8b**)* Orange–yellow solid; yield: 25 %; m.p.: 140.7–141.6 °C; ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.21 (d, $J = 2.0$ Hz, 1H), 7.45 (dd, $J = 9.0$, 1.9 Hz, 1H), 6.74–6.64 (m, 4H), 4.17 (t, $J = 7.1$ Hz, 2H), 4.10 (q, $J = 7.1$ Hz, 2H), 3.01 (d, $J = 5.1$ Hz, 3H), 2.70 (t, $J = 7.1$ Hz, 2H), 1.23 (t, $J = 7.1$ Hz, 3H). FT-MS *m*/*z* Calcd. for $C_{19}H_{19}FN_3O_5$ ([M+H] +): 408.13655, Found: 408.13663.

*Ethyl 3-(4-(methylamino)-3-nitro-N-(3-(trifluoromethyl) phenyl)benzamido)propanoate (**8c**)* Orange–yellow oil; yield: 70 %; ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.17 (d, $J = 1.6$ Hz, 1H), 7.45–7.39 (m, 1H), 7.29–7.21 (m, 1H), 6.91 (td, $J = 6.3$, 1.6 Hz, 2H), 6.85 (dt, $J = 7.6$, 1.6 Hz, 1H), 6.62 (d, $J = 7.2$ Hz, 1H), 4.18 (t, $J = 5.8$ Hz, 2H), 4.07 (q, $J = 5.7$ Hz, 2H), 2.97 (d, $J = 4.1$ Hz, 3H), 2.69 (t, $J = 5.8$ Hz, 2H), 1.20 (t, $J = 5.7$ Hz, 3H). FT-MS *m*/*z* Calcd. for $C_{20}H_{20}F_3N_3O_5$ ([M+H] +): 440.14278, Found: 440.14178.

*Ethyl 3-(4-(methylamino)-3-nitro-N-(4-(trifluoromethyl) phenyl)benzamido)propanoate (**8d**)* Orange–yellow oil; yield: 45 %; ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.21 (d, $J = 1.9$ Hz, 1H), 7.57 (d, $J = 8.3$ Hz, 2H), 7.41 (dd, $J = 9.0$, 1.7 Hz, 1H), 7.29–7.25 (m, 2H), 6.65 (d, $J = 9.0$ Hz, 1H), 4.23 (t, $J = 7.1$ Hz, 2H), 4.09 (q, $J = 7.1$ Hz, 2H), 3.01 (d, $J = 5.1$ Hz, 3H), 2.73 (t, $J = 7.1$ Hz, 2H), 1.22 (t, $J = 7.1$ Hz, 3H). FT-MS *m*/*z* Calcd. for $C_{20}H_{20}F_3N_3O_5$ ([M+H] +): 440.14278, Found: 440.14373.

*Ethyl 3-(4-(methylamino)-3-nitro-N-(4-(trifluoromethoxy) phenyl)benzamido)propanoate (**8e**)* Orange–yellow oil; yield: 77 %; ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.15 (d, $J = 2.0$ Hz, 1H), 7.41 (dd, $J = 9.0$, 1.9 Hz, 1H), 7.16 (s, 4H), 6.63 (d, $J = 9.0$ Hz, 1H), 4.19 (t, $J = 7.1$ Hz, 2H), 4.06 (q, $J = 7.1$ Hz, 2H), 2.99 (d, $J = 5.1$ Hz, 3H), 2.71 (t, $J = 7.1$ Hz, 2H), 1.21 (t, $J = 7.1$ Hz, 3H). FT-MS *m*/*z* Calcd. for $C_{20}H_{20}F_3N_3O_6$ ([M+H] +): 456.13770, Found: 456.14187.

*General procedure for the synthesis of ethyl 3-(3-amin o-N-(fluorinated-phenyl)-4-(methylamino) benzamido) propanoate (**9a**–**e**)*

A round bottom flask was charged with a solution of compound 8 (20 mmol) in tetrahydrofuran/water (40/80 mL). Zn powder (0.1 mol) and NH4Cl (10 mmol) were added in turn. The reaction mixture was refluxed at 80 °C overnight under the atmosphere of nitrogen. The reaction process was monitored by thin layer chromatography using ethyl acetate: petroleum ether (1:1) as the solvent system. The mixture was filtered through a pad to remove the Zn powder.

The residue solution was extracted with dichloromethane three times. The combined organic layer was washed with an aqueous solution of sodium chloride solution three times and dried over anhydrous sodium sulfate. The crude product was purified by silica gel chromatography eluting 1:1 ethyl acetate: petroleum ether to give product 9.

*Ethyl 3-(3-amino-N-(3-fluorophenyl)-4-(methylamino)benzamido)propanoate (**9a**)* Brown oil; yield: 90 %; ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.19 (q, $J = 7.5$ Hz, 1H), 6.85 (d, $J = 10.5$ Hz, 4H), 6.77–6.73 (m, 1H), 6.32 (d, $J = 8.3$ Hz, 1H), 4.17 (t, $J = 7.3$ Hz, 2H), 4.08 (q, $J = 7.1$ Hz, 2H), 3.35 (s, 3H), 2.80 (s, 3H), 2.71 (t, $J = 7.3$ Hz, 2H), 1.22 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for C19H22FN3O3 ([M+H] +): 360.17180, Found: 360.17403.

*Ethyl 3-(3-amino-N-(3,5-difluorophenyl)-4-(methylamino) benzamido)propanoate (**9b**)* Red–brown oil; yield: 89 %; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.02 (t, $J = 9.2$ Hz, 1H), 6.94–6.86 (m, 2H), 6.72 (s, 1H), 6.48 (dd, $J = 8.2$, 1.6 Hz, 1H), 6.16 (d, $J = 8.3$ Hz, 1H), 4.57 (s, 2H), 4.06 (t, $J = 7.0$ Hz, 2H), 3.99 (q, $J = 7.1$ Hz, 2H), 2.68 (d, $J = 4.7$ Hz, 3H), 2.59 (t, $J = 7.0$ Hz, 2H), 1.14 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{19}H_{21}F_2N_3O_3$ ([M+H] +): 378.16237, Found: 378.16437.

*Ethyl 3-(3-amino-4-(methylamino)-N-(3-(trifluoromethyl) phenyl)benzamido)propanoate (**9c**)* Gray–green oil; yield: 93 %; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.49 (dd, $J = 15.5$, 7.7 Hz, 3H), 7.36 (d, $J = 7.3$ Hz, 1H), 6.72–6.67 (m, 1H), 6.31 (dd, $J = 8.2$, 1.8 Hz, 1H), 6.08 (d, $J = 8.3$ Hz, 1H), 5.02 (s, 1H), 4.52 (s, 2H), 4.07 (t, $J = 6.9$ Hz, 2H), 3.94 (q, $J = 7.1$ Hz, 2H), 2.64 (s, 3H), 2.56 (t, $J = 6.9$ Hz, 2H), 1.11 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{20}H_{22}F_3N_3O_3$ ([M+H] +): 410.16860, Found: 410.16733.

*Ethyl 3-(3-amino-4-(methylamino)-N-(4-(trifluoromethyl) phenyl)benzamido)propanoate (**9d**)* Gray–green solid; yield: 83 %; m.p.: 91.3–92.0 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.63 (d, $J = 8.4$ Hz, 2H), 7.30 (d, $J = 8.3$ Hz, 2H), 6.69 (d, $J = 1.8$ Hz, 1H), 6.36 (dd, $J = 8.2$, 1.7 Hz, 1H), 6.09 (d, $J = 8.3$ Hz, 1H), 5.05 (s, 1H), 4.53 (s, 2H), 4.07 (t, $J = 7.0$ Hz, 2H), 3.95 (q, $J = 7.1$ Hz, 2H), 2.65 (d, $J = 3.6$ Hz, 3H), 2.56 (t, $J = 7.0$ Hz, 2H), 1.11 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{20}H_{22}F_3N_3O_3$ ([M+H] +): 410.16860, Found: 410.16827.

*Ethyl 3-(3-amino-4-(methylamino)-N-(4-(trifluoromethoxy) phenyl)benzamido)propanoate (**9e**)* Red–brown oil; yield: 84 %; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.27 (d, $J = 8.8$ Hz, 2H), 7.22 (d, $J = 9.0$ Hz, 2H), 6.69 (d, $J = 1.8$ Hz, 1H), 6.34 (dd, $J = 8.2$, 1.8 Hz, 1H), 6.08 (d, $J = 8.3$ Hz, 1H), 5.02 (s, 1H), 4.51 (s, 2H), 4.03 (t, $J = 7.0$ Hz, 2H), 3.94 (q, $J = 7.1$ Hz, 2H), 2.65 (s, 3H), 2.56 (t, $J = 7.0$ Hz, 2H), 1.11 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{20}H_{22}F_3N_3O_4$ ([M+H] +): 426.16352, Found: 426.16751.

*General procedure for the synthesis of compounds **10a–e***

A round bottom flask was charged with a solution of 2-((4-cyanophenyl) amino) acetic acid (12 mmol), EDCI (12 mmol) and HoBt (12 mmol) in tetrahydrofuran/*N*,*N*-dimethylformamide (70/10 mL). The reaction mixture was stirred at ice bath for 35 min. Then a solution of compound 9 (10 mmol) in tetrahydrofuran (50 mL) was added dropwise and kept under stirring at room temperature. The reaction solution was kept stirred for overnight. The reaction solution was evaporated in vacuo to dryness. The residual was dissolved in dichloromethane (100 mL), washed with water three times, and dried over anhydrous sodium sulfate. The organic layer was evaporated in vacuo to dryness. Then the residual was dissolved in acetic acid (70 mL) and refluxed at 120 °C for 2–3 h. The reaction solution was adjusted to pH 9–10 with ammonium hydroxide and stirred for 30 min. The reaction solution was extracted with dichloromethane three times, washed with water three times, and dried over anhydrous sodium sulfate. The reaction process was monitored by thin layer chromatography using dichloromethane: methyl alcohol (20:1) as the solvent system. The crude product was purified by silica gel chromatography eluting 2:1 ethyl acetate: petroleum ether to give product 10.

*Ethyl 3-(2-(((4-cyanophenyl)amino)methyl)-N-(3-fluorophenyl) -1-methyl-1H-benzo[d]imidazole-5-carboxamido)propanoate (**10a**)* White–gray solid; yield: 89 %; m.p.: 168.3–168.9 °C; 1H NMR (500 MHz, DMSO-d_6) δ 7.51 (s, 1H), 7.47 (d, $J = 8.7$ Hz, 2H), 7.40 (d, $J = 8.4$ Hz, 1H), 7.31–7.26 (m, 1H), 7.24–7.21 (m, 1H), 7.17 (d, $J = 10.2$ Hz, 1H), 6.98 (dt, $J = 11.2$, 5.1 Hz, 2H), 6.83 (d, $J = 8.8$ Hz, 2H), 5.76 (s, 1H), 4.59 (d, $J = 5.5$ Hz, 2H), 4.11 (t, $J = 7.1$ Hz, 2H), 3.99 (q, $J = 7.1$ Hz, 2H), 3.75 (s, 3H), 2.67–2.58 (m, 2H), 1.14 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{28}H_{26}FN_5O_3$ ([M+H] +): 500.20924, Found: 500.21132.

*Ethyl 3-(2-(((4-cyanophenyl)amino)methyl)-N-(3,5-difluorophenyl)-1-methyl-1H-benzo[d]imidazole-5-carboxamido)propanoate (**10b**)* Light yellow solid; yield: 78 %; m.p.: 171.2–172.0 °C; ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.68–7.64 (m, 1H), 7.41 (d, $J = 8.7$ Hz, 2H), 7.28 (dd, $J = 8.4$, 1.4 Hz, 1H), 7.11 (d, $J = 8.5$ Hz, 1H), 6.70 (d, $J = 8.7$ Hz, 2H), 6.62 (dd, $J = 7.4$, 2.0 Hz, 2H), 6.57 (tt, $J = 8.6$, 2.1 Hz, 1H), 5.67 (s, 1H), 4.48 (d, $J = 4.6$ Hz, 2H), 4.19 (t, $J = 7.1$ Hz, 2H), 4.09 (q, $J = 7.1$ Hz, 2H), 3.69 (s, 3H), 2.71 (t, $J = 7.1$ Hz, 2H), 1.23–1.19 (m, 3H). FT-MS *m/z* Calcd. for $C_{28}H_{25}F_2N_5O_3$ ([M+H] +): 518.19982, Found: 518.20475.

*Ethyl 3-(2-(((4-cyanophenyl)amino)methyl)-1-methyl-N-(3-(trifluoromethyl)phenyl)-1H-benzo[d]imidazole-5-carboxamido)propanoate (**10c**)* Red oil; yield: 63 %; ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.65 (s, 1H), 7.45–7.39 (m, 3H), 7.36 (d, $J = 8.7$ Hz, 2H), 7.30 (dd, $J = 14.3$, 6.6 Hz, 1H), 7.27–7.20 (m, 2H), 7.08 (d, $J = 8.2$ Hz, 1H), 6.71 (d, $J = 8.7$ Hz, 2H), 5.63 (s, 1H), 4.48 (d, $J = 4.4$ Hz, 2H), 4.26 (t, $J = 7.1$ Hz, 2H), 4.08 (q, $J = 7.1$ Hz, 2H), 3.69 (s, 3H), 2.74 (t, $J = 7.1$ Hz, 2H), 1.22 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{29}H_{26}F_3N_5O_3$ ([M+H] +): 550.20605, Found: 550.20209.

*Ethyl 3-(2-(((4-cyanophenyl)amino)methyl)-1-methyl-N-(4-(trifluoromethyl)phenyl)-1H-benzo[d]imidazole-5-carboxamido)propanoate (**10d**)* Orange oil; yield: 75 %; ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.67 (s, 1H), 7.47–7.38 (m, 4H), 7.23 (dt, $J = 8.3$, 2.5 Hz, 1H), 7.18 (d, $J = 8.3$ Hz, 2H), 7.07 (dd, $J = 8.4$, 5.0 Hz, 1H), 6.71 (d, $J = 8.7$ Hz, 2H), 5.60 (s, 1H), 4.48 (d, $J = 4.5$ Hz, 2H), 4.26 (t, $J = 7.1$ Hz, 2H), 4.07 (q, $J = 7.1$ Hz, 2H), 3.68 (d, $J = 4.1$ Hz, 3H), 2.73 (t, $J = 7.1$ Hz, 2H), 1.22–1.19 (m, 3H). FT-MS *m/z* Calcd. for $C_{29}H_{26}F_3N_5O_3$ ([M+H] +): 550.20605, Found: 550.20365.

*Ethyl 3-(2-(((4-cyanophenyl)amino)methyl)-1-methyl-N-(4-(trifluoromethoxy)phenyl)-1H-benzo[d]imidazole-5-carboxamido)propanoate (**10e**)* Pink oil; yield: 81 %; ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.65 (s, 1H), 7.41 (d, $J = 8.4$ Hz, 2H), 7.20 (d, $J = 8.3$ Hz, 1H), 7.09 (d, $J = 8.8$ Hz, 2H), 7.04 (t, $J = 8.7$ Hz, 3H), 6.70 (d, $J = 8.5$ Hz, 2H), 5.66 (s, 1H), 4.46 (d, $J = 4.1$ Hz, 2H), 4.22 (t, $J = 7.1$ Hz, 2H), 4.06 (q, $J = 7.1$ Hz, 2H), 3.67 (s, 3H), 2.71 (t, $J = 7.0$ Hz, 2H), 1.21 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{29}H_{26}F_3N_5O_4$ ([M+H] +): 550.20605, Found: 566.20678.

*General procedure for the synthesis of compound **11a–e***

A round-bottom flask was charged with a solution of compound 10 (5 mmol), hydroxylamine hydrochloride (10 mmol), and triethylamine (10 mmol) in ethanol (50 mL). The reaction mixture was refluxed at 80 °C for 4–5 h. Then the reaction mixture was evaporated in vacuo to dryness to afford the crude intermediate which was used without further purification in the next step. The crude intermediate was dissolved in acetic acid (50 mL). Then Pd/C (5 %, 1.0 g) and ammonium formate (15 mmol) were added in turn. The reaction mixture was refluxed at 120 °C for 5–6 h under the protection of nitrogen. The reaction process was monitored by thin layer chromatography using dichloromethane: methyl alcohol (10:1) as the solvent system. The crude product was purified by silica gel chromatography eluting 7:1 dichloromethane: methyl alcohol to give product 11.

*Ethyl 3-(2-(((4-carbamimidoylphenyl)amino)methyl)-N-(3-fluorophenyl)-1-methyl-1H-benzo[d]imidazole-5-carboxamido)propanoate (**11a**)* White solid; yield: 47 %; m.p.: 108.4–109.1 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.91 (s, 3H), 7.70 (d, $J = 8.1$ Hz, 2H), 7.51 (s, 1H), 7.40 (d, $J = 8.4$ Hz, 1H), 7.24 (q, $J = 7.9$ Hz, 1H), 7.18 (t, $J = 10.1$ Hz, 2H), 7.01–6.94 (m, 2H), 6.87 (d, $J = 8.7$ Hz, 2H), 4.63 (d, $J = 5.5$ Hz, 2H), 4.10 (t, $J = 7.1$ Hz, 2H), 3.98 (q, $J = 7.1$ Hz, 2H), 3.77 (s, 3H), 2.61 (t, $J = 7.1$ Hz, 2H), 1.31–1.22 (m, 3H). FT-MS *m/z* Calcd. for $C_{28}H_{29}FN_6O_3$ ([M+H] +): 517.23579, Found: 517.23672.

*Ethyl3-(2-(((4-carbamimidoylphenyl)amino)methyl)-N-(3,5-difluorophenyl)-1-methyl-1H-benzo[d]imidazole-5-carboxamido)propanoate (**11b**)* White solid; yield: 57 %; m.p.: 103.5–104.6 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.89 (s, 1H), 8.68 (s, 1H), 7.66 (d, $J = 8.7$ Hz, 2H), 7.55 (s, 1H), 7.47–7.42 (m, 2H), 7.22 (d, $J = 8.4$ Hz, 1H), 7.05 (dd, $J = 13.2$, 4.3 Hz, 2H), 6.87 (d, $J = 8.7$ Hz, 2H), 4.65 (d, $J = 5.4$ Hz, 2H), 4.10 (t, $J = 7.0$ Hz, 2H), 4.00 (q, $J = 7.1$ Hz, 2H), 3.78 (s, 3H), 2.64 (t, $J = 7.0$ Hz, 2H), 1.14 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{28}H_{28}F_2N_6O_3$ ([M+H] +): 535.22637, Found: 535.22759.

*Ethyl 3-(2-(((4-carbamimidoylphenyl)amino)methyl)-1-methyl-N-(3-(trifluoromethyl)phenyl)-1H-benzo[d]imidazole-5-carboxamido)propanoate (**11c**)* White solid; yield: 46 %; m.p.: 169.8–170.4 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.88 (s, 4H), 7.67 (d, $J = 8.5$ Hz, 3H), 7.53–7.48 (m, 2H), 7.44 (d, $J = 6.1$ Hz, 2H), 7.40 (d, $J = 8.5$ Hz, 1H), 7.17 (d, $J = 8.4$ Hz, 1H), 6.86 (d, $J = 8.7$ Hz, 2H), 4.64 (d, $J = 5.2$ Hz, 2H), 4.14 (t, $J = 6.8$ Hz, 2H), 3.76 (s, 3H), 2.63 (t, $J = 6.8$ Hz, 2H), 1.11 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{29}H_{29}F_3N_6O_3$ ([M+H] +): 567.23260, Found: 567.23802.

*Ethyl 3-(2-(((4-carbamimidoylphenyl)amino)methyl)-1-methyl-N-(4-(trifluoromethyl)phenyl)-1H-benzo[d]imidazole-5-carboxamido)propanoate (**11d**)* White solid; yield: 41 %; m.p.: 154.1–155.0 °C; 1H NMR (501 MHz, DMSO-d_6) δ 7.61 (dd, $J = 8.4$, 3.8 Hz, 4H), 7.53 (s, 1H), 7.40 (t, $J = 7.7$ Hz, 3H), 7.20 (d, $J = 8.5$ Hz, 1H), 6.84 (d, $J = 8.7$ Hz, 2H), 4.62 (d, $J = 5.4$ Hz, 2H), 4.15 (t, $J = 7.0$ Hz, 2H), 3.97 (q, $J = 7.1$ Hz, 2H), 3.76 (s, 3H), 2.64 (t, $J = 7.0$ Hz, 2H), 1.12 (t, $J = 7.1$ Hz, 3H). FT-MS *m/z* Calcd. for $C_{29}H_{29}F_3N_6O_3$ ([M+H] +): 567.23260, Found: 567.23573.

*Ethyl 3-(2-(((4-carbamimidoylphenyl)amino)methyl)-1-methyl-N-(4-(trifluoromethoxy)phenyl)-1H-benzo[d]imidazole-5-carboxamido)propanoate (**11e**)* White solid; yield: 43 %; m.p.: 152.6–153.8 °C; ^{1}H NMR (500 MHz,

DMSO-d_6) δ 8.87 (s, 4H), 7.66 (d, J = 6.2 Hz, 2H), 7.51 (s, 1H), 7.40 (d, J = 8.4 Hz, 1H), 7.31 (d, J = 8.8 Hz, 2H), 7.25 (d, J = 8.5 Hz, 2H), 7.18 (d, J = 8.4 Hz, 1H), 6.87 (d, J = 8.6 Hz, 2H), 4.64 (d, J = 5.4 Hz, 2H), 4.11 (t, J = 6.9 Hz, 2H), 3.96 (q, J = 7.1 Hz, 2H), 3.77 (s, 3H), 2.62 (t, J = 6.9 Hz, 2H), 1.12 (t, J = 7.1 Hz, 3H). FT-MS *m/z* Calcd. for $C_{29}H_{29}F_3N_6O_4$ ([M+H] +): 583.22752, Found: 583.23286.

*General procedure for the synthesis of compound **12a–e***

A round-bottom flask was charged with a solution of compound 11 (0.2 mmol), sodium hydroxide (0.6 mmol) in 6 mL of water and 3 mL of ethanol. The reaction mixture was stirred at ambient temperature for 2 h. The mixture was neutralized with acetic acid. The precipitate was isolated and washed with water and ether to afford the zwitterionic title compound 12.

*3-(2-(((4-Carbamimidoylphenyl)amino)methyl)-N-(3-fluorophenyl)-1-methyl-1H-benzo[d]imidazole-5-carboxamido)propanoic acid (**12a**)* White solid; yield: 99 %; m.p.: 243.4–246.3 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.12 (d, J = 5.3 Hz, 1H), 8.89 (d, J = 4.0 Hz, 1H), 7.85 (d, J = 8.7 Hz, 1H), 7.74 (d, J = 8.8 Hz, 2H), 7.70 (s, 1H), 7.48 (d, J = 8.7 Hz, 1H), 7.29–7.24 (m, 2H), 7.06–7.00 (m, 2H), 6.93 (d, J = 8.9 Hz, 2H), 5.01 (s, 2H), 4.06 (t, J = 7.3 Hz, 2H), 3.97 (s, 3H), 2.57 (t, J = 7.4 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.47, 169.07, 164.29–163.97 (m), 163.09, 160.66, 153.41, 152.55, 144.63, 144.53, 130.67, 130.58, 129.67, 124.40, 115.21, 114.98, 113.88, 113.67, 111.97, 110.90, 46.14, 32.07, 30.77. FT-MS *m/z* Calcd. for $C_{26}H_{25}FN_6O_3$ ([M+H] +): 489.20449, Found: 489.20405. Anal. calcd for $C_{26}H_{25}FN_6O_3$ (488.20): C, 63.92; H, 5.16; N,17.20. Found: C, 63.75; H, 5.07; N, 16.93.

*3-(2-(((4-Carbamimidoylphenyl)amino)methyl)-N-(3,5-difluorophenyl)-1-methyl-1H-benzo[d]imidazole-5-carboxamido)propanoic acid (**12b**)* White solid; yield: 57 %; m.p.: 229.1–232.6 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.13 (s, 1H), 8.89 (s, 1H), 7.88 (d, J = 8.7 Hz, 1H), 7.75 (d, J = 8.6 Hz, 3H), 7.51 (d, J = 8.6 Hz, 1H), 7.19–7.06 (m, 3H), 6.94 (d, J = 8.8 Hz, 2H), 5.02 (s, 2H), 4.06 (t, J = 7.2 Hz, 2H), 3.98 (s, 3H), 2.60 (t, J = 7.3 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.44, 168.28, 164.14 (q, J = 7.7 Hz), 163.30, 163.15, 160.85, 160.70, 153.64, 152.21, 145.12, 133.62, 133.42, 129.72, 129.20, 125.57, 114.54, 114.47, 112.18, 111.91, 102.88, 46.03, 32.00, 31.52, 20.99. FT-MS *m/z* Calcd. for $C_{26}H_{24}F_2N_6O_3$ ([M+H] +): 507.19507, Found: 507.19301. Anal. calcd for $C_{26}H_{24}F_2N_6O_3$ (506.19): C, 61.65; H, 4.78; N,16.59. Found: C, 61.60; H, 4.86; N, 16.63.

*3-(2-(((4-Carbamimidoylphenyl)amino)methyl)-1-methyl-N-(3-(trifluoromethyl)phenyl)-1H-benzo[d]imidazole-5-carboxamido)propanoic acid (**12c**)* White solid; yield: 86 %; m.p.: 235.2–237.7 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.12 (s, 1H), 8.88 (s, 1H), 7.85 (d, J = 8.5 Hz, 1H), 7.78 (s, 1H), 7.74 (d, J = 8.2 Hz, 3H), 7.52 (t, J = 9.1 Hz, 2H), 7.46 (d, J = 7.4 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H), 5.02 (s, 2H), 4.11–4.06 (m, 2H), 3.97 (s, 3H), 2.58 (t, J = 7.0 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.45, 168.57, 164.12 (q, J = 7.7 Hz), 153.54, 152.18, 143.12, 133.66, 133.46, 132.46, 130.35, 130.05, 129.71, 129.38, 125.47, 124.87, 124.72, 123.74, 122.16, 114.58, 114.51, 114.42, 112.24, 55.92, 46.18, 32.03, 31.50. FT-MS *m/z* Calcd. for $C_{27}H_{25}F_3N_6O_3$ ([M+H] +):539.20130, Found: 539.19987. Anal. calcd for $C_{27}H_{25}F_3N_6O_3$ (538.19): C, 60.22; H, 4.68; N, 15.61. Found: C, 60.26; H, 4.63; N, 15.11.

*3-(2-(((4-Carbamimidoylphenyl)amino)methyl)-1-methyl-N-(4-(trifluoromethyl)phenyl)-1H-benzo[d]imidazole-5-carboxamido)propanoic acid (**12d**)* White solid; yield: 81 %; m.p.: 254.6–256.6 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.13 (s, 1H), 8.86 (s, 1H), 7.84 (d, J = 8.7 Hz, 1H), 7.75–7.72 (m, 3H), 7.62 (d, J = 8.5 Hz, 2H), 7.45 (dd, J = 13.8, 8.6 Hz, 3H), 6.92 (d, J = 8.9 Hz, 2H), 5.02 (s, 2H), 4.09 (t, J = 7.2 Hz, 2H), 3.97 (s, 3H), 2.56 (t, J = 7.3 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.36, 168.36, 164.13, 164.05, 163.98, 153.59, 152.10, 146.12, 133.56, 133.51, 129.69, 128.89, 128.62, 127.13, 126.81, 126.34, 125.80, 125.10, 122.40, 112.36, 112.27, 55.91, 46.21, 32.01, 31.59, 18.30. FT-MS *m/z* Calcd. for $C_{27}H_{25}F_3N_6O_3$ ([M+H] +):539.20130, Found: 539.19900. Anal. calcd for $C_{27}H_{25}F_3N_6O_3$ (538.19): C, 60.22; H, 4.68; N,15.61. Found: C, 60.27; H, 4.65; N, 15.49.

*3-(2-(((4-Carbamimidoylphenyl)amino)methyl)-1-methyl-N-(4-(trifluoromethoxy)phenyl)-1H-benzo[d]imidazole-5-carboxamido)propanoic acid (**12e**)* White solid; yield: 98 %; m.p.: 215.3–216.9 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.12 (s, 1H), 8.85 (s, 0H), 7.83 (d, J = 8.4 Hz, 1H), 7.74–7.70 (m, 3H), 7.43 (d, J = 7.5 Hz, 1H), 7.38 (d, J = 8.5 Hz, 2H), 7.24 (d, J = 8.1 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 5.02 (s, 2H), 4.03 (t, J = 6.0 Hz, 2H), 3.97 (s, 3H), 2.54 (t, J = 7.0 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.37, 168.28, 164.13, 164.06, 163.98, 153.51, 152.12, 146.53, 141.43, 133.76, 133.37, 129.98, 129.69, 128.88, 125.71, 123.62, 121.71, 121.07, 118.52, 114.54, 112.24, 46.23, 31.97, 31.57. FT-MS *m/z* Calcd. for $C_{27}H_{25}F_3N_6O_4$ ([M+H] +):555.19621, Found: 555.19270. Anal. calcd for $C_{27}H_{25}F_3N_6O_4$ (554.19): C, 58.48; H, 4.54; N,15.16. Found: C, 58.88; H, 4.57; N, 15.09.

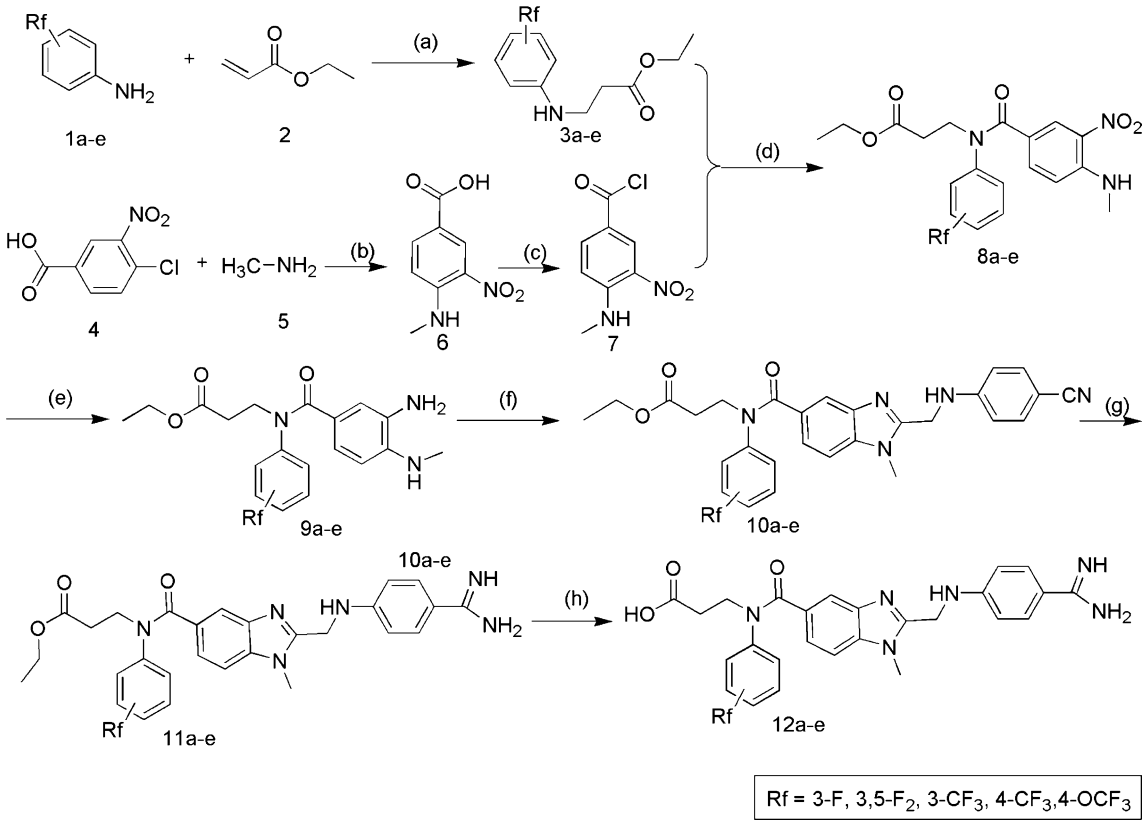
Thrombin assay

Different dilutions of the test compounds dissolved in DMSO were preincubated for 10 min at 37 °C. The national standard, lyophilized human thrombin (5.4 μg/mL), which was purified from human blood was purchased. After that, Ac-FVR-AMC (5 μM), a specific fluorogenic thrombin substrate, was added to the above system. We detected the dynamic changes in relative fluorescence intensity using an Envision microplate reader (PerkinElmer) at room temperature within 10 min. The slope of the linear enzyme dynamics curve during the initial stage of the reaction was referred to as the initial velocity of enzyme reaction. The known thrombin inhibitor dabigatran was used as a positive control. Instrument settings included excitation wavelength, 355 nm; and emission wavelength, 460 nm. Each well was measured 20 times every 20 s for about 10 min. The change in fluorescence within a predetermined time was measured under these conditions. The reaction kinetic curve slope (V_{max}) was as an activity indicator, and the concentration that induced a 50 % inhibition of thrombin activity (IC_{50}) was calculated. All measurements were performed by six experiments; the mean values of both determinations are presented.

Results and discussion

Chemistry

Given the high pharmacological activity profile of thrombin inhibitory drugs and fluorinated compounds, we designed and synthesized fluorinated thrombin inhibitor derivatives through the synthesis process depicted in Scheme 1. The main intermediate ethyl 3-((fluorinated-phenyl)-amino) propanoate (**3**) was synthesized from commercially available fluorinated anilines (**1**) in excess ethyl acrylate with the catalyst trifluoromethanesulfonic acid at 100 °C. Compound 4-(substituted-amino)-3-nitrobenzoic acid (**6**) was synthesized in good yield by the reaction between 4-chloro-3-nitrobenzoic acid and substituted amino derivatives in water without a solvent [21]. The intermediate



Scheme 1 Reagents, conditions, and range of yields: (*a*) trifluoromethanesulfonic acid, yields: 38–98 %. (*b*) Reflux. (*c*) Thionyl chloride, *N*,*N*-dimethylformamide, dichloromethane, yields: 100 %. (*d*) Triethylamine, dichloromethane, yields: 24–79 %. (*e*) Zn powder, ammonium chloride, tetrahydrofuran/water, yields: 59–98 %. (*f*) (I) 2-((4-cyanophenyl) amino) acetic acid, EDCI, HoBt, tetrahydrofuran/*N*,*N*-dimethylformamide. (II) Acetic acid, ammonium hydroxide, yields: 46–89 %. (*g*) (I) hydroxylamine hydrochloride, triethylamine, ethanol. (II) Pd/C, ammonium formate, acetic acid, nitrogen, yields: 54–88 %. (*h*) Sodium hydroxide, ethanol/water, yields: 42–99 %

Table 1 The results obtained from docking studies and in vitro evaluations of compounds **12a–e** on inhibitory activities of Thrombin

Compound no.	Rf	Total-score	H–B[a]	aa H–B[b]	H–B length[c]	IC_{50}(mean ± SD)[d]/(nM/L)
12a	3-F	10.3459	4	Asp189,Gly219,Thr172,Gly216	2.05,1.92, 2.42,2.79	5.41 ± 0.082
12b	3,5-F_2	10.3356	3	Asp189,Gly219, Tyr60A	2.04,1.99, 2.30	10.49 ± 1.24
12c	3-CF_3	10.8250	3	Asp189,Gly219, Tyr60A	1.98,1.98, 2.70	12.3 ± 0.71
12d	4-CF_3	10.2052	2	Asp189,Gly219	2.01,1.91	228.3 ± 14.07
12e	4-OCF_3	10.3130	2	Asp189,Gly219	2.02,1.94	249.7 ± 26.29
Dabigatran[e]		11.5436	2	Asp189,Gly219	1.99,1.96	2.61 ± 0.84

[a] The number of hydrogen bond

[b] Amino acids involving in hydrogen bond

[c] The distance between amino acids and compounds **4a–e**

[d] Values are expressed as the mean ± standard error of the mean of six experiments

[e] Reference drug

4-(substituted-amino)-3-nitrobenzoyl chloride (**7**) was synthesized from compound (**6**) and thionyl chloride in dichloromethane using *N*,*N*-dimethylformamide as a catalyst. The important intermediate ethyl 3-(*N*-(fluorinated-phenyl)-4-(substituted-amino)-3-nitrobenz-amido) propanoate (**8**) was synthesized from the intermediate compound (**3**) and compound (**7**) in dichloromethane using triethylamine as base. The main intermediate ethyl 3-(3-amino-*N*-(fluorinated-phenyl)-4-(substituted-amino)-benzamido) propanoate (**9**) was obtained using the reductant zinc powder from compound (**8**) in tetrahydrofuran with water as solvent. The main intermediate compound (**10**) was obtained from compounds (**9**) by condensation reactions [3]. However, the most important intermediate compound (**11**) was obtained from intermediate (**10**) through Pinner reaction [22]. The target compound (**12**) was obtained from compound (**11**) by hydrolysis. The structures of products **12a–e** were determined from their ^{1}H NMR and ^{13}C NMR spectra, FT-ICR-MS data, as well as elemental analysis data.

Biological evaluation based on statistical analysis and *P* values

With dabigatran as reference, thrombin inhibitory activities of synthesized fluorinated dabigatran analogs were explored using a commercially available chromogenic assay according to a reported assay [3]. Moreover, all the mentioned compounds were stable enough in biological conditions, and no decomposition was observed. The corresponding IC_{50} values for thrombin inhibition are shown in Table 1. Screening data revealed that all test compounds inhibit thrombin with IC_{50} values in the nanomole range. They showed inhibition toward thrombin, but this inhibition was weaker than the compared dabigatran. Among the synthesized compounds, **12a** was the most potent inhibitor against thrombin (IC_{50} = 5.41 nM), with activity value similar to the reference drug dabigatran (IC_{50} = 2.61 nM). Table 1 shows the half-maximal inhibitory (IC_{50}) value of the test compounds. When fluorine group was attached at the *meta* and *para* positions of benzene nucleus, thrombin inhibitory activities decreased, as observed in **12b**, **12c**, **12d**, and **12e** with IC_{50} values of 10.49, 12.3, 228.3, and 249.7 nM, respectively. When the electron-withdrawing group (–CF_3) was placed at the *meta* and *para* positions of the modified benzene nucleus, inhibitory activities decreased to 12.3 and 228.3 nM. Compound **12e** had a long-range withdrawing group (–OCF_3) at the para position and remarkably decreased IC_{50} values of 249.7 nM.

Molecular modeling

To get a better understand of the binding mode of the inhibitors in the active site of thrombin protein, molecular docking was performed using the Surflex-Dock module in SYBYL-X 2.0 (Tripos, Inc., USA). Initially, the performance of docking software was tested by redocking experiment. For this purpose, crystal structure of protein with the cocrystallized ligand was redocked. In Fig. 3a, it can be recognized that the redocking result and the cocrystallized structure are basically similar, except the slight rotation of carboxylic acid moiety at the end of pyridine ring. It can be inferred that the rational of the program and the docking results are reliable. The crystal structure of the thrombin receptor complex was retrieved from the RCSB Protein Data Bank (PDB entry code: 1KTS) [3]. Before the docking process, the original ligand was extracted, and the other natural ligands and water molecules were removed from the crystal structure. Subsequently, the protein was prepared by

using the Biopolymer module implemented in Sybyl. The polar hydrogen atoms were added, and Gasteiger–Huckel charges were assigned to protein atoms. All the dabigatran analogs were optimized using Tripos force field [23] and Gasteiger–Huckel charges [24]; the structural energy minimization was terminated when using Powell gradient algorithm with a convergence criterion of 0.005 kcal/(mol*A) reached [25] and a maximum of 10,000 iterations. Then, the ligands docked in the corresponding protein's binding site by an empirical scoring function in Surflex-Dock. Surflex-Dock total scores, which were expressed in –log10(Kd) units to represent binding affinities, were applied to estimate the ligand–receptor interactions of newly designed molecules.

The results of docking process including total-score, hydrogen bond, amino acids involving in hydrogen bond, and the distance between the amino acid residues and the compounds **12a–e** are outlined in Table 1. The in vitro data acquired for these compounds demonstrated that thrombin inhibition would be improved by varying the benzene cores and their substitution. Dabigatran analogs **12a–e** docked into thrombin by means of total-score fitness function in Sybyl-X2.0. Among the docking structures, the best docking solutions for each compound were considered (Figs. 3, 4, and 5). It is noted that total-score of dabigatran was 11.5436, which was greater than the other scores (Table 1). The results also show there is an appropriate relationship between IC_{50} and the docking score for compounds **12a–e**. Moreover, the investigation of the ligand interaction mode of the docked analogs clearly demonstrates that the number of hydrogen bonds and hydrogen bond distances appear to play major role in thrombin inhibition; especially, all the compounds formed strong hydrogen bonds with Asp189 and Gly219. As shown in Figs. 3, 4, and 5, dabigatran and compounds **12a–e** were docked in the binding cavity, and this series of compound shared a similar binding mode with thrombin. The benzimidazole ring formed π–π stacking interactions with the conserved Trp60D and by a hydrophobic interaction in the same P-pocket, which may be crucial to the binding of dabigatran analogs with thrombin protein. Electrostatic interactions and hydrogen bonds were observed between the amidino and the following important amino acids: Asp189 and Gly219. Remarkably, we found that different substituents in benzene ring lead to a different biological activity, due

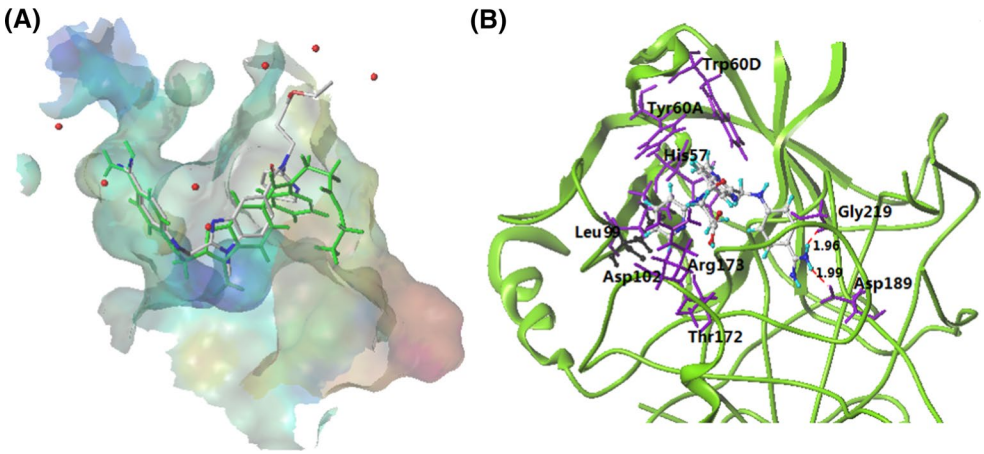


Fig. 3 **a** Redocking result of dabigatran (atom type *color*) into the binding site of thrombin protease (PDB entry: 1KTS) and the cocrystallized structure (*green*) for compound complex. **b** The docked structure of dabigatran in the active site pocket of thrombin (PDB entry: 1KTS) in stick views. Carbon, oxygen, nitrogen, and hydrogen atoms are distinguished by *white*, *red*, *blue*, and *cyan colors*, respectively. The hydrogen bonds are displayed by *red dashed lines*

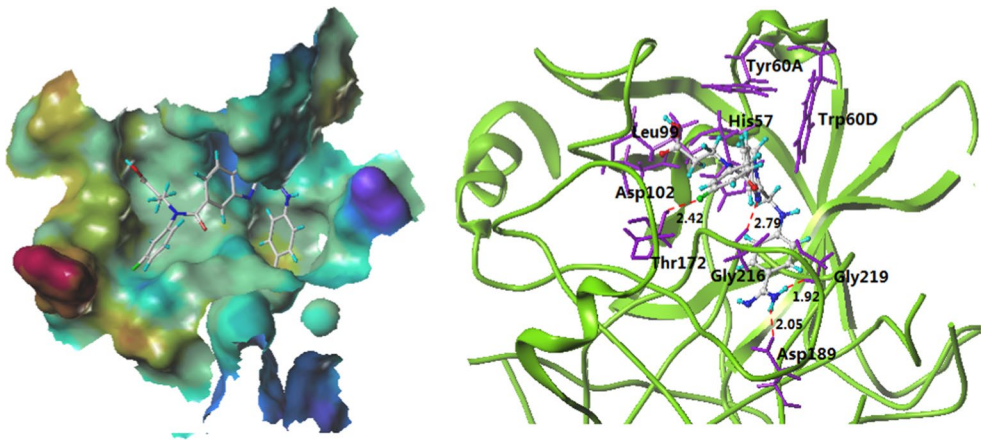


Fig. 4 A mode of compound **12a** (IC_{50} = 5.41 nM) binding in the thrombin protease (PDB code 1KTS). Fluorine is distinguished by *green color*

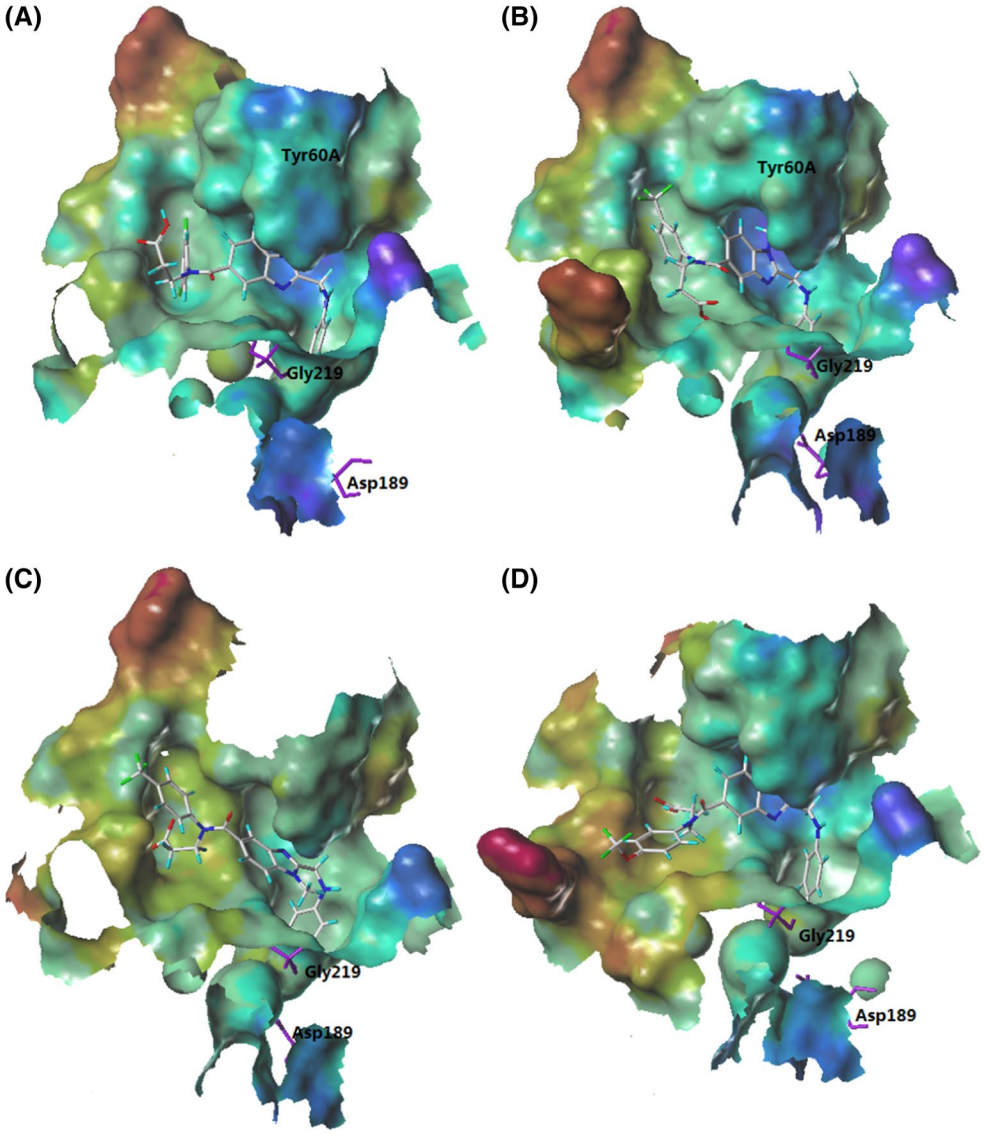


Fig. 5 The docked structure of **12b** (**a**), **12c** (**b**), **12d** (**c**) and **12e** (**d**) in the active site pocket of thrombin (PDB entry: 1KTS) in solvent surface views

to its steric effects. As can be seen clearly from Fig. 5a–d, with the increasing volume of the substituents group (3,5-F_2 < 3-CF_3 < 4-OCF_3), the whole substituted benzene ring gradually moved out of the activity pocket. The same substituent (3-CF_3) in *para* positions of benzene nucleus had far greater influence on activity than in the meta position (**12c**, 12.3 nM > **12d**, 228.3 nM). In the *para* position, the biological activity of compound **12e** is lower than the activity of **12d** because of the bulky –OCF_3 group.

Conclusions

In summary, we developed an easy methodology for the synthesis of fluorinated dabigatran analogs. As the synthesis routine, compounds (**12a**–**12e**) were synthesized and evaluated for their thrombin inhibitory activity in vitro. Biological results revealed that the target compounds exhibited antithrombin activity at nanomolar concentration. Notably, compounds **12a** showed comparable thrombin inhibitory activity (IC_{50} = 5.41 nM) in contrast to dabigatran (IC_{50} = 2.6 nM). Furthermore, the molecular docking was carried out, and the results of docking process including total-score, hydrogen bond, amino acids involving in hydrogen bond, and the distance between the residues and the compounds **12a**–**12e** are calculated. Based on the theoretical and experimental results, the bulky group substituted in benzene ring would decrease the biological activity of the anticoagulant, especially in the para position. The hydrogen bond interaction of the nitrogen of the amidino in compounds in the active site of the thrombin appears to play major role in thrombin inhibition.

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