FULL PAPER

Design, Synthesis, and Thrombin Inhibitory Activity Evaluation of Some Novel Benzimidazole Derivatives

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Some benzimidazole derivatives were designed and screened *via* molecular docking. Six compounds which obtained high scores were selected for synthesis and all compounds were characterized by ^{1}H- and ^{13}C-NMR, and HR-ESI-MS. Subsequently, these compounds were evaluated for their inhibitory activities on thrombin. Compound **5a** (IC_{50} 3.11 nM) showed a better activity than the reference argatroban (IC_{50} 9.88 nM). These results, along with related molecular model studies, indicated that **5a** could be a potential thrombin inhibitor for further research.

Keywords: Benzimidazole derivatives, Molecular docking, Anticoagulation activity, Reduction.

Introduction

Thromboembolic diseases are among the most significant causes of morbidity and mortality worldwide. Venous thrombus is estimated to cause over 500,000 deaths each year in Europe alone [1]. Anticoagulant drugs inhibit blood coagulation and thrombosis by inhibiting or destroying the activity of the coagulation factor or thrombin [2]. For decades, warfarin, a representative indirect thrombin inhibitor, is the most common oral anticoagulant for the prevention and treatment of thromboembolic events. However, this drug poses disadvantages, such as narrow therapeutic index and the necessity for frequent monitoring to ensure a therapeutic anticoagulant effect [3 – 5]. In recent years, direct thrombin inhibitors (DTIs), including melagatran, argatroban, and dabigatran, have been the focus of anticoagulant drug development [2][6]. These drugs not only exhibit high specificity for thrombin but also show predictable anticoagulant effect. For instance, argatroban is the first clinically used DTI. This drug can reduce the activity of thrombin by not only reducing fibrin formation but also inhibiting thrombin-induced platelet activation. The short half life and predictable dose-dependent therapeutic effect make argatroban preferable to other agents in the treatment of heparin-induced thrombocytopenia [7][8]. However, safety and efficiency are the major concerns regarding the clinical use of DTIs. The RE-LY trial showed that high-dose dabigatran increases the risk of hemorrhage by 0.4% [9]; in 2006, melagatran was withdrawn from the market because it induced severe kidney toxicity [10][11]. A new drug with enhanced efficacy may contribute to the decrease in side effects by reducing drug dosage. Therefore, finding better bioactive lead compounds is significant to the discovery of novel potential anticoagulant drugs.

Heterocyclic nitrogen compounds present a broad spectrum of biological activities. This nitrogen form is often included as an important component of drugs, such as benzimidazole in dabigatran and quinoline in argatroban [12 – 14]. We studied the pharmacological strategies for inhibition of thrombin activity (*Fig. 1*). The tested drugs exhibited an amidino group interacting with Asp189, which is important for the inhibition of thrombin [15]. Therefore, an amidino group as specific binding site is necessary for the DTIs. To identify natural substrates, such as fibrinogen or thrombin receptors, regions distant from the active site should also be considered because the distal pocket can harbor hydrophobic and aromatic moieties [16][17]. Thus, an aromatic group occupies the distal pocket and may favor activity. On the basis of the above approaches, we designed some novel compounds by linking benzamidine and hydrophobic aromatic moieties with the benzimidazole skeleton.

Computer-aided drug design is extensively applied in the drug-design process. High efficiency and less-time consumption rendered this approach important in designing and screening compounds [18 – 20]. In this article, all designed compounds were screened *via* molecular docking. Six compounds which obtained high scores were selected for synthesis, characterization, and evaluation of their inhibitory activities on thrombin. We would expect to find a better potential thrombin inhibitor.

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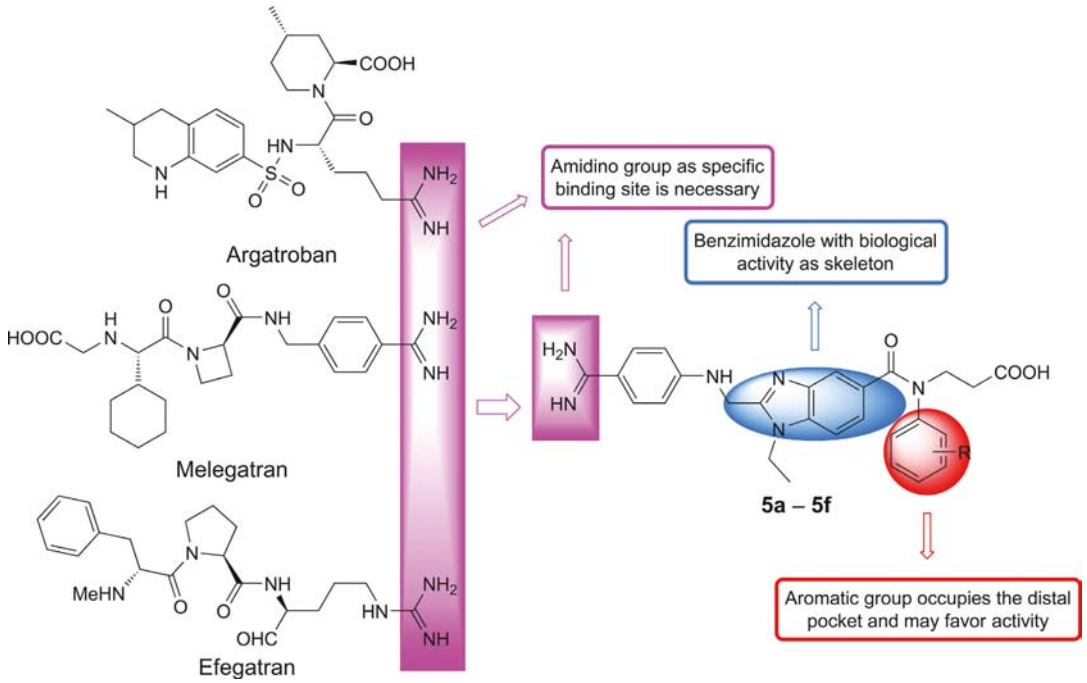


Fig. 1. Structure feature of some known direct thrombin inhibitors and the target compounds **5a** – **5f**

Results and Discussion

Molecular Docking

In this work, all molecular modeling calculations were performed in Surflex-Dock (Sybyl 2.0), the X-ray crystal structure of argatroban (PDB code: 1KTS) was retrieved from the RCSB Protein Data Bank. All designed compounds were screened *via* molecular docking; six high-scored compounds were selected for synthesis and exhibited in *Table 1*.

Chemistry

The synthetic route to benzimidazole derivatives is presented in the *Scheme*. All derivatives were characterized by ^{1}H- and ^{13}C-NMR, and HR-ESI-MS. Compounds **5a** – **5f** were synthesized using corresponding amines according to the literature [21]. Zn powder as an effective and cheap reductant is widely used in the reduction reaction [22][23]. *Khan et al.* [24] used Zn as reductant and clarified the intermediate process of the reaction.

Table 1. Docking results of the selected compounds **5a** – **5f**

Compound	Total Score	Crash	Number of H-Bonds
5a	11.6726	−2.0997	3
5b	11.9567	−2.5240	4
5c	11.1104	−2.2977	4
5d	9.9584	−3.2600	7
5e	12.8963	−2.2737	4
5f	11.1623	−3.7131	4
Argatroban	7.2927	−2.9474	4

Compounds **2a** – **2f** were obtained by the reduction of **1a** – **1f**; we tried Zn/NH_4Cl as reductant and THF/H_2O as solvent in the reaction. However, in this case the reaction time is long (more than 10 h) and compounds must be purified by column chromatography (55 – 71% yield). Through a large number of experiments, we adopted Zn as reducing agent and AcOH/H_2O as solvent in this study. The reaction time for this method was only 1 h. Additionally, **2a** – **2f** were purified by recrystallization from AcOEt and petroleum ether (PE) (80 – 90% yield, 97 – 99% purity), and this method may be suitable for industrial production. This is because Zn shows high reducibility in acidic environment. Therefore, the reaction was performed well.

Inhibition Rate Test

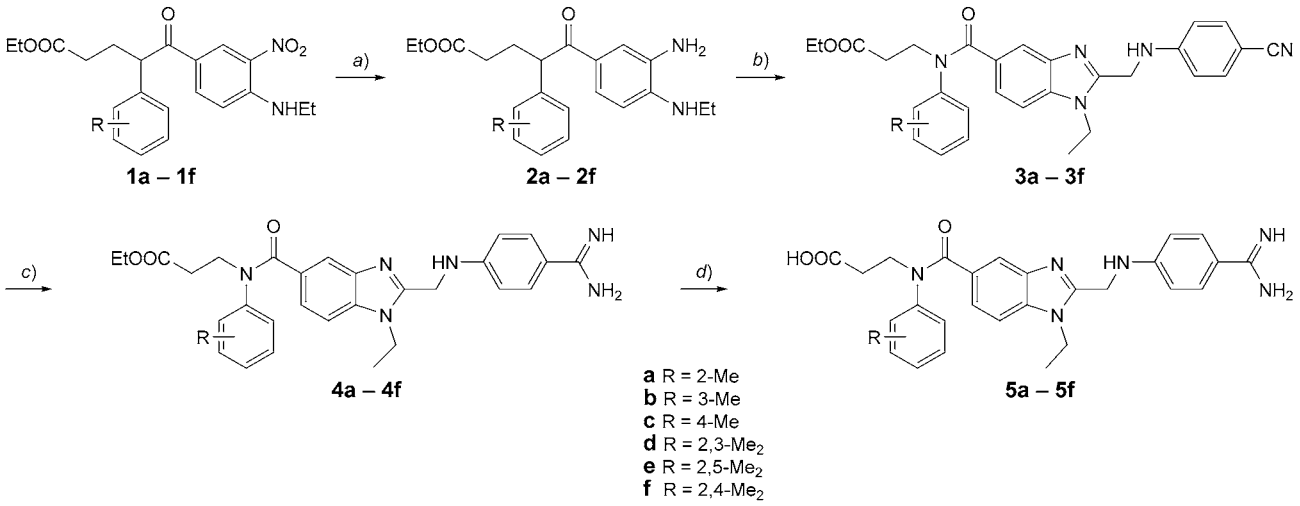
The thrombin inhibition rates of **5a** – **5f** were tested at a concentration of 1 μg/ml and the results are shown in *Table 2*.

Table 2 shows that all novel compounds exhibit inhibitory activities on thrombin as they demonstrate high inhibition rates (> 90%). Compound **5a** exhibited the highest inhibition rate. Among **5a** – **5c**, inhibition rates showed the order **5a** > **5b** > **5c**. Additionally, the inhibition rates of **5a** and **5d** (Me substituted in *o*-position) were higher than those of **5c** and **5f** (Me substituted in *p*-position).

Anticoagulant Activity In Vitro

Thrombin plays a central role in the generation of a thrombus. Thrombin's principal function is to convert soluble fibrinogen to insoluble fibrin, while also stimulating

Scheme. Synthetic route of benzimidazole derivatives.



a) Zn, AcOH/H_2O, r.t., 1 h. b) EDCI, HOBt, THF/DMF, r.t., 10 h. c) 1. $NH_2OH \cdot HCl$, Et_3N, EtOH, 80°, 3 h; 2. $HCOONH_4$, Pd/C, AcOH, N_2, 120°, 5 h. d) 1. NaOH, EtOH/H_2O, r.t., 1 h; 2. AcOH, pH 5 – 6.

Table 2. Thrombin inhibition rates of **5a** – **5f**

Compound	Inhibition Rate (%)
5a	93.83 ± 0.22
5b	92.87 ± 0.33
5c	90.34 ± 0.69
5d	92.81 ± 0.41
5e	92.82 ± 0.59
5f	91.42 ± 0.82

platelet activation. Thrombin can be inhibited directly or indirectly by the binding of thrombin-inhibiting drugs [7]. DTIs bind directly to thrombin and do not require a cofactor. DTIs can inhibit both soluble thrombin and fibrin-bound thrombin [25]. In order to save resources and improve efficiency, according to the inhibition rates of previous compounds, **5a** and **5f** were selected as representative compounds to test thrombin inhibition activity *in vitro* with argatroban used as reference. The results are exhibited in *Table* 3.

Table 3 showed that the inhibitory activity of **5a** on thrombin was better than that of argatroban (> threefold), but **5f** was worse than argatroban. Additionally, the activity of **5a** was better than that of **5f**, this result was consistent with the result of the inhibition rate test. This may result from the different binding affinity between the compounds and amino acid residues in the active site of the thrombin protein.

Table 3. *In vitro* thrombin inhibition activities of **5a** and **5f**

Compound	Mean IC_{50} ± SD (nM)
5a	3.11 ± 0.21
5f	16.45 ± 1.72
Argatroban	9.88 ± 2.26

Molecular Modeling Studies

Molecular docking could simulate the bonding form between molecules and target enzymes [26][27]. In order to identify the recognition processes of argatroban and the promising compound **5a**, we studied the molecular modeling between argatroban/**5a** and the thrombin protein, and performed it in Surflex-Dock (Sybyl 2.0; *Fig.* 2). The X-ray crystal structure of thrombin inhibitor complex (PDB code: 1KTS) was retrieved from the RCSB Protein Data Bank.

As shown in *Fig.* 2, argatroban and **5a** were docked into the binding cavity. All amidine groups were located in the same pocket, two H-bonds were formed between the N–H H-atoms of the amidino group and the residues Asp189 and Gly219. Compared to argatroban, the benzimidazole ring of **5a** forms $\pi\cdots\pi$ stacking interactions with Trp60D, which is crucial for the binding of derivatives with the thrombin protein [15]. The residues Arg173, Ile174, and Arg175 were relatively far away from argatroban but close to **5a**. From the docking results, we conclude that the amidino group plays an important role in the inhibition activity on thrombin, and **5a** had a firmer binding affinity than argatroban.

Conclusions

All the designed compounds were screened *via* molecular docking. Six highly scored compounds were selected for synthesis and characterization by ^{1}H- and ^{13}C-NMR, and HR-ESI-MS. Zn powder and AcOH/H_2O were used as reductant and solvent, respectively, in the synthesis of **2a** – **2f**. This method not only improved the efficiency but

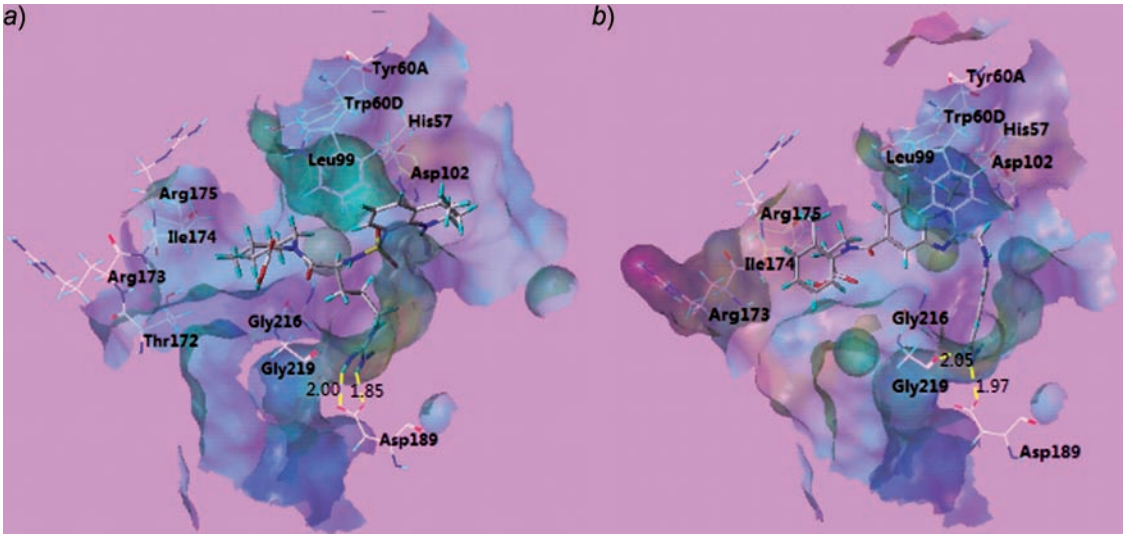


Fig. 2. *a*) View of the cognate ligand (red) and redocking result (blue) in the docked complex by superimposing the coordinates of protein together. *b*) A molecular surface of the active site of inhibitor–thrombin complexes depicted by lipophilic potential

also reduced pollution. Furthermore, **2a** – **2f** were purified *via* recrystallization from AcOEt and PE. Thus, the compounds bestow cost reduction and suitability for industrial production. The synthesized compounds were evaluated in terms of thrombin inhibition rate and thrombin inhibition activity *in vitro*. Compound **5a** exhibited better thrombin inhibition activity than the reference argatroban. Molecular modeling studies also indicated that the binding force between **5a** and thrombin protein is stronger than the binding force between argatroban and thrombin protein. Therefore, **5a** can be a potential thrombin inhibitor for further research.

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Experimental Part

General

All reagents were AR grade and purchased from *Shanghai Chemical Reagent Company* (Shanghai, P. R. China). M.p.: *WRS-1B*. Thin-layer chromatography (TLC): silica gel plates GF_{254} (SiO_2); visualized by UV light at 254 nm or exposure to I_2. Column chromatography (CC): SiO_2. ^{1}H- and ^{13}C-NMR spectra: *Bruker Avance 500* (500 MHz for ^{1}H) or *Bruker Avance 400* (400 and 125 MHz for ^{1}H and ^{13}C, resp.); δ in ppm rel. to Me_4Si as internal standard, J in Hz. HR-ESI-MS: *SolariX-70FT-MS Bruker* (Karlsruhe, Germany) spectrometer; in m/z. Reactions were monitored by TLC.

General Procedure for the Synthesis of **2a** – **2f**

Compounds **1a** – **1f** (20 mmol) were dissolved in AcOH/H_2O (50:50 ml). Subsequently, Zn powder (80 mmol) was added and stirred at room temperature for 1 h. Then, the mixture was filtered, extracted with CH_2Cl_2 (3 × 50 ml), and purified by recrystallization from AcOEt and PE.

Ethyl 5-[3-Amino-4-(ethylamino)phenyl]-4-(2-methylphenyl)-5-oxopentanoate (**2a**). Yield: 86.3%. White solid. M.p. 133 – 135°. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.14 (*d*, *J* = 9.1, 3 H); 7.07 (*d*, *J* = 6.0, 1 H); 6.86 (*s*, 1 H); 6.67 (*d*, *J* = 7.3, 1 H); 6.28 (*d*, *J* = 7.6, 1 H); 4.38 – 4.30 (*m*, 1 H); 4.07 (*q*, *J* = 7.1, 2 H); 3.88 – 3.81 (*m*, 1 H); 3.08 (*q*, *J* = 6.4, 2 H); 2.76 – 2.71 (*m*, 2 H); 2.20 (*s*, 3 H); 1.27 – 1.21(*m*, 6 H).

Ethyl 5-[3-Amino-4-(ethylamino)phenyl]-4-(3-methylphenyl)-5-oxopentanoate (**2b**). Yield: 83.7%. White solid. M.p. 148 – 149°. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.11 (*t*, *J* = 7.7, 1 H); 6.97 (*d*, *J* = 7.5, 1 H); 6.93 (*s*, 1 H); 6.89 (*s*, 1 H); 6.83 (*d*, *J* = 7.7, 1 H); 6.73 (*d*, *J* = 8.3, 1 H); 6.31 (*d*, *J* = 8.3, 1 H); 4.17 (*t*, *J* = 7.4, 2 H); 4.07 (*q*, *J* = 7.1, 2 H); 3.09 (*q*, *J* = 7.1, 2 H); 2.71 (*t*, *J* = 7.4, 2 H); 2.29 (*s*, 3 H); 1.24 (*dt*, *J* = 17.1, 7.1, 6 H).

Ethyl 5-[3-Amino-4-(ethylamino)phenyl]-4-(4-methylphenyl)-5-oxopentanoate (**2c**). Yield: 86.5%. White solid. M.p. 133 – 136°. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.02 (*d*, *J* = 8.1, 2 H); 6.93 (*d*, *J* = 8.2, 2 H); 6.84 (*d*, *J* = 1.8, 1 H); 6.71 (*dd*, *J* = 8.3, 1.8, 1 H); 6.29 (*d*, *J* = 8.3, 1 H); 4.14 (*t*, *J* = 7.4, 2 H); 4.04 (*q*, *J* = 7.1, 2 H); 3.06 (*q*, *J* = 7.1, 2 H); 2.67 (*t*, *J* = 7.4, 2 H); 2.27 (*s*, 3 H); 1.21 (*dt*, *J* = 14.6, 7.1, 6 H).

Ethyl 5-[3-Amino-4-(ethylamino)phenyl]-4-(2,3-dimethylphenyl)-5-oxopentanoate (**2d**). Yield: 83.3%. White solid. M.p. 119 – 120°. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.99 (*d*, *J* = 10.8, 2 H); 6.86 (*d*, *J* = 7.1, 2 H); 6.60 (*d*, *J* = 8.1, 1 H); 6.24 (*d*, *J* = 8.0, 1 H); 4.39 – 4.29 (*m*, 1 H); 4.04 (*q*, *J* = 7.1, 2 H); 3.78 – 3.68 (*m*, 1 H); 3.05 (*q*, *J* = 7.1, 2 H); 2.78 – 2.61 (*m*, 2 H); 2.22 (*s*, 3 H); 2.09 (*s*, 3 H); 1.24 – 1.21 (*m*, 3 H); 1.19 (*t*, *J* = 5.3, 3 H).

Ethyl 5-[3-Amino-4-(ethylamino)phenyl]-4-(2,5-dimethylphenyl)-5-oxopentanoate (**2e**). Yield: 81.2%. White solid. M.p. 124 – 126°. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.99 (*d*,

J = 7.2, 1 H); 6.92 (*d*, J = 7.8, 1 H); 6.87 (*d*, J = 5.2, 2 H); 6.64 (*d*, J = 7.1, 1 H); 6.24 (*d*, J = 8.0, 1 H); 4.30 – 4.19 (*m*, 1 H); 4.05 (*q*, J = 7.1, 2 H); 3.89 – 3.78 (*m*, 1 H); 3.05 (*q*, J = 6.9, 2 H); 2.70 (*t*, J = 7.4, 2 H); 2.24 (*s*, 3 H); 2.08 (*s*, 3 H); 1.21 (*dt*, J = 14.4, 7.3, 6 H).

Ethyl 5-[3-Amino-4-(ethylamino)phenyl]-4-(2,4-dimethylphenyl)-5-oxopentanoate (**2f**). Yield: 86.1%. White solid. M.p. 121 – 122°. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.97 (*d*, J = 8.1, 3 H); 6.92 (*s*, 1 H); 6.68 (*d*, J = 8.1, 1 H); 6.29 (*d*, J = 8.2, 1 H); 4.37 – 4.29 (*m*, 1 H); 4.09 (*q*, J = 7.1, 2 H); 3.86 – 3.79 (*m*, 1 H); 3.08 (*q*, J = 6.8, 2 H); 2.76 – 2.71 (*m*, 2 H); 2.29 (*s*, 3 H); 2.16 (*s*, 3 H); 2.09 (*s*, 3 H); 1.31 – 1.18 (*m*, 6 H).

General Procedure for the Synthesis of **3a** – **3f**

A mixture of [(4-cyano-2-substituted-phenyl)amino]acetic acid (15 mmol), 1-hydroxy-1*H*-1,2,3-benzotriazole (HOBt; 15 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI; 15 mmol), and DMF (4 ml) was dissolved in 30 ml THF, and the mixture was stirred under ice cooling for 30 min, then, a THF (40 ml) soln. of **2a** – **2f** (13 mmol) was slowly added at room temperature, and finally, the mixture was stirred for 8 h. After completion of the reaction, the solvent was removed by vacuum distillation. Then, the mixture was extracted with CH_2Cl_2 (3 × 50 ml) and concentrated by vacuum distillation. To the residue, AcOH (50 ml) was added and the mixture was heated under reflux for 2.5 h. After completion of the reaction, the solvent AcOH was removed by vacuum distillation. Then, $NH_3 \cdot H_2O$ was added until pH 8 – 9, the mixture was extracted with CH_2Cl_2 (3 × 50 ml) and the CH_2Cl_2 layer was dried (Na_2SO_4) and concentrated by vacuum distillation. The residue was subjected to CC (SiO_2; CH_2Cl_2/MeOH 100:1) to obtain **3a** – **3f**.

Ethyl 3-{[(2-{[(4-Cyanophenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2-methylphenyl)amino}propanoate (**3a**). Yield: 83.0%. White solid. M.p. 175 – 176°. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.63 (*s*, 1 H); 7.48 (*d*, J = 8.4, 2 H); 7.37 (*d*, J = 8.6, 1 H); 7.17 – 7.07 (*m*, 5 H); 6.73 (*d*, J = 8.4, 2 H); 5.44 (*s*, 1 H); 4.48 (*d*, J = 4.0, 2 H); 4.17 – 4.12 (*m*, 2 H); 4.09 (*q*, J = 7.0, 2 H); 2.85 – 2.71 (*m*, 2 H); 2.26 (*s*, 3 H); 1.68 (*s*, 1 H); 1.40 (*t*, J = 7.1, 3 H); 1.24 (*t*, J = 7.0, 3 H).

Ethyl 3-{[(2-{[(4-Cyanophenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](3-methylphenyl)amino}propanoate (**3b**). Yield: 83.7%. White solid. M.p. 187 – 188°. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.72 (*s*, 1 H); 7.48 (*d*, J = 8.4, 2 H); 7.37 (*d*, J = 8.4, 1 H); 7.16 (*d*, J = 8.5, 1 H); 7.07 (*t*, J = 7.7, 1 H); 6.96 (*s*, 1 H); 6.94 (*d*, J = 7.7, 1 H); 6.83 (*d*, J = 7.9, 1 H); 6.73 (*d*, J = 8.5, 2 H); 5.48 (*s*, 1 H); 4.50 (*d*, J = 4.4, 2 H); 4.25 (*t*, J = 7.4, 2 H); 4.15 (*q*, J = 7.2, 2 H); 4.10 (*q*, J = 7.2, 2 H); 2.74 (*t*, J = 7.4, 2 H); 2.26 (*s*, 3 H); 1.40 (*t*, J = 7.3, 3 H); 1.24 (*t*, J = 7.1, 3 H).

Ethyl 3-{[(2-{[(4-Cyanophenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](4-methylphenyl)amino}propanoate (**3c**). Yield: 82.4%. White solid. M.p. 158 – 159°. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.69 (*s*, 1 H); 7.47 (*d*, J = 8.5, 2 H); 7.35 (*d*, J = 8.4, 1 H); 7.15 (*d*, J = 8.5, 1 H); 7.01 (*d*, J = 8.0, 2 H); 6.97 (*d*, J = 7.9, 2 H); 6.73 (*d*, J = 8.5, 2 H); 5.51 (*s*, 1 H); 4.49 (*d*, J = 4.3, 2 H); 4.23 (*t*, J = 7.3, 2 H); 4.15 (*q*, J = 7.2, 2 H); 4.09 (*q*, J = 7.1, 2 H); 2.73 (*t*, J = 7.3, 2 H); 2.25 (*s*, 3 H); 1.40 (*t*, J = 7.2, 3 H); 1.23 (*t*, J = 7.1, 3 H).

Ethyl 3-{[(2-{[(4-Cyanophenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2,3-dimethylphenyl)amino}propanoate (**3d**). Yield: 87.6%. White solid. M.p. 150 – 151°. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.63 (*s*, 1 H); 7.46 (*d*, J = 8.5, 2 H); 7.32 (*d*, J = 8.5, 1 H); 7.12 (*d*, J = 8.4, 1 H); 6.99 (*d*, J = 7.2, 1 H); 6.95 (*t*, J = 7.6, 1 H); 6.89 (*d*, J = 7.5, 1 H); 6.72 (*d*, J = 8.5, 2 H); 5.51 (*s*, 1 H); 4.47 (*d*, J = 4.1, 2 H); 4.11 – 4.15 (*m*, 2 H); 4.10 – 4.05 (*m*, 2 H); 2.67 – 2.84 (*m*, 2 H); 2.20 (*s*, 3 H); 2.18 (*s*, 3 H); 1.38 (*t*, J = 7.2, 3 H); 1.23 (*t*, J = 7.1, 3 H).

Ethyl 3-{[(2-{[(4-Cyanophenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2,5-dimethylphenyl)amino}-propanoate (**3e**). Yield: 84.2%. White solid. M.p. 163 – 165°. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.65 (*s*, 1 H); 7.47 (*d*, J = 8.3, 2 H); 7.36 (*d*, J = 9.0, 1 H); 7.14 (*d*, J = 8.9, 1 H); 6.98 (*d*, J = 7.5, 1 H); 6.91 (*d*, J = 5.1, 2 H); 6.73 (*d*, J = 8.6, 2 H); 5.49 (*s*, 1 H); 4.48 (*d*, J = 4.1, 2 H); 4.43 – 4.34 (*m*, 1 H); 4.14 (*q*, J = 6.4, 2 H); 4.11 – 4.06 (*m*, 2 H); 3.94 – 3.86 (*m*, 1 H); 2.78 (*t*, J = 6.3, 2 H); 2.23 (*s*, 3 H); 2.16 (*s*, 3 H); 1.39 (*t*, J = 7.3, 3 H); 1.24 (*t*, J = 7.0, 3 H).

Ethyl 3-{[(2-{[(4-Cyanophenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2,4-dimethylphenyl)amino}-propanoate (**3f**). Yield: 85.5%. White solid. M.p. 155 – 157°. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.64 (*s*, 1 H); 7.46 (*d*, J = 6.8, 2 H); 7.35 (*d*, J = 7.8, 1 H); 7.13 (*d*, J = 7.5, 1 H); 6.96 (*d*, J = 7.9, 1 H); 6.92 (*s*, 1 H); 6.87 (*d*, J = 7.6, 1 H); 6.72 (*d*, J = 8.3, 2 H); 5.50 (*s*, 1 H); 4.48 (*d*, J = 3.5, 2 H); 4.46 – 4.40 (*m*, 1 H); 4.17 – 4.11 (*m*, 2 H); 4.11 – 4.06 (*m*, 2 H); 3.85 – 3.79 (*m*, 1 H); 2.84 – 2.68 (*m*, 2 H); 2.22 (*s*, 3 H); 2.20 (*s*, 3 H); 1.39 (*t*, J = 5.8, 3 H); 1.23 (*t*, J = 7.1, 3 H).

General Procedure for the Synthesis of **4a** – **4f**

A mixture of **3a** – **3f** (5.7 mmol), $NH_2OH{\cdot}HCl$ (24 mmol), and Et_3N (24 mmol) was dissolved in 30 ml EtOH and the mixture was heated under reflux for 3 h. The solvent was distilled under reduced pressure. To a soln. of the residue in AcOH (20 ml), 10% Pd/C (24 mmol) and ammonium formate ($HCOONH_4$; 34 mmol) were added, and the mixture was stirred under reflux under N_2 atmosphere for 5 h. The mixture was filtered and the filtrate was concentrated by vacuum distillation. The residue was subjected to CC (SiO_2; CH_2Cl_2/MeOH 7:1) to obtain solid compounds **4a** – **4f**.

Ethyl 3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2-methylphenyl)-amino}propanoate (**4a**). Yield: 77.3%. White solid. M.p. 200 – 201°. ^{1}H-NMR (500 MHz, (D_6)DMSO): 7.61 (*d*, *J* = 8.4, 2 H); 7.39 (*d*, *J* = 11.4, 2 H); 7.29 (*s*, 1 H); 7.23 (*t*, *J* = 7.4, 2 H); 7.13 (*d*, *J* = 8.7, 2 H); 6.83 (*d*, *J* = 8.5, 2 H); 4.61 (*d*, *J* = 4.4, 2 H); 4.23 (*d*, *J* = 6.8, 2 H); 3.99 (*q*, *J* = 7.0, 2 H); 3.74 – 3.66 (*m*, 2 H); 2.64 (*t*, *J* = 7.5, 2 H); 2.14 (*s*, 3 H); 1.79 (*s*, 3 H); 1.23 (*t*, *J* = 6.7, 3 H); 1.14 (*t*, *J* = 7.0, 3 H); 0.94 (*t*, *J* = 7.2, 1 H).

Ethyl 3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](3-methylphenyl)-amino}propanoate (**4b**). Yield: 71.5%. White solid. M.p. 199 – 200°. ^{1}H-NMR (500 MHz, (D_6)DMSO): 7.61 (*d*, *J* = 8.6, 2 H); 7.50 (*s*, 1 H); 7.41 (*d*, *J* = 8.4, 1 H); 7.22 (*d*, *J* = 8.4, 1 H); 7.11 (*s*, 1 H); 7.08 (*d*, *J* = 7.8, 1 H); 6.96 (*d*, *J* = 7.6, 1 H); 6.88 (*d*, *J* = 7.8, 1 H); 6.84 (*d*, *J* = 8.6, 2 H); 4.62 (*d*, *J* = 5.3, 2 H); 4.25 (*q*, *J* = 7.0, 2 H); 4.07 (*t*, *J* = 7.2, 2 H); 4.00 (*q*, *J* = 14.2, 7.2, 2 H); 2.60 (*t*, *J* = 7.2, 2 H); 2.21 (*s*, 3 H); 1.24 (*t*, *J* = 7.1, 3 H); 1.14 (*t*, *J* = 7.1, 3 H).

Ethyl 3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](4-methylphenyl)-amino}propanoate (**4c**). Yield: 76.6%. White solid. M.p. 200 – 201°. ^{1}H-NMR (500 MHz, (D_6)DMSO): 7.62 (*d*, *J* = 8.7, 2 H); 7.48 (*s*, 1 H); 7.40 (*d*, *J* = 8.5, 1 H); 7.21 (*d*, *J* = 8.4, 1 H); 7.08 – 7.03 (*m*, 4 H); 6.85 (*d*, *J* = 8.7, 2 H); 4.62 (*d*, *J* = 5.3, 2 H); 4.25 (*q*, *J* = 7.2, 2 H); 4.05 (*t*, *J* = 7.1, 2 H); 4.00 (*q*, *J* = 7.1, 2 H); 2.58 (*t*, *J* = 7.1, 2 H); 2.19 (*s*, 3 H); 1.24 (*t*, *J* = 7.1, 3 H); 1.14 (*t*, *J* = 7.1, 3 H).

Ethyl 3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2,3-dimethylphenyl)-amino}propanoate (**4d**). Yield: 74.8%. White solid. M.p. 200 – 202°. ^{1}H-NMR (500 MHz, (D_6)DMSO): 7.60 (*d*, *J* = 8.5, 2 H); 7.41 (*s*, 1 H); 7.38 (*d*, *J* = 8.5, 1 H); 7.19 (*d*, *J* = 8.7, 1 H); 7.03 – 6.96 (*m*, 3 H); 6.83 (*d*, *J* = 8.3, 2 H); 4.61 (*d*, *J* = 5.1, 2 H); 4.23 (*q*, *J* = 7.4, 2 H); 3.99 (*q*, *J* = 7.0, 2 H); 2.70 – 2.53 (*m*, 2 H); 2.15 (*s*, 3 H); 2.10 (*s*, 3 H); 1.23 (*t*, *J* = 6.8, 3 H); 1.14 (*t*, *J* = 7.0, 3 H); 0.94 (*t*, *J* = 7.1, 1 H).

Ethyl 3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2,5-dimethylphenyl)-amino}propanoate (**4e**). Yield: 73.3%. White solid. M.p. 183 – 184°. ^{1}H-NMR (500 MHz, (D_6)DMSO): 7.61 (*d*, *J* = 7.6, 2 H); 7.43 (*s*, 1 H); 7.40 (*d*, *J* = 8.3, 1 H); 7.25 (*d*, *J* = 8.6, 1 H); 7.12 (*s*, 1 H); 7.00 (*d*, *J* = 6.4, 1 H); 6.93 (*d*, *J* = 7.3, 1 H); 6.84 (*d*, *J* = 8.3, 2 H); 4.61 (*d*, *J* = 3.8, 2 H); 4.25 – 4.21 (*m*, 2 H); 4.00 (*q*, *J* = 6.7, 2 H); 3.74 (*m*, 2 H); 2.64 (*t*, *J* = 8.9, 2 H); 2.20 (*s*, 3 H); 2.04 (*s*, 3 H); 1.23 (*t*, *J* = 5.7, 3 H); 1.14 (*t*, *J* = 6.9, 3 H); 0.94 (*t*, *J* = 7.1, 1 H).

Ethyl 3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2,4-dimethylphenyl)-amino}propanoate (**4f**). Yield: 78.1%. White solid. M.p. 200 – 202°. ^{1}H-NMR (500 MHz, (D_6)DMSO): 7.62 (*d*, *J* = 8.5, 2 H); 7.41 (*s*, 1 H); 7.40 (*d*, *J* = 8.9, 1 H); 7.23 (*d*, *J* = 7.4, 1 H); 7.11 (*d*, *J* = 7.8, 1 H); 6.95 (*s*, 1 H); 6.93 (*d*, *J* = 8.1, 1 H); 6.84 (*d*, *J* = 8.2, 2 H); 4.61 (*d*, *J* = 4.7, 1 H); 4.27 – 4.22 (*m*, 1 H); 4.00 (*q*, *J* = 7.0, 1 H); 3.67 – 3.63 (*m*, 1 H); 2.62 (*t*, *J* = 7.7, 1 H); 2.16 (*s*, 1 H); 2.10 (*s*, 1 H); 1.24 (*t*, *J* = 6.5, 2 H); 1.14 (*t*, *J* = 6.8, 2 H).

General Procedure for the Synthesis of **5a** – **5f**

Compounds **4a** – **4f** (2 mmol) were dissolved in EtOH/H_2O (5:5 ml), and a soln. of NaOH (6 mmol) was added, then, stirring was continued at room temperature for 1 h. The mixture was acidized with AcOH to pH 6 – 7 and the precipitated white solid was filtered, washed with H_2O, and dried to obtain **5a** – **5f**.

3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2-methylphenyl)amino}-propanoic Acid (**5a**). Yield: 90.3%. White solid. M.p. 259 – 260°. HPLC: 98.3%. ^{1}H-NMR (400 MHz, (D_6) DMSO + DCl): 8.91 (*s*, 1 H); 8.66 (*s*, 1 H); 7.66 (*d*, *J* = 8.7, 2 H), 7.47 (*s*, 1 H); 7.40 (*d*, *J* = 11.7, 2 H); 7.25 (*t*, *J* = 9.6, 2 H); 7.13 (*d*, *J* = 5.9, 2 H); 6.86 (*d*, *J* = 8.7, 2 H); 4.63 (*s*, 2 H); 4.23 (*t*, *J* = 12.9, 2 H); 3.82 – 3.56 (*m*, 2 H); 2.59 (*dd*, *J* = 11.8, 7.6, 2 H); 2.14 (*s*, 3 H); 1.23 (*t*, *J* = 7.0, 3 H). ^{13}C-NMR (100 MHz, (D_6)DMSO + DCl): 173.05; 170.34; 164.71; 153.37; 152.97; 142.24; 135.88; 135.22; 131.70; 130.40; 130.18; 129.99; 128.14; 127.37; 123.46; 119.11; 113.72; 113.27; 112.15; 110.04; 56.46; 45.97; 38.99; 32.38; 18.01; 15.23. HR-ESI-MS: 499.24786 ([*M* + H]$^+$, $C_{28}H_{31}N_6O_3^+$; calc. 499.24522).

3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](3-methylphenyl)amino}-propanoic Acid (**5b**). Yield: 93.3%. White solid. M.p. 252 – 254°. HPLC: 98.6%. ^{1}H-NMR (400 MHz, (D_6) DMSO + DCl): 8.93 (*s*, 1 H); 8.69 (*s*, 1 H); 7.67 (*d*, *J* = 8.6, 2 H); 7.53 (*s*, 1 H); 7.46 (*d*, *J* = 7.3, 1 H); 7.25 (*d*, *J* = 8.4, 1 H); 7.13 (*s*, 1 H); 7.09 (*t*, *J* = 7.7, 1 H); 6.96 (*d*, *J* = 7.6, 1 H); 6.88 (*t*, *J* = 9.0, 3 H); 4.69 (*s*, 2 H); 4.29 (*q*, *J* = 6.6, 2 H); 4.03 (*t*, *J* = 7.5, 2 H); 2.55 (*t*, *J* = 7.6, 2 H); 2.21 (*s*, 3 H); 1.25 (*t*, *J* = 7.0, 3 H). ^{13}C-NMR (100 MHz, (D_6)DMSO + DCl): 172.96; 169.30; 164.62; 153.01; 152.64; 141.31; 138.84; 134.11; 133.09; 130.92; 130.28; 129.79; 127.81; 126.59; 125.51; 115.06; 114.87; 113.70; 112.62; 112.43; 56.43; 45.93; 32.03; 20.44; 14.80; 14.55. HR-ESI-MS: 499.24750 ([*M* + H]$^+$, $C_{28}H_{31}N_6O_3^+$; calc. 499.24522).

3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](4-methylphenyl)amino}-propanoic Acid (**5c**). Yield: 88.7%. White solid. M.p. 246 – 247°. HPLC: 99.2%. ^{1}H-NMR (400 MHz, (D_6) DMSO + DCl): 8.91 (*s*, 1 H); 8.65 (*s*, 1 H); 7.66 (*d*, *J* = 8.8, 2 H); 7.50 (*s*, 1 H); 7.42 (*d*, *J* = 8.3, 1 H); 7.22 (*d*, *J* = 8.4, 1 H); 7.11 – 7.02 (*m*, 4 H); 6.87 (*d*, *J* = 8.9, 2 H); 4.66 (*s*, 2 H); 4.27 (*q*, *J* = 6.8, 2 H); 4.02 (*t*, *J* = 7.5, 2 H); 2.57 – 2.52 (*m*, 2 H); 2.19 (*s*, 3 H); 1.24 (*t*, *J* = 7.1, 3 H). ^{13}C-NMR (100 MHz, (D_6)DMSO + DCl): 173.08; 170.26; 164.72; 154.88; 153.26; 152.97; 141.05; 139.17; 136.43; 135.45; 131.11; 130.32; 130.19; 128.21; 124.04; 119.04; 113.88; 113.32; 112.23; 110.43; 46.87; 32.57; 21.50; 20.89;

15.53; 15.16. HR-ESI-MS: 499.24792 ($[M + H]^+$, $C_{28}H_{31}N_6O_3^+$; calc. 499.24522).

3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2,3-dimethylphenyl)amino}-propanoic Acid (5d). Yield: 87.6%. White solid. M.p. 255 – 256°. HPLC: 97.6%. ^{1}H-NMR (400 MHz, (D_6) DMSO + DCl): 8.97 (*s*, 1 H); 8.74 (*s*, 1 H); 7.68 (*d*, *J* = 8.6, 2 H); 7.52 (*d*, *J* = 8.3, 1 H); 7.49 (*s*, 1 H); 7.26 (*d*, *J* = 8.5, 1 H); 7.00 (*d*, *J* = 6.7, 2 H); 6.97 (*d*, *J* = 7.6, 1 H); 6.87 (*d*, *J* = 8.8, 2 H); 4.74 (*s*, 2 H); 4.35 – 4.28 (*m*, 2 H); 2.63 (*m*, 2 H); 2.13 (*d*, *J* = 13.0, 6 H); 1.26 (*t*, *J* = 7.1, 3 H). ^{13}C-NMR (100 MHz, (D_6) DMSO + DCl): 173.05; 170.02; 164.71; 153.19; 152.99; 143.49; 139.23; 135.19; 131.43; 130.20; 129.38; 128.53; 127.96; 125.80; 124.29; 118.62; 114.02; 113.31; 112.27; 110.67; 56.46; 46.91; 32.59; 21.27; 18.90; 15.08. HR-ESI-MS: 513.26341 ($[M + H]^+$, $C_{29}H_{33}N_6O_3^+$; calc. 513.26087).

3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2,5-dimethylphenyl)amino}-propanoic Acid (5e). Yield: 89.3%. White solid. M.p. 248 – 249°. HPLC: 98.8%. ^{1}H-NMR (400 MHz, (D_6) DMSO + DCl): 8.89 (*s*, 1 H); 8.70 (*s*, 1 H); 7.65 (*d*, *J* = 8.7, 2 H); 7.44 (*s*, 1 H); 7.40 (*d*, *J* = 8.5, 1 H); 7.25 (*d*, *J* = 8.4, 1 H); 7.15 (*s*, 1 H); 7.00 (*d*, *J* = 7.8, 1 H); 6.93 (*d*, *J* = 7.6, 1 H); 6.86 (*d*, *J* = 8.7, 2 H); 4.62 (*s*, 2 H); 4.34 – 4.20 (*m*, 2 H); 4.20 – 4.10 (*m*, 1 H); 3.75 – 3.66 (*m*, 1 H); 2.59 (*t*, *J* = 7.0, 2 H); 2.20 (*s*, 3 H); 2.03 (*s*, 3 H); 1.23 (*t*, *J* = 7.0, 3 H). ^{13}C-NMR (100 MHz, (D_6)DMSO + DCl): 173.10; 170.32; 164.72; 153.44; 152.96; 142.17; 140.79; 136.62; 136.14; 131.94; 131.44; 130.17; 128.85; 123.42; 119.50; 113.62; 113.29; 112.13; 109.83; 46.31; 38.88; 32.40; 20.85; 17.60; 15.29. HR-ESI-MS: 513.26344 ($[M + H]^+$, $C_{29}H_{33}N_6O_3^+$; calc. 513.26087).

3-{[(2-{[(4-Carbamimidoylphenyl)amino]methyl}-1-ethyl-1*H*-benzimidazol-5-yl)carbonyl](2,4-dimethylphenyl)amino}-propanoic Acid (5f). Yield: 91.7%. White solid. M.p. 250 – 251°. HPLC: 97.3%. ^{1}H-NMR (400 MHz, (D_6) DMSO + DCl): 8.94 (*s*, 1 H); 8.71 (*s*, 1 H); 7.67 (*d*, *J* = 8.7, 2 H); 7.48 (*d*, *J* = 12.0, 2 H); 7.27 (*d*, *J* = 8.3, 1 H); 7.14 (*d*, *J* = 7.8, 1 H); 6.94 (*d*, *J* = 10.9, 2 H); 6.87 (*d*, *J* = 8.8, 2 H); 4.70 (*s*, 2 H); 4.36 – 4.25 (*m*, 2 H); 3.71 – 3.56 (*m*, 2 H); 2.59 – 2.54 (*m*, 2 H); 2.13 (*d*, *J* = 23.1, 6 H); 1.26 (*t*, *J* = 7.1, 3 H). ^{13}C-NMR (100 MHz, (D_6)DMSO + DCl): 173.09; 170.36; 164.78; 153.27; 152.96; 139.57; 137.34; 135.56; 134.84; 132.23; 130.94; 130.18; 129.77; 127.99; 123.68; 118.61; 113.93; 113.31; 112.23; 110.31; 45.98; 32.34; 21.50; 20.88; 17.91; 15.16. HR-ESI-MS: 513.26259 ($[M + H]^+$, $C_{29}H_{33}N_6O_3^+$; calc. 513.26087).

Thrombin Assay

The test compounds dissolved in DMSO were added to a soln. of lyophilized human thrombin (5.4 mg/ml) and preincubated for 10 min at 37°. Subsequently, Ac-FVR-AMC (5 μM), a specific fluorogenic thrombin substrate, was added. DMSO was used as negative control in the assay. The dynamic changes of fluorescence intensity were detected by an envision microplate reader (*PerkinElmer*, Waltham Mass, USA) 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 thrombin inhibitor argatroban was used as positive control. The following instrument settings were included: excitation wavelength, 355 nm; emission wavelength, 460 nm. Each well was measured 20 times for every 20 s for 10 min. The change in fluorescence within a predetermined time was measured under these conditions.

The reaction kinetic curve slope (V_{max}) was an activity indicator. The concentration that induced a 50% inhibition of thrombin activity (IC_{50}) was calculated. All measurements were performed in duplicate; the mean values of both determinations are presented.

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