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RESEARCH ARTICLE

Synthesis and Evaluation of *In Vitro* Antiplatelet Aggregation Activities of 2-Methoxy-5-Aminobenzamides

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Abstract: ***Objective*:** According to the principles of drug design, the structures of picotamide and betrixaban were combined to design novel series of 2-methoxy-5-aminobenzamides. A total of twenty new compounds **1a-1t** have been synthesized and evaluated for their antiplatelet aggregation activities *in vitro*.

***Methods*:** In the structural design of target compounds **1a-1t**, the betrixaban was retained group characteristics and the picotamide was retained its 1, 3, 4-substitution position. With 2-methoxybenzoic acid as starting material, compounds **1a-1t** were synthesized after 5 steps of nitration, acylation, ammoniation, reduction and secondary ammoniation. And their antiplatelet aggregation activities *in vitro* were assessed by the Born test with ADP, arachidonic acid and collagen as inducing agents, respectively, and with aspirin and picotamide as two reference drugs.

***Results*:** The compound **1f** (46.14%±0.07) had the highest activity for ADP and its IC_{50} value was 0.17 μM, far better than the two control drugs aspirin (0.44 μM) and picotamide (0.47 μM). The IC_{50} value of four compounds **1i** (0.24 μM), **1j** (0.22 μM), **1r** (0.25 μM) and **1t** (0.24 μM), displayed higher antiplatelet activities *in vitro* for AA than aspirin (0.43 μM) and picotamide (0.34 μM). Evaluation of cytotoxicity activity of the compounds against L929 cells line revealed that at lower concentration of 10 $\mu mol \cdot L^{-1}$, compound **1p** had lower effect on L929 cells, and its cell survival rate (88.24%±4.16) was higher than that (82.35%±4.16) of picotamide.

***Conclusion*:** Novel series of 2-methoxy-5-aminobenzamides has shown higher *in vitro* antiplatelet activities and lower effect on L929 cells at lower concentration.

Keywords: Drug design, 2-methoxy-5-aminobenzamides, picotamide, betrixaban, structural modification, antiplatelet aggregation activities.

1. INTRODUCTION

Thromboembolic diseases is a leading cause of cardiovascular morbidity and death [1-2]. The antiplatelet aggregation drug picotamide (Fig. **1**), as the selective TXA_2 synthetase inhibitor was developed by Smail company and listed for the first time in 1987 in Italy, which can specifically inhibit the synthesis and release of TXA_2 which stimulates the proliferation of vascular endothelial cells, smooth muscle cell migration and vasoconstriction, and promote the release of vasodilatory substances PGI_2, with dual antithrombotic effect [3, 4]. Since 1996, our laboratory has been performed structural modification to picotamide, more than 800 derivatives with structures of aromatic esters, aromatic amides, derivatives with structures of aromatic esters, aromatic amides, aromatic sulfonamides and aromatic hydrazones were synthesized. Among them, some compounds had higher antiplatelet aggregation activities and lower toxicity than the positive control drug picotamide [5-10]. Betrixaban (Fig. **1**) is an oral, highly selective factor Xa inhibitor developed by Portola Pharmaceutical company and the only oral anticoagulant drug for patients with renal insufficiency [11-13], which was approved for listing by the US FDA in June 2017 [14]. In this paper, based on the principle of drug design integration and years of laboratory work, the two side chains on N^1- and N^3- positions of picotamide were modified again, using substituted phenylamino groups instead of N^3- side chain 3-pyridinemethylamino, combining the structures of picotamide and betrixaban, then using substituted benzoylamide groups instead of N^1- side chain 3-pyridinemethylamino, twenty new 2-methoxy-5-aminobenzamides **1a-1t** were designed and synthesized. In the previous structural analogs of picotamide, the 1, 3 – position other

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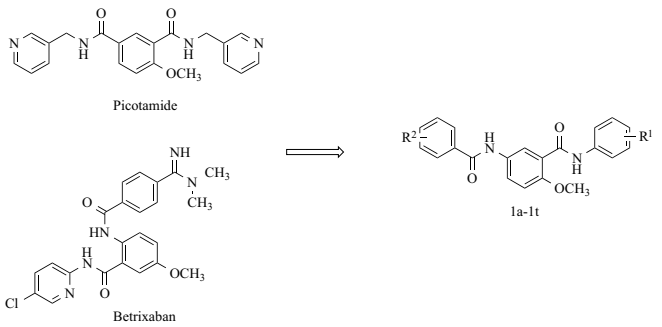


Fig. (1). The design idea of novel target compounds **1a-1t.**

than methoxy were the same two groups, while the corresponding two groups of the target compounds in this study were no longer the same: one was an amide group; the other was aminoacyl group. In contrast, the structures of these 2-methoxy-5-aminobenzamides retained the 1, 3, 4-trisubstituted positional characteristics of picotamide and didn't proceed according to the 1, 2, 4-position substitution position of betrixaban, although the group feature of betrixaban was retained. The structures of compounds **1a-1t** were confirmed by ^{1}H-NMR, IR, MS and ^{13}C-NMR. And their antiplatelet aggregation activities *in vitro* were assessed by the Born test with ADP, AA and collagen as inducing agents and with picotamide and aspirin as two reference drugs. The results have shown that among **1a-1t**, most compounds had different degrees of antiplatelet activities, and two compounds **1b** and **1f** for ADP and three compounds **1i**, **1j** & **1r** for AA displayed more excellent antiplatelet activities *in vitro*.

Referenced to relevant literatures and methods, this paper determined the synthetic route of the target compounds: with 2-methoxybenzoic acid as starting material, followed by nitration and acylation reaction, 2-methoxy-5-nitrobenzoyl chloride (3) was obtained, then ammoniated with substituted anilines to give compounds (4), reduced the nitro group to get compounds (5), and ammoniated with substituted benzoyl chlorides again to obtain the target compounds **1a-1t**. The synthetic routes and chemical structures of the target compounds are shown in Scheme **1**.

2. EXPERIMENTAL

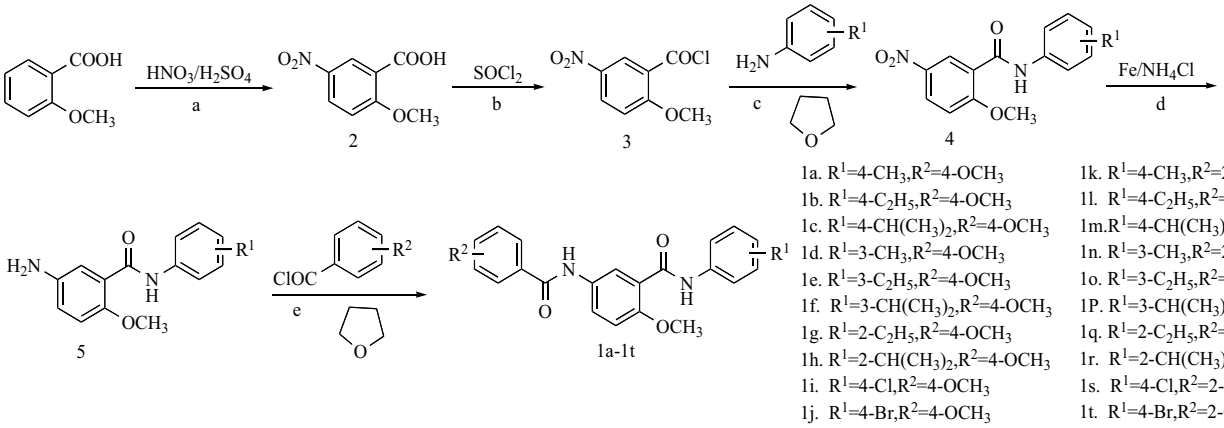
2.1. General

The NMR spectra were recorded on a Bruker ARX-400MHZ spectrometer using tetramethylsilane (TMS) as an internal standard. IR spectra were obtained using an Avatar 370 spectrometer. Mass spectra were recorded on Agilent 6310 Ion Trap and Shimadzu LCMS. Melting points were determined with a X-4 digital display micro melting point tester and the thermometer was uncorrected. Reactions were monitored by TLC on silica gel plates (RSGF 254) using UV light as the visualizing agent.

2.2. Chemistry

2.2.1. Preparation of 2-Methoxy-5-Nitrobenzoic Acid (2)

In a round-bottom flask (100 mL) equipped with a magnetic stirrer, concentrated nitric acid (6 mL) was added in an ice salt bath. When the temperature dropped below 0℃, concentrated sulfuric acid (6 mL) was added dropwise and the temperature was kept at 0-5℃. 2-Methoxybenzoic acid (3.0 g, 20 mmol) was added to the reaction solution and the temperature was kept at 0-8℃. After addition, the reaction mixture was stirred at 0-8℃ for 30 min. The ice salt bath was removed. After stirring at room temperature for 30 min, the system was stirred at 40℃ for another 30 min. After monitoring by thin layer chromatography, the reaction solution was poured into a beaker of crushed ice, stirred, and a pale yellow solid was precipitated. The resulting precipitate was filtrated and washed with water to obtain a white crude product. Recrystallization from ethyl acetate to obtain 2.4 g 2-methoxy-5-nitrobenzoic acid as white crystals. Yield: 62 %.



Reagents and conditions: (**a**) 68%HNO_3, 98%H_2SO_4; (**b**) $SOCl_2$, reflux, 8h; (**c**) THF, $N(CH_2CH_3)_3$, 18%HCl, 5%NaOH, H_2O; (**d**) C_2H_5OH, H_2O, NH_4Cl, Fe, reflux, 3h, CH_2Cl_2, 18%HCl, 20%NaOH, $MgSO_4$; (**e**) THF, $N(CH_2CH_3)_3$, 18%HCl, 5%NaOH, H_2O.

Scheme (1). Synthetic routes and chemical structures of target compounds **1a-1t**.

2.2.2. Preparation of 2-Methoxy-5-Nitrobenzoyl Chloride (3)

In a round-bottom flask (100mL) equipped with a magnetic stirrer, 2-methoxy-5-nitrobenzoic acid (1.1g, 5.6mmol) was added, along with $SOCl_2$ (10mL). The reaction mixture was refluxed and stirred at 80℃ for 8h. The excess $SOCl_2$ was evaporated under reduced pressure to obtain a white solid 1.1g. Yield: 92 %.

2.2.3. Preparation of N- (4-isopropylphenyl)-2-Methoxy-5-Nitrobenzamide (4)

2-Methoxy-5-nitrobenzoyl chloride (1.1g, 5.1 mmol) was placed in a round-bottomed flask (100 mL) and dissolved in tetrahydrofuran (15 mL). 4-Isopropylaniline (0.69 g, 5.1mmol) was dissolved in tetrahydrofuran (15 mL) and then added dropwise in the solution. After addition, the solution was pale yellow and turbid. Triethylamine (0.72 mL) was added and a large amount of white gas was produced. The mixture was stirred at room temperature for 12h. After monitoring by thin layer chromatography, the solvent was evaporated under reduced pressure. The residue was washed with dilute hydrochloric acid, 5 % sodium hydroxide solution and water until neutral in an ice bath , filtered and dried to give a crude yellow product. Recrystallization from ethanol to obtain a white floc 1.3 g. Yield: 81 %.

2.2.4. Preparation of 5-Amino-N- (4-isopropylphenyl)-2-Methoxybenzamide (5)

In a 250mL three-necked flask equipped with a thermometer and a condensation reflux device, N- (4-isopropylphenyl)-2-methoxy-5-nitrobenzamide (1.3 g, 4.1mmol) and ethanol (35 mL) were added. When the temperature was raised to 70 to 80℃, the solution became clear and the solid was completely dissolved. A solution of saturated NH_4Cl (1.6g, 29mmol) was added and then a white solid in the three- neck flask was precipitated. After 0.5 h, the solution also became clear. Fe (0.69 g, 12 mmol) was added and stirred at 80℃ for 3h. After monitoring by thin layer chromatography, filtered, rinsed the filter cake with hot CH_2Cl_2, the filtrate was collected and washed with water three times to retain the organic layer. The organic layer was extracted with dilute hydrochloric acid in an ice bath, a solution of saturated NaCl was added to demulsify and the aqueous phase was retained. 20 % NaOH solution was added to adjust to pH=10, CH_2Cl_2 was added to extract organic layers and then dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure at room temperature to obtain a light gray solid 1.1g. Yield: 92 %.

2.2.5. Preparation of N- (4-isopropylphenyl)-2-Methoxy-5-(4-methoxybenzamido)Benzamide (1c)

4-Methoxybenzoyl chloride (0.63 g, 3.7 mmol) was placed in a round-bottomed flask (100mL) and dissolved in tetrahydrofuran (15 mL). 5-Amino-N- (4-isopropylphenyl)-2-methoxybenzamide (1.1g, 3.7mmol) was dissolved in tetrahydrofuran (15mL) and then added dropwise in the solution. After addition, the solution was brown and turbid. Triethylamine (0.51mL) was added and a large amount of white gas was produced. The mixture was stirred at room temperature for 12 h. After monitoring by thin layer chromatography, the solvent was evaporated under reduced pressure. The residue was washed with dilute hydrochloric acid, 5 % sodium hydroxide solution and water until neutral in an ice bath, filtered and dried to give a crude brown product. Recrystallization from ethanol to obtain a brown needle crystal 0.84 g. Yield: 56 %.

Compounds **1a**, **1b** and **1d-1t** were prepared in the similar manner as above. The physical properties and spectral data of the target compounds **1a-1t** have been shown below.

2.2.6. N- (p-tolyl)-2-Methoxy-5-(4-methoxybenzamido) Benzamide (1a)

Yield: 60 %; m.p: 209.0-210.2°C; MS (m/z): 391.1657 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3336.19 (υ_{NH}), 3302.63 (υ_{NH}), 1671.46 ($\upsilon_{C=O}$), 1636.80 ($\upsilon_{C=O}$), 1605.54, 1532.71, 1504.33, 1457.68, 1315.28, 1253.43 (υ_{C-N}), 1176.75, 1018.35, 817.69; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.84 (s, 1H, -CONH-), 8.40 (dd, *J* = 8.9, 2.8 Hz, 1H, Ar-H), 8.14 (s, 1H, -CONH-), 8.10 (d, *J* = 2.8 Hz, 1H, Ar-H), 7.85 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.53 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.15 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.07 (d, *J* = 9.0 Hz, 1H, Ar-H), 6.93 (d, *J* = 8.5 Hz, 2H, Ar-H), 4.07 (s, 3H, -OCH_3), 3.86 (s, 3H, -OCH_3), 2.33 (s, 3H, -CH_3); ^{13}C NMR (101 MHz, $CDCI_3$) δ 165.54 (C=O), 162.92 (C=O), 162.48, 153.71, 135.66, 134.06, 132.80, 129.58, 129.15, 126.92, 125.77, 124.05, 121.98, 120.74, 113.91, 112.45, 56.72 (-OCH_3), 55.52 (-OCH_3), 21.03 (- CH_3).

2.2.7. N- (4-ethylphenyl)-2-Methoxy-5-(4-methoxybenzamido)Benzamide (1b)

Yield: 61%; m.p: 224.0-224.8°C; MS (m/z): 405.1812$[M+H]^+$; IR (KBr, σ/cm^{-1}): 3340.98 (υ_{NH}), 3290.00 (υ_{NH}), 1672.46 ($\upsilon_{C=O}$), 1638.64 ($\upsilon_{C=O}$), 1605.37, 1542.98, 1505.13, 1458.01, 1410.10, 1315.94, 1253.43 (υ_{C-N}), 1178.18, 1019.13, 837.79; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.84 (s, 1H, -CONH-), 8.40 (dd, *J* = 9.0, 2.8 Hz, 1H, Ar-H), 8.13 (s, 1H, -CONH-), 8.09 (d, *J* = 2.9 Hz, 1H, Ar-H), 7.85 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.56 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.18 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.07 (d, *J* = 9.1 Hz, 1H, Ar-H), 6.93 (d, *J* = 8.8 Hz, 2H, Ar-H), 4.07 (s, 3H, -OCH_3), 3.86 (s, 3H, -OCH_3), 2.63 (q, *J* = 7.6 Hz, 2H, -CH_2-), 1.23 (t, *J* = 7.6 Hz, 3H, -CH_2CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.51 (C=O), 162.88 (C=O), 162.51, 153.74, 140.57, 135.84, 132.77, 129.13, 128.42, 126.92, 125.74, 124.01, 122.01, 120.85, 113.96, 112.50, 56.74 (-OCH_3), 55.54 (-OCH_3), 28.47 (-CH_2-), 15.81 (-CH_3).

2.2.8. N- (4-isopropylphenyl)-2-Methoxy-5-(4-methoxybenzamido)Benzamide (1c)

Yield: 54 %; m.p: 175.4-176.5°C; MS (m/z): 419.1958 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3352.89 (υ_{NH}), 3279.35 (υ_{NH}), 1667.61 ($\upsilon_{C=O}$), 1635.79 ($\upsilon_{C=O}$), 1605.06, 1532.08, 1506.60, 1460.37, 1312.62, 1255.33 (υ_{C-N}),1178.52, 1021.94, 679.98; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.83 (s, 1H, -CONH-), 8.39 (dd, *J* = 9.2, 2.7 Hz, 1H, Ar-H), 8.28 (s, 1H, -CONH-), 8.13 (d, *J* = 2.6 Hz, 1H, Ar-H), 7.84 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.55 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.19 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.06 (d, *J* = 9.0 Hz, 1H, Ar-H), 6.89 (d, *J* = 8.3 Hz, 2H, Ar-H), 4.05 (s, 3H, -OCH_3), 3.84 (s, 3H, -OCH_3), 2.89 (sept, *J* = 6.9 Hz, 1H, -CH-), 1.24 (d, *J* = 6.9 Hz, 6H, -CH (CH_3)$_2$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.55 (C=O), 162.95 (C=O), 162.47, 153.73, 145.20, 135.89, 132.82, 129.18, 126.96, 126.91,

125.82, 124.11, 122.00, 120.88, 113.91, 112.47, 56.73 (-OCH_3), 55.51 (-OCH_3), 33.73 (-CH-), 24.15 (2×-CH_3).

2.2.9. N- (m-tolyl)-2-Methoxy-5-(4-methoxybenzamido) Benzamide (1d)

Yield: 53%; m.p: 176.4-178.3°C; MS (m/z): 391.1665 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3311.39 (υ_{NH}), 1640.06 ($\upsilon_{C=O}$), 1608.63, 1553.27, 1505.67, 1307.50, 1258.45 (υ_{C-N}), 1220.95, 1179.91, 1032.33, 770.80, 678.26; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.84 (s, 1H, -CONH-), 8.48 (s, 1H, -CONH-), 8.37 (d, *J* = 8.5 Hz, 1H, Ar-H), 8.18 (s, 1H, Ar-H), 7.84 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.46 (s, 1H, Ar-H), 7.42 (d, *J* = 8.1 Hz, 1H, Ar-H), 7.20 (t, *J* = 7.7 Hz, 1H, Ar-H), 7.03 (d, *J* = 8.6 Hz, 1H, Ar-H), 6.91 (d, *J* = 7.5 Hz, 1H, Ar-H), 6.84 (d, *J* = 8.0 Hz, 2H, Ar-H), 4.04 (s, 3H, -OCH_3), 3.81 (s, 3H, -OCH_3), 2.33 (s, 3H, -CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.70 (C=O), 163.16 (C=O), 162.26, 153.56, 138.85, 138.02, 132.84, 129.23, 128.80, 126.78, 125.97, 125.20, 124.28, 121.68, 121.28, 117.77, 113.65, 112.20, 56.60 (-OCH_3), 55.39 (-OCH_3), 21.55 (-CH_3).

2.2.10. N- (3-ethylphenyl)-2-Methoxy-5-(4-methoxybenzamido)Benzamide (1e)

Yield: 45 %; m.p: 151.2-152.4°C; MS (m/z): 405.1826$[M+H]^+$; IR (KBr, σ/cm^{-1}): 3345.96 (υ_{NH}), 3278.63(υ_{NH}), 1665.20($\upsilon_{C=O}$), 1636.78 ($\upsilon_{C=O}$), 1607.82, 1558.48, 1502.10, 1300.45, 1253.15(υ_{C-N}), 1212.73, 1177.96, 1020.84, 691.55; ^{1}H NMR (400 MHz, DMSO) δ 10.16 (s, 1H, -CONH-), 10.12 (s, 1H, -CONH-), 8.04 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.99 (s, 1H, Ar-H), 7.96 (d, *J* = 3.6 Hz, 1H, Ar-H), 7.93 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.60 (s, 1H, Ar-H), 7.57 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.25 (t, *J* = 7.8 Hz, 1H, Ar-H), 7.18 (d, *J* = 9.0 Hz, 1H, Ar-H), 7.06 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.95 (d, *J* = 7.4 Hz, 1H, Ar-H), 3.91 (s, 3H, -OCH_3), 3.84 (s, 3H, -OCH_3), 2.60 (q, *J* = 7.6 Hz, 2H, -CH_2-), 1.19 (t, *J* = 7.6 Hz, 3H, -CH_2CH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.58 (C=O), 163.91 (C=O), 161.84, 152.55, 144.29, 138.91, 132.51, 129.47, 128.57, 126.76, 124.32, 124.04, 123.03, 122.08, 119.14, 117.23, 113.56, 112.22, 56.18 (-OCH_3), 55.38 (-OCH_3), 28.26 (-CH_2-), 15.53 (-CH_3).

2.2.11. N- (3-isopropylphenyl)-2-Methoxy-5-(4-methoxy benzamido)Benzamide (1f)

Yield: 54 %; m.p: 168.1-169.2°C; MS (m/z): 419.1964 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3341.85 (υ_{NH}), 1674.65 ($\upsilon_{C=O}$), 1640.01 ($\upsilon_{C=O}$), 1609.93, 1556.01, 1501.39, 1306.17, 1253.11 (υ_{C-N}), 1177.12, 1021.80, 702.67; ^{1}H NMR (400 MHz, DMSO) δ 10.14 (s, 1H, -CONH-), 10.10 (s, 1H, -CONH-), 8.06 – 8.02 (m, 1H, Ar-H), 7.98 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.93 (dd, *J* = 8.8, 2.7 Hz, 1H, Ar-H), 7.62 (s, 1H, Ar-H), 7.57 (d, *J* = 8.1 Hz, 1H, Ar-H), 7.25 (t, *J* = 7.8 Hz, 1H, Ar-H), 7.18 (d, *J* = 9.0 Hz, 1H, Ar-H), 7.06 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.98 (d, *J* = 7.6 Hz, 1H, Ar-H), 3.91 (s, 3H, -OCH_3), 3.84 (s, 3H, -OCH_3), 2.87 (sept, *J* = 6.7 Hz, 1H, -CH-), 1.21 (d, *J* = 6.9 Hz, 6H, -$CH(CH_3)_2$); ^{13}C NMR (101 MHz, DMSO) δ 164.59(C=O), 163.98(C=O), 161.85, 152.53, 149.00, 138.93, 132.49, 129.48, 128.59, 126.76, 124.45, 123.98, 122.02, 121.56, 117.77, 117.39, 113.59, 112.20, 56.18(-OCH_3), 55.41(-OCH_3), 33.51(-CH-), 23.86(2×-CH_3).

2.2.12. N- (2-ethylphenyl)-2-Methoxy-5-(4-methoxybenzamido)Benzamide (1g)

Yield: 61 %; m.p: 219.8-221.0°C; MS (m/z): 405.1827 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3362.82 (υ_{NH}), 3283.69(υ_{NH}), 1642.62 ($\upsilon_{C=O}$), 1605.24, 1535.63, 1500.25, 1456.25, 1305.36, 1254.50 (υ_{C-N}), 1177.80, 1019.50, 755.69; ^{1}H NMR (400 MHz, DMSO) δ 10.20 (s, 1H, -CONH-), 9.94 (s, 1H, -CONH-), 8.30 (d, *J* = 2.7 Hz, 1H, Ar-H), 8.04 (dd, *J* = 9.0, 2.8 Hz, 1H, Ar-H), 8.00 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.94 (d, *J* = 8.0 Hz, 1H, Ar-H), 7.30 – 7.22 (m, 3H, Ar-H), 7.14 (t, *J* = 7.4 Hz, 1H, Ar-H), 7.07 (d, *J* = 8.6 Hz, 2H, Ar-H), 4.01 (s, 3H, -OCH_3), 3.84 (s, 3H, -OCH_3), 2.69 (q, *J* = 7.5 Hz, 2H, -CH_2-), 1.22 (t, *J* = 7.5 Hz, 3H, -CH_2CH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.60(C=O), 162.99(C=O), 161.86, 152.96, 135.89, 135.26, 132.87, 129.51, 128.63, 126.71, 126.20, 124.96, 124.87, 123.50, 123.15, 122.17, 113.57, 112.50, 56.50 (-OCH_3), 55.40 (-OCH_3), 24.10 (-CH_2-), 14.10 (-CH_3).

2.2.13. N- (2-isopropylphenyl)-2-Methoxy-5-(4-methoxybenzamido)Benzamide (1h)

Yield: 49%; m.p: 157.2-158.1°C; MS (m/z): 419.1976 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3378.81 (υ_{NH}), 3287.48 (υ_{NH}), 1667.10 ($\upsilon_{C=O}$), 1637.63 ($\upsilon_{C=O}$), 1606.39, 1533.56, 1502.62, 1449.49, 1301.28, 1253.78 (υ_{C-N}), 1205.78, 1176.24, 1019.16, 759.25; ^{1}H NMR (400 MHz, DMSO) δ 10.19 (s, 1H, -CONH-), 9.90 (s, 1H, -CONH-), 8.27 (d, *J* = 2.8 Hz, 1H, Ar-H), 8.04 (dd, *J* = 9.0, 2.8 Hz, 1H, Ar-H), 8.00 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.76 (dd, *J* = 7.3, 2.0 Hz, 1H, Ar-H), 7.36 (dd, *J* = 7.1, 2.2 Hz, 1H, Ar-H), 7.27 – 7.18 (m, 3H, Ar-H), 7.06 (d, *J* = 8.9 Hz, 2H, Ar-H), 4.00 (s, 3H, -OCH_3), 3.84 (s, 3H, -OCH_3), 3.20 (sept, *J* = 6.8 Hz, 1H, -CH-), 1.24 (d, *J* = 6.8 Hz, 6H, -$CH(CH_3)_2$); ^{13}C NMR (101 MHz, DMSO) δ 164.54 (C=O), 163.28 (C=O), 161.81, 152.92, 140.70, 134.99, 132.78, 129.43, 126.70, 125.79, 125.39, 125.26, 124.79, 124.74, 123.04, 122.41, 113.51, 112.42, 56.44 (-OCH_3), 55.33 (-OCH_3), 27.35 (-CH-), 22.79 (2×-CH_3).

2.2.14. N- (4-chlorophenyl)-2-methoxy-5- (4-methoxybenzamido) Benzamide (1i)

Yield: 39 %; m.p: 178.3-179.2°C; MS (m/z): 411.1105 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3314.08 (υ_{NH}), 1674.56 ($\upsilon_{C=O}$), 1638.31 ($\upsilon_{C=O}$), 1595.72, 1541.62, 1499.36, 1457.78, 1412.23, 1314.65, 1254.16 (υ_{C-N}), 1174.11, 1014.35, 828.28; ^{1}H NMR (400 MHz, DMSO) δ 10.28 (s, 1H, -CONH-), 10.14 (s, 1H, -CONH-), 8.02 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.98 (s, 1H, Ar-H), 7.95 (d, *J* = 9.5 Hz, 2H, Ar-H), 7.79 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.41 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.18 (d, *J* = 9.0 Hz, 1H, Ar-H), 7.06 (d, *J* = 8.8 Hz, 2H, Ar-H), 3.90 (s, 3H, -OCH_3), 3.84 (s, 3H, -OCH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.62 (C=O), 164.29 (C=O), 161.88, 152.51, 137.94, 132.52, 129.51, 128.63, 127.12, 126.73, 124.25, 124.14, 121.94, 121.29, 113.61, 112.23, 56.16(-OCH_3), 55.43(-OCH_3).

2.2.15. N- (4-bromophenyl)-2-Methoxy-5-(4-methoxybenzamido)Benzamide (1j)

Yield: 58 %; m.p: 209.2-210.8°C; MS (m/z): 457.0584 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3324.67 (υ_{NH}), 1672.83 ($\upsilon_{C=O}$), 1640.70 ($\upsilon_{C=O}$), 1595.73, 1537.46, 1502.12, 1408.95, 1310.41, 1252.16 (υ_{C-N}), 1176.92, 1017.75, 819.22; ^{1}H NMR (400 MHz, DMSO) δ 10.27 (s, 1H, -CONH-), 10.14 (s, 1H, -

CONH-), 8.02 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.98 (s, 1H, Ar-H), 7.97 – 7.93 (m, 2H, Ar-H), 7.73 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.53 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.18 (d, *J* = 9.0 Hz, 1H, Ar-H), 7.06 (d, *J* = 8.8 Hz, 2H, Ar-H), 3.89 (s, 3H,-OCH_3), 3.84 (s, 3H, -OCH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.60 (C=O), 164.29 (C=O), 161.87, 152.50, 138.33, 132.50, 131.52, 129.49, 126.72, 124.23, 124.14, 121.92, 121.65, 115.14, 113.59, 112.23, 56.16 (-OCH_3), 55.41 (-OCH_3).

2.2.16. N- (p-tolyl)-2-Methoxy-5-(2-methoxyben zamido) Benzamide (1k)

Yield: 63 %; m.p: 172.8-174.3°C; MS (m/z): 391.1666 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3349.80 (υ_{NH}), 1661.16 ($\upsilon_{C=O}$), 1600.86, 1541.18, 1489.32, 1312.62, 1229.88 (υ_{C-N}), 1177.13, 1016.58, 747.54; ^{1}H NMR (400 MHz, DMSO) δ 10.14 (s, 1H, -CONH-), 10.10 (s, 1H, -CONH-), 8.00 (s, 1H, Ar-H), 7.88 (dd, *J* = 8.9, 2.7 Hz, 1H, Ar-H), 7.63 (d, *J* = 7.9 Hz, 3H, Ar-H), 7.50 (t, *J* = 7.8 Hz, 1H, Ar-H), 7.19 – 7.13 (m, 4H, Ar-H), 7.06 (t, *J* = 7.4 Hz, 1H, Ar-H), 3.89 (s, 6H, 2×-OCH_3), 2.28 (s, 3H, -CH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.34 (C=O), 163.94 (C=O), 156.49, 152.50, 136.57, 132.53, 132.32, 132.03, 129.70, 129.15, 124.92, 124.80, 123.27, 121.31, 120.49, 119.71, 112.27, 111.96, 56.22 (-OCH_3), 55.94 (-OCH_3), 20.58 (-CH_3).

2.2.17. N- (4-ethylphenyl)-2-Methoxy-5-(2-methoxybenza mido)Benzamide (1l)

Yield: 64 %; m.p: 174.0-175.3°C; MS (m/z): 405.1812 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3345.14 (υ_{NH}), 1660.02($\upsilon_{C=O}$), 1601.13, 1545.80, 1491.34, 1316.34, 1288.40, 1232.99 (υ_{C-N}), 1178.99, 1017.94, 752.28; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.93 (s, 1H, -CONH-), 9.88 (s, 1H, -CONH-), 8.54 (dd, *J* = 8.8, 2.8 Hz, 1H, Ar-H), 8.31 (d, *J* = 7.6 Hz, 1H, Ar-H), 7.99 (d, *J* = 2.8 Hz, 1H, Ar-H), 7.60 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.52 (t, *J* = 7.7 Hz, 1H, Ar-H), 7.22 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.15 (t, *J* = 7.6 Hz, 1H, Ar-H), 7.07 (t, *J* = 8.2 Hz, 2H, Ar-H), 4.09 (s, 3H, -OCH_3), 4.08 (s, 3H, -OCH_3), 2.66 (q, *J* = 7.6 Hz, 2H, -CH_2-), 1.25 (t, *J* = 7.6 Hz, 3H, -CH_2CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.45 (C=O), 162.81 (C=O), 157.47, 153.73, 140.52, 136.05, 133.45, 132.90, 132.63, 128.46, 125.88, 123.90, 122.05, 121.74, 121.65, 120.71, 112.59, 111.66, 56.78 (-OCH_3), 56.42 (-OCH_3), 28.51 (-CH_2-), 15.83 (-CH_3).

2.2.18. N- (4-isopropylphenyl)-2-Methoxy-5- (2-metho xybenzamido)Benzamide (1m)

Yield: 62 %; m.p: 184.5-185.7°C; MS (m/z): 419.1964 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3342.61 (υ_{NH}), 1660.30 ($\upsilon_{C=O}$), 1600.27, 1546.39, 1490.59, 1316.37, 1289.40, 1232.71 (υ_{C-N}), 1178.14, 1017.63, 753.60; ^{1}H NMR (400 MHz, DMSO) δ 10.14 (s, 1H, -CONH-), 10.10 (s, 1H, -CONH-), 7.99 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.88 (dd, *J* = 9.0, 2.7 Hz, 1H, Ar-H), 7.63 (t, *J* = 7.1 Hz, 3H, Ar-H), 7.50 (t, *J* = 7.9 Hz, 1H, Ar-H), 7.21 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.17 (dd, *J* = 8.7, 5.4 Hz, 2H, Ar-H), 7.06 (t, *J* = 7.5 Hz, 1H, Ar-H), 3.89 (s, 3H, -OCH_3), 3.89 (s, 3H, -OCH_3), 2.86 (sept, *J* = 6.9 Hz, 1H, -CH-), 1.19 (d, *J* = 6.8 Hz, 6H, -$CH(CH_3)_2$); ^{13}C NMR (101 MHz, DMSO) δ 164.20(C=O), 163.79(C=O), 156.46, 152.50, 143.64, 136.70, 132.24, 131.94, 129.65, 126.35, 124.79, 124.68, 123.30, 121.35, 120.41, 119.81, 112.29, 111.94, 56.17 (-OCH_3), 55.86 (-OCH_3), 32.87 (-CH-), 23.92(2×-CH_3).

2.2.19. N- (m-tolyl)-2-Methoxy-5-(2-methoxybenz amido)Benzamide (1n)

Yield: 67%; m.p: 178.6-180.0°C; MS (m/z): 391.1657 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3334.32 (υ_{NH}), 1660.09 ($\upsilon_{C=O}$), 1607.75, 1550.53, 1492.69, 1312.74, 1235.16 (υ_{C-N}), 1179.57, 1020.94, 774.44; ^{1}H NMR (400 MHz, DMSO) δ 10.13 (s, 1H, -CONH-), 10.09 (s, 1H, -CONH-), 8.00 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.88 (dd, *J* = 8.9, 2.8 Hz, 1H, Ar-H), 7.63 (dd, *J* = 7.6, 1.8 Hz, 1H, Ar-H), 7.58 (s, 1H, Ar-H), 7.56 – 7.47 (m, 2H, Ar-H), 7.22 (t, *J* = 7.8 Hz, 1H, Ar-H), 7.17 (dd, *J* = 8.7, 3.7 Hz, 2H, Ar-H), 7.06 (t, *J* = 7.5 Hz, 1H, Ar-H), 6.91 (d, *J* = 7.5 Hz, 1H, Ar-H), 3.90 (s, 6H, 2×-OCH_3), 2.31 (s, 3H, -CH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.15(C=O), 163.86 (C=O), 156.46, 152.52, 138.81, 137.83, 132.23, 131.92, 129.65, 128.45, 124.72, 124.58, 124.18, 123.38, 121.38, 120.40, 120.20, 116.90, 112.31, 111.94, 56.18 (-OCH_3), 55.85 (-OCH_3), 21.11 (-CH_3).

2.2.20. N- (3-ethylphenyl)-2-Methoxy-5- (2-methoxybenz amido)Benzamide (1o)

Yield: 59%; m.p: 160.2-161.5°C; MS (m/z): 405.1830 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3344.62 (υ_{NH}), 1664.15 ($\upsilon_{C=O}$), 1594.57, 1551.09, 1494.95, 1281.27, 1235.48 (υ_{C-N}), 1177.47, 1015.05, 749.21, 640.54; ^{1}H NMR (400 MHz, DMSO) δ 10.13 (s, 1H, -CONH-), 10.11 (s, 1H, -CONH-), 8.00 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.88 (dd, *J* = 9.0, 2.7 Hz, 1H, Ar-H), 7.63 (dd, *J* = 7.5, 1.8 Hz, 1H, Ar-H), 7.61 (s, 1H, Ar-H), 7.56 (d, *J* = 8.1 Hz, 1H, Ar-H), 7.53 – 7.47 (m, 1H, Ar-H), 7.24 (t, *J* = 7.8 Hz, 1H, Ar-H), 7.18 (d, *J* = 3.6 Hz, 1H, Ar-H), 7.16 (d, *J* = 4.3 Hz, 1H, Ar-H), 7.06 (t, *J* = 7.5 Hz, 1H, Ar-H), 6.95 (d, *J* = 7.5 Hz, 1H, Ar-H), 3.90 (s, 6H, 2×-OCH_3), 2.60 (q, *J* = 7.6 Hz, 2H, -CH_2-), 1.19 (t, *J* = 7.6 Hz, 3H, -CH_2CH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.19 (C=O), 163.95 (C=O), 156.47, 152.51, 144.28, 138.93, 132.24, 131.95, 129.66, 128.55, 124.77, 124.68, 123.33, 123.01, 121.34, 120.42, 119.08, 117.17, 112.29, 111.95, 56.18 (-OCH_3), 55.86 (-OCH_3), 28.25 (-CH_2-), 15.52 (-CH_3).

2.2.21. N- (3-isopropylphenyl)-2-Methoxy-5-(2-methoxyben zamido)Benzamide (1p)

Yield: 55 %; m.p: 160.4-161.3°C; MS (m/z): 419.1977 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3351.36 (υ_{NH}), 1663.76 ($\upsilon_{C=O}$), 1594.12, 1548.84, 1497.18, 1439.28, 1276.46, 1235.45 (υ_{C-N}), 1179.35, 1014.93, 753.59, 650.37; ^{1}H NMR (400 MHz, DMSO) δ 10.12 (s, 1H, -CONH-), 10.11 (s, 1H, -CONH-), 7.99 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.88 (dd, *J* = 8.9, 2.7 Hz, 1H, Ar-H), 7.66 – 7.62 (m, 2H, Ar-H), 7.56 (dd, *J* = 7.8, 2.1 Hz, 1H, Ar-H), 7.53 – 7.47 (m, 1H, Ar-H), 7.25 (t, *J* = 7.9 Hz, 1H, Ar-H), 7.18 (d, *J* = 4.1 Hz, 1H, Ar-H), 7.16 (d, *J* = 4.8 Hz, 1H, Ar-H), 7.06 (t, *J* = 7.5 Hz, 1H, Ar-H), 6.98 (d, *J* = 7.6 Hz, 1H, Ar-H), 3.90 (s, 6H, 2×-OCH_3), 2.88 (sept, *J* = 6.9 Hz, 1H, -CH-), 1.21 (d, *J* = 6.9 Hz, 6H, -$CH(CH_3)_2$); ^{13}C NMR (101 MHz, DMSO) δ 164.19 (C=O), 163.99 (C=O), 156.46, 152.49, 148.97, 138.93, 132.23, 131.96, 129.66, 128.54, 124.78, 123.28, 121.54, 121.30, 120.43, 117.70, 117.32, 112.28, 111.95, 56.17 (-OCH_3), 55.87 (-OCH_3), 33.48 (-CH-), 23.82 (2×-CH_3).

2.2.22. N- (2-ethylphenyl)-2-Methoxy-5- (2-methoxybenzamido)Benzamide (1q)

Yield: 45 %; m.p: 190.7-191.8°C; MS (m/z): 405.1808 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3363.94 (υ_{NH}), 3335.67(υ_{NH}), 1672.21 ($\upsilon_{C=O}$), 1591.13, 1548.04, 1499.32, 1450.02, 1409.42, 1285.35, 1235.62 (υ_{C-N}), 1176.43, 1014.74, 751.93, 643.77; ^{1}H NMR (400 MHz, DMSO) δ 10.17 (s, 1H, -CONH-), 9.92 (s, 1H, -CONH-), 8.28 (d, *J* = 2.8 Hz, 1H, Ar-H), 7.96 – 7.90 (m, 2H, Ar-H), 7.63 (dd, *J* = 7.6, 1.8 Hz, 1H, Ar-H), 7.50 (t, *J* = 7.1 Hz, 1H, Ar-H), 7.28 (d, *J* = 8.1 Hz, 1H, Ar-H), 7.24 (t, *J* = 8.1 Hz, 2H, Ar-H), 7.18 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.14 (t, *J* = 6.7 Hz, 1H, Ar-H), 7.06 (t, *J* = 7.4 Hz, 1H, Ar-H), 4.00 (s, 3H, -OCH_3), 3.90 (s, 3H, -OCH_3), 2.69 (q, *J* = 7.5 Hz, 2H, -CH_2-), 1.22 (t, *J* = 7.5 Hz, 3H, -CH_2CH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.28 (C=O), 162.97 (C=O), 156.45, 152.95, 135.82, 135.34, 132.56, 131.90, 129.61, 128.58, 126.13, 124.86, 124.33, 123.56, 122.49, 122.44, 120.38, 112.57, 111.94, 56.49 (-OCH_3), 55.85 (-OCH_3), 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.90, 24.04 (-CH_2-), 14.06 (-CH_3).

2.2.23. N- (2-isopropylphenyl)-2-Methoxy-5- (2-methoxy ben zamido)Benzamide (1r)

Yield: 55 %; m.p: 181.0-182.5°C; MS (m/z): 419.1988 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3349.28 (υ_{NH}), 1662.77 ($\upsilon_{C=O}$), 1595.10, 1556.97, 1535.69, 1491.61, 1449.20, 1301.21, 1231.63 (υ_{C-N}), 1175.87, 1018.15, 747.60, 628.00; ^{1}H NMR (400 MHz, DMSO) δ 10.16 (s, 1H, -CONH-), 9.88 (s, 1H, -CONH-), 8.24 (d, *J* = 2.8 Hz, 1H, Ar-H), 7.93 (dd, *J* = 8.9, 2.8 Hz, 1H, Ar-H), 7.74 (dd, *J* = 7.4, 2.1 Hz, 1H, Ar-H), 7.63 (dd, *J* = 7.5, 1.8 Hz, 1H, Ar-H), 7.53 – 7.47 (m, 1H, Ar-H), 7.36 (dd, *J* = 7.0, 2.4 Hz, 1H, Ar-H), 7.24 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.21 – 7.16 (m, 2H, Ar-H), 7.06 (t, *J* = 7.4 Hz, 1H, Ar-H), 3.99 (s, 3H, -OCH_3), 3.90 (s, 3H, -OCH_3), 3.20 (sept, *J* = 6.8 Hz, 1H, -CH-), 1.24 (d, *J* = 6.9 Hz, 6H, -$CH(CH_3)_2$); ^{13}C NMR (101 MHz, DMSO) δ 164.28 (C=O), 163.34 (C=O), 156.46, 152.94, 140.83, 134.99, 132.52, 131.90, 129.61, 125.82, 125.46, 125.30, 124.86, 124.19, 122.71, 122.39, 120.39, 112.53, 111.93, 56.47 (-OCH_3), 55.85 (-OCH_3), 27.36 (-CH-), 22.84 (2×-CH_3).

2.2.24. N- (4-chlorophenyl)-2-Methoxy-5- (2-methoxybenzamido)Benzamide (1s)

Yield: 67 %; m.p: 199.6-200.8°C; MS (m/z): 411.1110 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3355.93 (υ_{NH}), 3322.93 (υ_{NH}), 1666.58 ($\upsilon_{C=O}$), 1601.65, 1548.47, 1495.99, 1315.14, 1231.94 (υ_{C-N}), 1178.52, 1013.53, 823.51, 749.24; ^{1}H NMR (400 MHz, DMSO) δ 10.29 (s, 1H, -CONH-), 10.12 (s, 1H, -CONH-), 7.98 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.88 (dd, *J* = 8.9, 2.7 Hz, 1H, Ar-H), 7.78 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.63 (dd, *J* = 7.6, 1.8 Hz, 1H, Ar-H), 7.50 (t, *J* = 7.8 Hz, 1H, Ar-H), 7.40 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.18 (d, *J* = 2.4 Hz, 1H, Ar-H), 7.16 (d, *J* = 3.0 Hz, 1H, Ar-H), 7.06 (t, *J* = 7.5 Hz, 1H, Ar-H), 3.90 (s, 3H, -OCH_3), 3.88 (s, 3H, -OCH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.32 (C=O), 164.25 (C=O), 156.46, 152.45, 137.94, 132.23, 131.99, 129.65, 128.60, 127.07, 124.79, 124.61, 123.40, 121.23, 120.44, 112.30, 111.96, 56.15 (-OCH_3), 55.88 (-OCH_3).

2.2.25. N- (4-bromophenyl)-2-Methoxy-5- (2-methoxybenzamido)Benzamide (1t)

Yield: 68 %; m.p: 209.0-210.0°C; MS (m/z): 457.0593 $[M+H]^+$; IR (KBr, σ/cm^{-1}): 3355.24 (υ_{NH}), 3321.31 (υ_{NH}), 1665.97 ($\upsilon_{C=O}$), 1600.46, 1545.19, 1494.27, 1314.41, 1232.06(υ_{C-N}), 1178.70, 1013.23, 821.53, 749.71; ^{1}H NMR (400 MHz, DMSO) δ 10.29 (s, 1H, -CONH-), 10.12 (s, 1H, -CONH-), 7.98 (d, *J* = 2.7 Hz, 1H, Ar-H), 7.88 (dd, *J* = 8.9, 2.8 Hz, 1H, Ar-H), 7.73 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.63 (dd, *J* = 7.6, 1.8 Hz, 1H, Ar-H), 7.53 (d, *J* = 8.9 Hz, 2H, Ar-H), 7.49 (d, *J* = 7.3 Hz, 1H, Ar-H), 7.17 (dd, *J* = 8.7, 3.0 Hz, 2H, Ar-H), 7.06 (t, *J* = 7.4 Hz, 1H, Ar-H), 3.90 (s, 3H, -OCH_3), 3.88 (s, 3H, -OCH_3); ^{13}C NMR (101 MHz, DMSO) δ 164.34 (C=O), 164.25 (C=O), 156.46, 152.45, 138.34, 132.23, 131.99, 131.50, 129.65, 124.79, 124.61, 123.41, 121.60, 121.15, 120.44, 115.12, 112.30, 111.96, 56.15 (-OCH_3), 55.88 (-OCH_3).

3. MATERIALS AND METHODS

3.1. *In Vitro Platelet Aggregation Assay*

The effect of the target compounds **1a-1t** on platelet aggregation in rabbits was determined by Born test [15-16]. The venous blood gathering from the ear vein of the rabbit was collected into buffered sodium citrate (3.8 %, w/v) as the anticoagulant at a ratio of 9:1 (v/v), followed by centrifugation at room temperature for 10 min (500–800 rpm) and absorbed the supernatant to obtain the platelet-rich plasma (PRP). The residual blood in the lower layer was centrifuged at 3000rpm for 10 min, the supernatant was aspirated with a pipettor and platelet-poor plasma (ppp) was obtained. Then, the platelet-poor plasma (ppp) was used to set zero of platelet aggregation analyzer. A solution of the target compounds (1.30 μmol·L^{-1}) in DMSO (5 μL) was added into PRP (200 μL), and the equal volume of DMSO was added to the solvent control group. After incubating for 2min, the platelet aggregation was assessed with 5 mmol·L^{-1} ADP (20 μL), 20 μmol·L^{-1} AA (20 μL) and 1 mg·mL^{-1} collagen (20 μL) as inducing agents respectively. The corresponding data of inhibition rates for ADP, AA and collagen of the compounds obtained after calculation and transformation according to the following formula. And some of the compounds with higher antiplatelet activities for ADP and AA were selected and their IC_{50} calculations have been performed. Inhibitory Concentration (IC_{50}) value was determined as the concentration of derivative (μM) able to inhibit 50 % of aggregation induced by ADP and AA.

$$Aggregation\% = \frac{PPP - PRP}{PPP} * 100\%$$

$$Inhibition\% = \frac{S - D}{S} * 100\%$$

S: the platelet aggregation in the presence of solvent.

D: the platelet aggregation in the presence of test compounds.

3.2. *In Vitro* Cytotoxicity Assay

Mouse fibroblast cells (L929) were chosen to evaluate the *in vitro* cytotoxicity of the target compounds via Cell Counting Kit-8 (CCK-8) assays [17]. L929 cells were seeded at a density of 1×10^4 cells per well on 96-well microplates. The cells in 10% fetal bovine serum (US) containing culture medium (100 μL of medium per well) were cultivated in a humidified 5% carbon dioxide atmosphere at 37°C for 24h to allow cells to attach [18]. Target compounds were formulated with DMSO to 10 μmol·L^{-1} and 100μmol L^{-1} concentrations and then added to the cells. After incubation at 37°C for 48 h, the medium was removed and the cells were washed with PBS, 100 μL of fresh culture medium was added per well. Then, the CCK-8 solution was added to the 96-well plates at 10 μL per well and incubated for 2 h, and the absorbance at 450 nm was measured on a microplate reader [19]. Non-treated cells were used as a control and cell viability was calculated as follows [20, 21].

$$\text{Cell Viability}(\%) = \frac{\text{Abs(test cell)}}{\text{Abs(controlled cell)}} * 100\%$$

4. RESULTS AND DISCUSSION

4.1. *In vitro* Platelet Aggregation Assay

The effect of the target compounds **1a-1t** on platelet aggregation in rabbits was determined by Born test. The corresponding data of inhibition rates for ADP, AA and collagen of the compounds are shown in Table **1**. Some of the compounds with higher antiplatelet activities for ADP and AA were selected and their IC_{50} calculations are shown in Table **1.**

It can be seen that two compounds (**1f**, **1b**) were able to inhibit the platelet aggregation induced by ADP in (46.14%±0.07), (40.07%±0.10), superior to picotamide (38.52%±0.12). The other two compounds **1i** (38.11%±0.23) and **1q** (37.89%±0.09) were slightly better but still lower than picotamide (38.52%±0.12). And compound **1f** displayed the highest activity for ADP and its IC_{50} value (0.17 μM) was far lower than two positive control drugs aspirin (0.44 μM) and picotamide (0.47 μM). At the concentration of 1.30 μmol·L^{-1}, being the inhibition rates of (42.98%±0.17), (37.93%±0.13) and (37.62%±0.13) respectively, three compounds **1r**, **1i** and **1j** displayed higher antiplatelet activities *in vitro* for AA than picotamide (37.29%±0.09). And the other two compounds **1t** (36.23%±0.13) and **1h** (35.02%±0.14) also had slightly better antiplatelet aggregation activities. Among them, compound **1j** had the lowest IC_{50} value (0.22 μM) and was far lower than two positive control drugs aspirin (0.43 μM) and picotamide (0.34 μM). All of the target compounds **1a-1t** had different degrees of antiplatelet activities for collagen-induced platelet aggregation. And four compounds **1f** (37.33%±0.11), **1m** (36.43%±0.07), **1l** (34.60%±0.24) and **1p** (34.53%±0.25) had slightly better antiplatelet aggregation activities but still lower than aspirin (39.78%±0.09) and picotamide (41.87%±0.10).

With a view to the structure-activity relationships (SAR) of the target compounds, some conclusions might be summarized as follows:

1. The comparison of inhibition rates of among compounds **1a-1t** for ADP is shown in Fig. **2**.

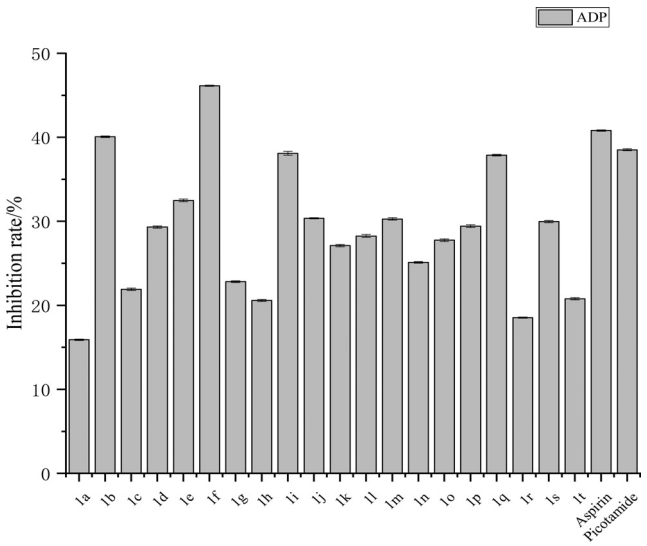


Fig. (2). The inhibition rates of compounds **1a-1t** for ADP.

① When the methoxy group was introduced into the para-position of the N^5- side chain, the activity order of the target compounds with different positions (ortho-, meta- and para-) of alkyl mono-substituted phenyl groups was: para-CH_3 (**1a**) < para- $CH(CH_3)_2$ (**1c**) < para-C_2H_5 (**1b**); meta-CH_3 (**1d**) < meta-C_2H_5 (**1e**) < meta-$CH(CH_3)_2$ (**1f**); ortho-$CH(CH_3)_2$ (**1h**) < ortho-C_2H_5 (**1g**), which indicated that the moderate volume of the para-position substituent groups was beneficial to the increase of activity and might also be applied to the ortho-position substituent groups. However, the antiplatelet activities tend to increase with the increasing volume of the meta-position substituent groups.

② When the methoxy group was introduced into the ortho-position of the N^5- side chain, the activity order of the target compounds with different positions (ortho-, meta- and para-) of alkyl mono-substituted phenyl groups was: para-CH_3 (**1k**) < para-C_2H_5 (**1l**) < para-CH $(CH_3)_2$ (**1m**); meta-CH_3 (**1n**) < meta-C_2H_5 (**1o**) < meta-$CH(CH_3)_2$ (**1p**); ortho-$CH(CH_3)_2$ (**1r**) < ortho-C_2H_5 (**1q**), which indicated that the antiplatelet activities tend to increase with the increasing volume of the para- and meta-position substituent groups and the moderate volume of the ortho-position substituent groups might be beneficial to the increase of activity.

③ No matter what methoxy group was introduced into the para- or ortho-position of the N^5- side chain, the halogen groups were introduced into the para-position, and the order of inhibition rates always was: para-Br (**1j**) < para-Cl (**1i**), para-Br (**1t**) < para-Cl (**1s**). Thus, in general, chlorine-substituted compounds were more potent than the bromine-substituted analogs.

Table 1. Effect of compounds 1a-1t on platelet aggregation induced by ADP, AA and collagen.

Compound	Administration dose/(μmol·L^{-1})	Inhibition rate/%(ADP)	IC_{50}/μM (ADP)	Inhibition rate/%(AA)	IC_{50}/μM (AA)	Inhibition rate/%(collagen)
Control	-	-	-	-	-	-
1a	1.30	15.92%±0.09	-	25.84%±0.19	-	31.02%±0.09
1b	1.30	40.07%±0.10	0.20	27.51%±0.13	-	24.94%±0.11
1c	1.30	21.92%±0.14	-	33.18%±0.14	-	32.19%±0.12
1d	1.30	29.33%±0.13	-	33.19%±0.11	-	15.25%±0.09
1e	1.30	32.50%±0.15	-	24.78%±0.10	-	23.77%±0.11
1f	1.30	46.14%±0.07	0.17	23.15%±0.11	-	37.33%±0.11
1g	1.30	22.83%±0.10	-	21.47%±0.12	-	18.58%±0.17
1h	1.30	20.60%±0.12	-	35.02%±0.14	0.36	16.83%±0.10
1i	1.30	38.11%±0.23	0.41	37.93%±0.13	0.24	15.69%±0.10
1j	1.30	30.38%±0.08	-	37.62%±0.13	0.22	21.92%±0.12
1k	1.30	27.12%±0.14	-	24.40%±0.10	-	22.40%±0.13
1l	1.30	28.27%±0.16	-	19.32%±0.06	-	34.60%±0.24
1m	1.30	30.29%±0.15	-	30.38%±0.12	-	36.43%±0.07
1n	1.30	25.13%±0.10	-	25.48%±0.09	-	20.68%±0.10
1o	1.30	27.77%±0.15	-	28.04%±0.13	-	28.43%±0.23
1p	1.30	29.43%±0.16	-	23.41%±0.10	-	34.53%±0.25
1q	1.30	37.89%±0.09	0.20	28.58%±0.14	-	29.42%±0.16
1r	1.30	18.55%±0.07*	-	42.98%±0.17	0.25	24.65%±0.15**
1s	1.30	29.98%±0.14	-	28.95%±0.14	-	25.73%±0.13
1t	1.30	20.80%±0.14*	-	36.23%±0.13	0.24	29.51%±0.15
Aspirin	1.30	40.82%±0.08	0.44	39.34%±0.09	0.43	39.78%±0.09
Picotamide	1.30	38.52%±0.12	0.47	37.29%±0.09	0.34	41.87%±0.10

* $P < 0.05$ VS Picotamide; ** $P < 0.01$ VS Picotamide

2. For AA-induced platelet aggregation, the comparison of inhibition rates of among compounds **1a-1t** was shown in Fig. (**3**).

When the methoxy group was introduced into the para-position of the N^5- side chain, introduced the alkyl groups into the para or meta-position, the order of inhibition rates was: para-CH_3 (**1a**) < para-C_2H_5 (**1b**) < para-CH $(CH_3)_2$ (**1c**); meta-$CH(CH_3)_2$ (**1f**) < meta-C_2H_5 (**1e**) < meta-CH_3 (**1d**), which indicated that the antiplatelet activities tend to increase with the increasing volume of the para-position substituent groups and the meta-position substituent groups were just opposite. The halogen groups were introduced into the para-position, and the order of inhibition rates was: picotamide < para-Br (**1j**) < para-Cl (**1i**). Thus, halogen-substituted compounds tend to increase activity. Whatever methoxy group was introduced into the para- or ortho-position of the N^5- side chain, the alkyl groups were introduced into the ortho-position, the order of inhibition rates always was: ortho-C_2H_5 (**1g**) < ortho-CH $(CH_3)_2$ (**1h**); ortho-C_2H_5 (**1q**) < ortho-$CH(CH_3)_2$ (**1r**), which indicated that isopropyl-substituted compounds tend to increase the activity.

3. Highly active compounds with inhibition rates for ADP and AA were selected and IC_{50} calculations were performed. Compounds **1b**, **1f**, **1i** and **1q** had lower IC_{50} value than two positive control drugs aspirin (0.44 μM) and picotamide (0.47 μM) induced by ADP. Compound **1f** had the lowest IC_{50} value (0.17 μM). Compounds **1i**, **1j**, **1r** and **1t** had lower IC_{50} value than two positive control drugs aspirin (0.43 μM) and picotamide (0.34 μM) induced by AA. Among them, compound **1j** had the lowest IC_{50} value (0.22 μM). Compound **1h** had lower IC_{50} value than positive control drug aspirin (0.43 μM).

4. For collagen-induced platelet aggregation, the comparison of inhibition rates of among compounds **1a-1t** was shown in Fig. (**4**).

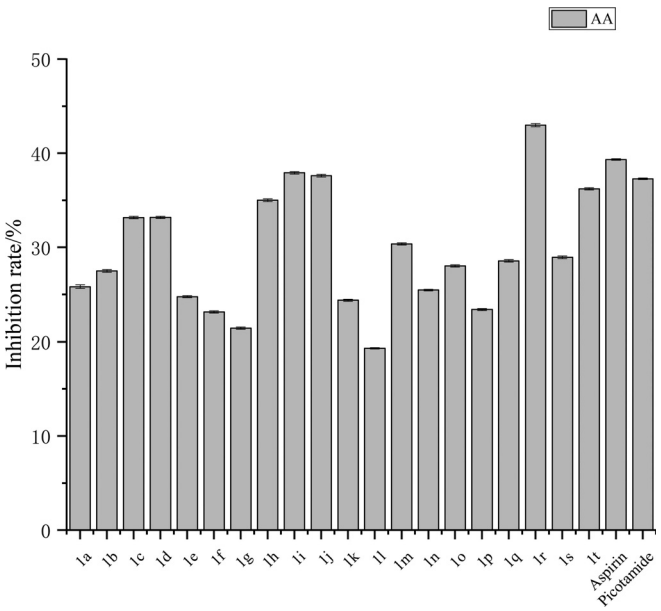


Fig. (3). The inhibition rates of compounds **1a-1t** for AA.

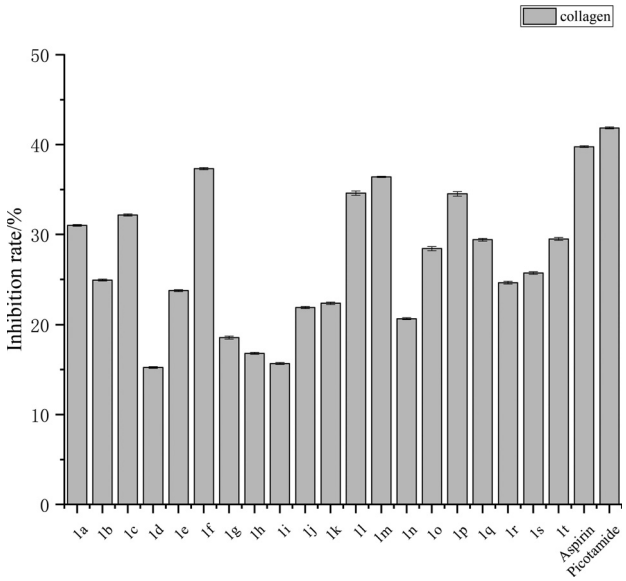


Fig. (4). The inhibition rates of compounds **1a-1t** for collagen.

Whatever methoxy group was introduced into the para- or ortho-position of the N^5- side chain, the alkyl groups were introduced into the para- or meta-position, and the order of inhibition rates was: para-C_2H_5 (**1b**) < para-CH_3 (**1a**) < para-CH $(CH_3)_2$ (**1c**); para-CH_3 (**1k**) < para-C_2H_5 (**1l**) < para-$CH(CH_3)_2$ (**1m**); meta-CH_3 (**1d**) < meta-C_2H_5 (**1e**) < meta-$CH(CH_3)_2$ (**1f**); meta-CH_3 (**1n**) < meta-C_2H_5 (**1o**) < meta-$CH(CH_3)_2$ (**1p**), which indicated that compounds with isopropyl group in para- and meta-position can help to promote the antiplatelet activities.

4.2. *In Vitro* Cytotoxicity Assay

A CCK-8 assay was employed to evaluate cell viability. Target compounds formulated with DMSO to 10μmol·L^{-1} and 100μmol L^{-1} concentrations. The measurement results are shown in Table **2**.

Evaluation of cytotoxicity activity of the compounds against L929 cells line revealed that at lower concentration of 10 μmol·L^{-1}, three compounds **1f** (76.47%±5.88), **1j** (70.59%±5.88) and **1p** (88.24%±4.16) had lower effect on L929 cells, and among them, the cell survival rate of **1p** was higher than the (82.35%±4.16) of picotamide. At the concentration of 100 μmol·L^{-1}, the cell survival rate of the three compounds was far lower than that of picotamide (Fig. **5**).

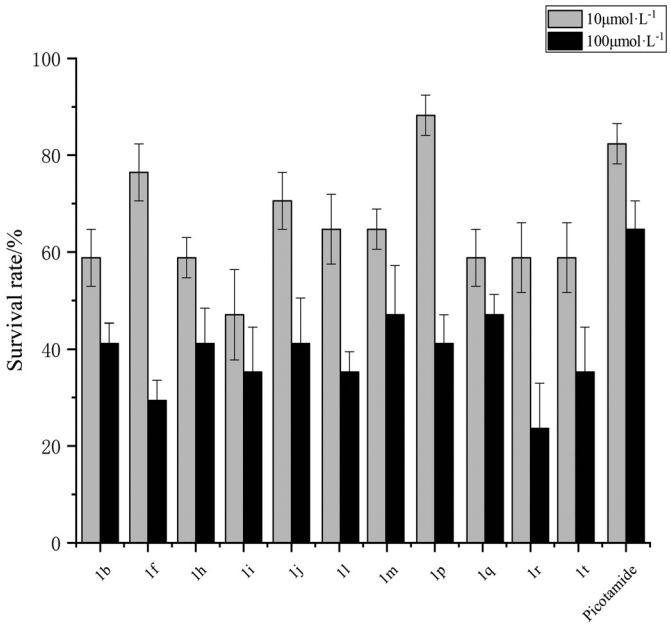


Fig. (5). Cytotoxicity effect of compounds **1b**, **1f**, **1h**, **1i**, **1j**, **1l**, **1m**, **1p**, **1q**, **1r** and **1t** on L929 cells.

CONCLUSION

In total, twenty 2-methoxy-5-aminobenzamides were designed and synthesized and the structures were confirmed by ^{1}H-NMR, IR, MS and ^{13}C-NMR. In the structural design of novel series of compounds **1a-1t**, the betrixaban retained group characteristics and picotamide retained its 1,3,4-substitution position. The effect of these compounds on platelet aggregation was assessed by the Born test with ADP, AA and collagen as inducing agents and with aspirin and picotamide as two reference drugs. The results showed that compound **1f** (46.14%±0.07) had the highest activity for ADP and its IC_{50} value was 0.17μM, far better than the two control drugs aspirin (0.44μM) and picotamide (0.47 μM). The IC_{50} value of four compounds **1i** (0.24μM), **1j** (0.22 μM), **1r** (0.25 μM) and **1t** (0.24 μM) displayed higher antiplatelet activities *in vitro* for AA than aspirin (0.43μM) and picotamide (0.34 μM). Evaluation of cytotoxicity activity of the compounds against L929 cells line revealed that at a lower concentration of 10μmol·L^{-1}, compound **1p** has a lower effect on L929 cells, and its cell survival rate (88.24%±4.16) was higher than that (82.35%±4.16) of picotamide. Although structurally different from picotamide and betrixaban, novel series of 2-methoxy-5-aminobenzamides have shown higher *in vitro* antiplatelet activities and lower effect on L929 cells at a lower concentration, and have the research value & contributed to the development of new antithrombotic drugs.

Table 2. Cytotoxicity effect of compounds 1b, 1f, 1h, 1i, 1j, 1l, 1m, 1p, 1q, 1r and 1t on L929 cells (n=3).

Compound	Dose ($\mu mol \cdot L^{-1}$)	Average Absorbance	Survival Rate (%)	Compound	Dose ($\mu mol \cdot L^{-1}$)	Average Absorbance	Survival Rate (%)
Blank	-	0.125	-	Picotamide	10	0.139	82.35±4.16
Control	-	0.142	-		100	0.136	64.71±5.89
1b	10	0.135	58.82±5.89	1m	10	0.136	64.71±4.16
	100	0.132	41.18±4.16		100	0.133	47.06±10.19
1f	10	0.138	76.47±5.88	1p	10	0.140	88.24±4.16
	100	0.130	29.41±4.16		100	0.132	41.18±5.89
1h	10	0.135	58.82±4.16	1q	10	0.135	58.82±5.89
	100	0.132	41.18±7.21		100	0.133	47.06±4.16
1i	10	0.133	47.06±9.30	1r	10	0.135	58.82±7.21
	100	0.131	35.29±9.24		100	0.129	23.63±9.34
1j	10	0.137	70.59±5.88	1t	10	0.135	58.82±7.21
	100	0.132	41.18±9.30		100	0.131	35.29±9.24
1l	10	0.136	64.71±7.21	-	-	-	-
	100	0.131	35.29±4.16		-	-	-

LIST OF ABBREVIATIONS

ADP = Adenosine Diphosphate

AA = Arachidonic Acid

IC_{50} = Half Maximal Inhibitory Concentration

PGI_2 = Epoprostenol

FDA = Food and Drug Administration

PRP = Platelet-rich Plasma

PPP = Platelet-poor Plasma

SAR = Structure-activity Relationship

TXA_2 = Thromboxane A_2

TMS = Tetramethylsilane

TLC = Thin-layer Chromatography

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the animal care committee of Shenyang pharmaceutical university (Ethical review number: SYP4-1AC4C-C2018-5-25-402).

HUMAN AND ANIMAL RIGHTS

No humans were used for studies All assays involving animals were conducted in accordance with the ethical guidance for investigation in laboratory animals.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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