Synthesis and Bioevaluation of 3,6-Diaryl-[1,2,4]Triazolo[4,3-b]Pyridazines as Antitubulin Agents

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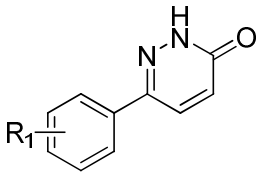
(1). Synthesis

Unless otherwise noted, all of the materials were obtained from commercially available sources and were used without purification. The progress of reactions was monitored by TLC using silica gel plates (250 μm, F-254) under UV light. The purification of products was performed using column chromatography (60 Å, 200-300 mesh, Qingdao Ocean Chemicals) or thin layer chromatography on silica gel plates (0.25 mm layer, Qingdao Ocean Chemicals) with the designated solvents. The microwave reactions were performed on a discover-sp single mode microwave reactor from CEM Corporation. Melting points were measured on a hot stage microscope (X-4, Beijing Taike Ltd.) and are uncorrected. Mass spectra (MS) were measured on an Agilent 1100-sl mass spectrometer with an electrospray ionisation source. NMR spectra were recorded on a Bruker AVANCE 400 (^{1}H, 400 MHz, 600 MHz; ^{13}C, 100 MHz, 150 MHz), in $CDCl_3$ or DMSO-d_6 (internal standard tetramethylsilane). Chemical shifts are expressed as parts per million downfield from tetramethylsilane. Splitting patterns have been designated as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet).

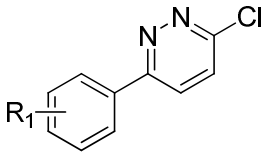
1. General procedure for the preparation of compounds 7



The substituted acetophenones **6** (1.0 mmol), glyoxylic acid monohydrate (1.0-1.3 mmol), and acetic acid (2.0 mL) were added to a round bottom flask equipped with a magnetic stir at room temperature. The mixture was heated and stirred under reflux for 2-10 h. After the reaction was completed, the reaction mixture cooled down to room temperature, 20 mL of water and ammonium hydroxide solution (25%) were added until the medium pH became 8. Then the reaction mixture was extracted with CH_2Cl_2 (3 × 25 mL). To the aqueous layer was added 10 mmol hydrazine hydrate and the reaction mixture was refluxed for 2 h. After completion of the reaction, the reaction mixture cooled down to room temperature. The resulting

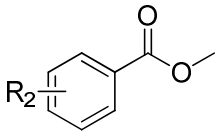
white precipitate was filtered and used for the next step without further purification.

2. General procedure for the preparation of compounds 8



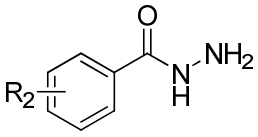
The compounds **7** (2.0 mmol) were added to a round bottom flask equipped with a magnetic stir at room temperature, and 2 mL $POCl_3$ was added dropwise over 10 min. Then, the mixture was stirred for 30-120 min at 110 °C. After the reaction was completed, the mixture was poured into ice and water. The precipitate was filtered and washed with water, which was used for the next step without further purification.

3. General procedure for the preparation of compounds 10



The substituted benzoic acids **9** (10 mmol) were dissolved in methanol (50 mL) and few drops of concentrated H_2SO_4 was added to the mixture. The mixture was heated to 70 °C within 3 min and then kept at that temperature for 27 min in a microwave reactor (discover-sp, CEM Corporation), irradiation power was 800 W. After microwave treatment, the mixture was cooled to room temperature and a saturated solution of sodium bicarbonate was added to the mixture to neutralise the benzoic acid. The precipitated product **10** was filtered, washed with water and recrystallized with methanol.

4. General procedure for the preparation of compounds 11



The substituted ethyl benzoate **10** (9 mmol), hydrazine hydrate (20 mmol), and methanol (100 mL) were added to a round bottom flask equipped with a magnetic stir at room temperature. The mixture was heated to 70 °C within 3 min and then kept at that temperature for 120 min in a microwave reactor, irradiation power was 800 W. After microwave treatment, the mixture was cooled to room temperature and excess

of methanol was distilled out. The crystals formed were filtered and washed thoroughly with water and dried.

5. General procedure for the preparation of target compounds

A mixture of chlorophthalazine **8** (1 mmol) and appropriate compounds **11** (1 mmol) in *n*-butyl alcohol (10 mL) was heated to 100 °C under microwave irradiation (discover-sp, CEM Corporation, 150 W). After the reaction was completed, the solid separated after concentrating and cooling, and recrystallized from the appropriate solvent to give most of the target compounds. Reduction of the nitro groups of **4g**, **4m**, **4p** and **5c** in a mixture of hydrazine hydrate, ferric chloride hexahydrate and activated carbon in methanol provided the corresponding **4h**, **4n**, **4q** and **5d**.

*3-(3,4,5-trimethoxyphenyl)-6-(4-methylphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4a**)*. Pale yellow solid; yield: 79%; M.p.: 177-179 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.22 (d, *J* = 7.9 Hz, 1H), 7.98 (s, 2H), 7.92 (d, *J* = 8.0 Hz, 2H), 7.60 (d, *J* = 7.9 Hz, 1H), 7.35 (d, *J* = 8.0 Hz, 2H), 4.00 (s, 6H), 3.95 (s, 3H), 2.46 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 153.5, 153.2 (2C), 148.1, 144.8, 141.6, 139.6, 131.6, 130.0 (2C), 127.0 (2C), 125.2, 121.7, 118.7, 104.8 (2C), 60.9, 56.2 (2C), 21.3; ESI-MS: m/z = 377.4 $[M+H]^+$.

*3-(3,4,5-trimethoxyphenyl)-6-(4-trifluoromethylphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4b**)*. Pale yellow solid; yield: 79%; M.p.: 189-190 ^{o}C; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 8.53 (d, *J* = 9.6 Hz, 1H), 8.33 (d, *J* = 7.8 Hz, 2H), 8.03 (d, *J* = 9.6 Hz, 1H), 7.97 (d, *J* = 7.8 Hz, 2H), 7.80 (s, 2H), 3.89 (s, 6H), 3.76 (s, 3H); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 153.4 (2C), 152.3, 147.0, 144.5, 139.3, 138.4, 131.2 (d, *J* = 32.3 Hz), 128.5 (2C), 126.5 (d, *J* = 3,1 Hz), 126.2, 125.2, 123.4, 121.7, 119.8, 104.7 (2C), 60.5, 56.3 (2C); ESI-MS: m/z = 431.1 $[M+H]^+$, 453.1 $[M+Na]^+$.

*3-(3,4,5-trimethoxyphenyl)-6-(4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4c**)*. Yellow solid; yield: 75%; M.p.: 162-165 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.19 (d, *J* = 9.4 Hz, 1H), 7.97 (d, *J* = 8.6 Hz, 2H), 7.95 (s, 2H), 7.57 (d, *J* = 9.4 Hz, 1H), 7.04 (d, *J* = 8.6 Hz, 2H), 3.99 (s, 6H), 3.94 (s, 3H), 3.89 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 162.0, 153.2 (2C), 153.2, 149.0, 143.7, 139.5, 128.6 (2C), 126.7, 125.1, 121.7, 118.6, 114.6 (2C), 104.8 (2C), 60.9, 56.2 (2C), 55.5; ESI-MS: m/z = 393.1 $[M+H]^+$,

415.4 $[M+Na]^+$.

3-(3,4,5-trimethoxyphenyl)-6-(4-fluorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine ***(4d)***. Pale yellow solid; yield: 78%; M.p.: 200-202 ^oC; ^1H-NMR (600 MHz, $CDCl_3$): δ 8.21 (s, 1H), 8.00 (s, 2H), 7.89 (s, 2H), 7.55 (s, 1H), 7.21-7.24 (m, 2H), 3.95 (s, 6H), 3.93 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 164.5 (d, *J* = 252.5Hz), 153.2 (2C), 152.7, 147.7, 144.1, 139.7, 130.6 (d, *J* = 2.8Hz), 129.2 (d, *J* = 8.5Hz, 2C), 125.6, 121.4, 118.6, 116.5 (d, *J* = 21.9Hz 2C), 104.8 (2C), 60.9, 56.2 (2C); ESI-MS: m/z = 381.0 $[M+H]^+$.

3-(3,4,5-trimethoxyphenyl)-6-(4-chlorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine ***(4e)***. Pale yellow solid; yield: 83%; M.p.: 190-191 ^oC; ^1H-NMR (600 MHz, $CDCl_3$): δ 8.21 (d, *J* = 9.6 Hz, 1H), 7.93 (d, *J* = 8.4 Hz, 2H), 7.88 (s, 2H), 7.54 (d, *J* = 9.6 Hz, 1H), 7.51 (d, *J* = 8.4 Hz, 2H), 3.95 (s, 6H), 3.93 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 153.2 (2C), 152.6, 147.6, 144.1, 139.7, 137.5, 132.8, 129.5 (2C), 128.3 (2C), 125.6, 121.3, 118.3, 104.7 (2C), 60.9, 56.1 (2C); ESI-MS: m/z = 397.0 $[M+H]^+$, 793.1 $[2M+H]^+$.

3-(3,4,5-trimethoxyphenyl)-6-(4-bromophenyl)-[1,2,4]triazolo[4,3-b]pyridazine ***(4f)***. Pale yellow solid; yield: 83%; M.p.: 205-206 ^oC; ^1H-NMR (600 MHz, $CDCl_3$): δ 8.20 (d, *J* = 8.8 Hz, 1H), 7.88 (s, 4H), 7.66 (d, *J* = 6.3 Hz, 2H), 7.53 (d, *J* = 8.8 Hz, 1H), 3.94 (s, 6H), 3.93 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 153.2 (2C), 152.6, 147.6, 144.1, 139.7, 133.3, 132.5 (2C), 128.5 (2C), 125.8, 125.6, 121.3, 118.2, 104.7 (2C), 60.9, 56.1 (2C); ESI-MS: m/z = 441.1 $[M+H]^+$, 463.1 $[M+Na]^+$.

3-(3,4,5-trimethoxyphenyl)-6-(4-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazine ***(4g)***. Yellow solid; yield: 82%; M.p.: 226-227 ^oC; ^1H-NMR (600 MHz, DMSO-d_6): δ 8.57 (d, *J* = 9.6 Hz, 1H), 8.42 (d, *J* = 8.8 Hz, 2H), 8.38 (d, *J* = 8.8 Hz, 2H), 8.06 (d, *J* = 9.6 Hz, 1H), 7.80 (s, 2H), 3.91 (s, 6H), 3.77 (s, 3H); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 153.4 (2C), 151.9, 149.2, 147.1, 144.5, 140.4, 139.4, 129.0 (2C), 126.3, 124.7 (2C), 121.6, 119.9, 104.7 (2C), 60.6, 56.3 (2C); ESI-MS: m/z =408.1 $[M+H]^+$, 430.1 $[M+Na]^+$.

3-(3,4,5-trimethoxyphenyl)-6-(4-aminophenyl)-[1,2,4]triazolo[4,3-b]pyridazine ***(4h)***. Pale yellow solid; yield: 59%; M.p.: 240-241 ^oC; ^1H-NMR (600 MHz, DMSO-d_6): δ

8.36 (d, *J* = 9.8 Hz, 1H), 7.91 (s, 2H), 7.88-7.91 (m, 3H), 6.71 (d, *J* = 8.6 Hz, 2H), 4.35 (s, 2H), 3.94 (s, 6H), 3.78 (s, 3H); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 153.8, 153.4 (2C), 152.3, 146.6, 144.6, 139.2, 128.9 (2C), 125.0, 122.2, 120.8, 119.6, 114.2 (2C), 104.7 (2C), 60.6, 60.1 (2C); ESI-MS: m/z = 378.2 $[M+H]^+$, 400.2 $[M+Na]^+$.

*3-(3,4,5-trimethoxyphenyl)-6-(4-methylthiophenyl)-[1,2,4]triazolo[4,3-b]pyridazine **(4i)**.* Pale yellow solid; yield: 78%; M.p.: 181-183 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.18 (d, *J* = 9.6 Hz, 1H), 7.92 (s, 3H), 7.90 (s, 1H), 7.55 (d, *J* = 9.6 Hz, 1H), 7.34 (d, *J* = 8.4 Hz, 2H), 3.97 (s, 6H), 3.93 (s, 3H), 2.54 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 153.2 (2C), 153.0, 147.6, 144.2, 143.3. 139.6, 130.5, 127.3 (2C), 126.0 (2C), 125.2, 121.5, 118.4, 104.7 (2C), 60.9, 56.2 (2C), 14.9; ESI-MS: m/z = 409.1 $[M+H]^+$, 431.1 $[M+Na]^+$.

*3-(3,4,5-trimethoxyphenyl)-6-(4-cyanophenyl)-[1,2,4]triazolo[4,3-b]pyridazine **(4j)**.* Pale yellow solid; yield: 71%; M.p.: 230-231 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.34 (d, *J* = 9.4 Hz, 1H), 8.14 (d, *J* = 7.8 Hz, 2H), 7.88 (s, 2H), 7.87 (d, *J* = 7.8 Hz, 2H), 7.61 (d, *J* = 9.4 Hz, 1H), 3.98 (s, 6H), 3.95 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 153.3 (2C), 151.9, 148.0, 144.1, 140.0, 138.6, 133.0 (2C), 127.7 (2C), 126.2, 121.1, 118.1, 117.8, 114.8, 105.0 (2C), 61.0, 56.2 (2C); ESI-MS: m/z = 388.1 $[M+H]^+$, 410.1 $[M+Na]^+$.

*3-(3,4,5-trimethoxyphenyl)-6-(3-chlorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine **(4k)**.* Pale yellow solid; yield: 78%; M.p.: 233-235 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.27 (s, 1H), 8.13 (s, 1H), 7.98 (s, 2H), 7.86 (s, 1H), 7.60 (d, *J* = 4.3 Hz, 1H), 7.54 (s, 1H), 7.51 (s, 1H), 4.02 (s, 6H), 3.96 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 153.3 (2C), 152.1, 147.7, 144.1, 139.7, 136.0, 135.5, 131.0, 130.5, 127.2, 125.8, 125.1, 121.3, 118.1, 104.6 (2C), 60.9, 56.2 (2C); ESI-MS: m/z = 397.1 $[M+H]^+$, 419.1 $[M+Na]^+$.

*3-(3,4,5-trimethoxyphenyl)-6-(3-bromophenyl)-[1,2,4]triazolo[4,3-b]pyridazine **(4l)**.* Yellow solid; yield: 76%; M.p.: 216-217 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.30-8.31 (m, 1H), 8.28 (d, *J* = 9.6 Hz, 1H), 7.99 (s, 2H), 7.90 (d, *J* = 7.8 Hz, 1H), 7.70 (dd, *J* = 7.8 Hz, *J* = 1.2 Hz, 1H), 7.60 (d, *J* = 9.6 Hz, 1H), 7.43-7.46 (m, 1H), 4.03 (s, 6H), 3.96 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 153.3 (2C), 152.0, 147.7, 144.1, 139.7, 136.2, 133.9, 130.7, 130.1, 125.8, 125.6, 123.5, 121.3, 118.0, 104.6 (2C),

60.9, 56.2 (2C); ESI-MS: m/z = 441.1 $[M+H]^+$, 463.0 $[M+Na]^+$.

3-(3,4,5-trimethoxyphenyl)-6-(3-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazine ***(4m)***. Brown solid; yield: 81%; M.p.: 209-211 ^oC; ^1H-NMR (600 MHz, $CDCl_3$): δ 8.99-9.00 (m, 1H), 8.43-8.45 (m, 1H), 8.35 (d, *J* = 9.6 Hz, 1H), 8.33 (d, *J* = 7.9 Hz, 1H), 7.95 (s, 2H), 7.78-7.81 (m, 1H), 7.68 (d, *J* = 9.6 Hz, 1H), 4.04 (s, 6H), 3.96 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 153.4 (2C), 151.4, 148.9, 148.0, 144.0, 139.9, 136.1, 132.6, 130.5, 126.3, 125.5, 122.0, 121.1, 117.8, 104.7 (2C), 61.0, 56.3 (2C); ESI-MS: m/z = 408.2 $[M+H]^+$, 430.2 $[M+Na]^+$.

3-(3,4,5-trimethoxyphenyl)-6-(3-aminophenyl)-[1,2,4]triazolo[4,3-b]pyridazine ***(4n)***. Pale yellow solid; yield: 77%; M.p.: 210-212 ^oC; ^1H-NMR (600 MHz, DMSO-d_6): δ 8.46 (d, *J* = 9.6 Hz, 1H), 7.89 (s, 2H), 7.84 (d, *J* = 9.6 Hz, 1H), 7.34 (s, 1H), 7.21-7.27 (m, 2H), 6.79 (d, *J* = 7.5 Hz, 1H), 5.38 (s, 2H), 3.93 (s, 6H), 3.77 (s, 3H); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 154.3, 153.4 (2C), 149.9, 146.9, 144.7, 139.2, 135.2, 130.1, 125.7, 122.0, 120.1, 116.9, 115.3, 112.3, 104.7 (2C), 60.6, 56.3 (2C); ESI-MS: m/z = 378.2 $[M+H]^+$, 400.2 $[M+Na]^+$.

3-(3,4,5-trimethoxyphenyl)-6-(3-fluoro-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyrid azine ***(4o)***. White solid; yield: 83%; M.p.: 210-211 ^oC; ^1H-NMR (600 MHz, $CDCl_3$): δ 8.22 (d, *J* = 9.6 Hz, 1H), 7.95 (s, 2H), 7.85 (d, *J* = 12.0 Hz, 1H), 7.74 (d, *J* = 8.4 Hz, 1H), 7.56 (d, *J* = 9.6 Hz, 1H), 7.09-7.12 (m, 1H), 4.01 (s, 6H), 3.99 (s, 3H), 3.95 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 153.3 (2C), 152.7 (d, *J* = 249.4 Hz), 152.1, 150.3 (d, *J* = 10.5 Hz), 147.7, 144.1, 139.7, 127.1 (d, *J* = 6.6 Hz), 125.4, 123.5, 121.4, 118.1. 114.6 (d, *J* = 19.9 Hz), 113.4, 104.8 (2C), 60.9, 56.3, 56.2 (2C); ESI-MS: m/z = 411.1 $[M+H]^+$, 433.1 $[M+Na]^+$.

3-(3,4,5-trimethoxyphenyl)-6-(3-nitro-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridaz ine ***(4p)***. Brown solid; yield: 82%; M.p.: 215-216 ^oC; ^1H-NMR (600 MHz, DMSO-d_6): δ 8.59 (s, 1H), 8.43 (d, *J* = 9.6 Hz, 1H), 8.36 (d, *J* = 8.6 Hz, 1H), 7.97 (d, *J* = 9.6 Hz, 1H), 7.76 (s, 2H), 7.52 (d, *J* = 8.6 Hz, 1H), 4.02 (s, 3H), 3.89 (s, 6H), 3.76 (s, 3H); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 154.3, 153.3 (2C), 151.4, 146.7, 144.4, 139.8, 139.2, 133.5, 126.4, 125.9, 123.9, 121.7, 119.2, 115.4, 104.4 (2C), 60.5, 57.5, 56.2 (2C); ESI-MS: m/z = 438.4 $[M+H]^+$.

*3-(3,4,5-trimethoxyphenyl)-6-(3-amino-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4q**).* Yellow solid; yield: 74%; M.p.: 198-199 ^{o}C; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 8.40 (d, *J* = 9.8 Hz, 1H), 7.88 (s, 2H), 7.83 (d, *J* = 9.8 Hz, 1H), 7.45 (d, *J* = 2.2 Hz, 1H), 7.35 (dd, *J* = 8.4 Hz, *J* = 2.2 Hz, 1H), 6.97 (d, *J* = 8.4 Hz, 1H), 5.01 (s, 2H), 3.93 (s, 6H), 3.86 (s, 3H), 3.76 (s, 3H); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 154.0, 153.4 (2C), 149.4, 146.8, 144.6, 139.2, 138.9, 127.0, 125.3, 122.1, 119.9, 116.7, 111.8, 110.9, 104.7 (2C), 60.5, 56.3 (2C), 55.9; ESI-MS: m/z = 408.1 $[M+H]^+$, 815.3 $[2M+H]^+$.

*3-(2,3,4-trimethoxyphenyl)-6-(4-methylphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**5a**).* Brown solid; yield: 55%; M.p.: 160-162 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.15 (d, *J* = 9.7 Hz, 1H), 7.80 (d, *J* = 8.2 Hz, 2H), 7.56 (d, *J* = 9.7 Hz, 1H), 7.42 (d, *J* = 8.6 Hz, 1H), 7.25 (d, *J* = 8.2 Hz, 2H), 6.82 (d, *J* = 8.6 Hz, 1H), 3.93 (s, 3H), 3.90 (s, 3H), 3.83 (s, 3H), 2.38 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 155.8, 153.1, 153.0, 142.2, 141.1, 131.5, 129.7 (2C), 127.0 (2C), 126.4, 126.3, 124.7, 119.0, 112.9, 107.5, 107.0, 61.5, 60.8, 56.0, 21.3; ESI-MS: m/z = 377.4 $[M+H]^+$.

*3-(2,3,4-trimethoxyphenyl)-6-(4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**5b**).* Brown solid; yield: 51%; M.p.: 157-159 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.07 (d, *J* = 9.7 Hz, 1H), 7.82 (d, *J* = 8.9 Hz, 2H), 7.50 (d, *J* = 9.7 Hz, 1H), 7.38 (d, *J* = 8.6 Hz, 1H), 6.90 (d, *J* = 8.9 Hz, 2H), 6.78 (d, *J* = 8.6 Hz, 1H), 3.89 (s, 3H), 3.87 (s, 3H), 3.80 (s, 3H), 3.77 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 161.6, 155.7, 153.1, 152.6, 142.1, 128.6 (2C), 128.5, 126.6, 126.2, 124.5, 118.9, 114.4, 114.3 (2C), 112.9, 107.0, 61.5, 60.8, 56.0, 55.3; ESI-MS: m/z = 393.1 $[M+H]^+$, 415.1 $[M+Na]^+$.

*3-(2,3,4-trimethoxyphenyl)-6-(3-nitro-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**5c**).* Yellow solid; yield: 57%; M.p.: 97-100 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.47 (d, *J* = 1.7 Hz, 1H), 8.23 (d, *J* = 9.6 Hz, 1H), 8.14 (dd, *J* = 8.7 Hz, *J* = 1.7 Hz, 1H), 7.57 (d, *J* = 9.6 Hz, 1H), 7.41 (d, *J* = 8.6 Hz, 1H), 7.20 (d, *J* = 8.7 Hz, 1H), 6.84 (d, *J* = 8.6 Hz, 1H), 4.02 (s, 3H), 3.95 (s, 3H), 3.94 (s, 3H), 3.80 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 156.0, 154.6, 153.1, 150.6, 147.9, 145.0, 142.2, 139.8, 132.5, 126.8, 126.2, 125.4, 124.6, 118.0, 114.0, 112.5, 107.2, 61.5, 60.9, 56.8, 56.1; ESI-MS: m/z = 438.4 $[M+H]^+$.

*3-(2,3,4-trimethoxyphenyl)-6-(3-amino-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**5d**)*. Brown solid; yield: 53%; M.p.: 114-116 ^{o}C; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.10 (d, *J* = 9.8 Hz, 1H), 7.52 (d, *J* = 9.8 Hz, 1H), 7.43 (d, *J* = 8.6 Hz, 1H), 7.32 (d, *J* = 2.1 Hz, 1H), 7.27 (dd, *J* = 8.6 Hz, *J* = 2.1 Hz, 1H), 6.83 (d, *J* = 8.5 Hz, 2H), 3.85 (s, 3H), 3.92 (s, 3H), 3.89 (s, 3H), 3.85 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 155.7, 153.2, 153.0, 149.5, 147.4, 143.7, 142.2, 136.8, 127.0, 126.3, 124.3, 119.1, 117.9, 113.1, 112.8, 110.1, 107.0, 61.5, 60.8, 56.0, 55.5; ESI-MS: m/z = 408.1 $[M+H]^+$, 815.3 $[2M+H]^+$.

(2). Biology

1. Cell line and culture conditions

The human gastric adenocarcinoma cell line SGC-7901, the human fibrosarcoma cell line HT-1080 and the human pulmonary carcinoma cell line A-549 were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences. All cells were maintained in RPMI-1640 medium containing 10% FBS, 100 U/mL streptomycin and 100 U/mL penicillin at 37 ^{o}C in humidified atmosphere with 5% CO_2.

2. MTT assay

The in vitro antiproliferative activities of CA-4 and all of the target compounds were determined by an MTT (Sigma) assay. Briefly, approximately 3 × 104 cells were seeded in a 96-well plate. After 24 h of incubation at 37 °C, cells were exposed to compounds of differing concentrations for 24 h. After treatment, cells were washed with 1X PBS followed by addition of 100 μL of 0.05% MTT reagent to each well, followed by incubation for 4 h at 37 °C. After incubation, the supernatant from each well was carefully removed and the formazan crystals were dissolved in 100 μL of DMSO. The colour density was measured spectrophotometrically at 490 nm using a microplate reader (SpectraMax Plus384, Molecular Devices Corp., USA). The data were calculated and plotted as percent viability compared to control.

3. Tubulin assembly assay

Tubulin polymerization assay was conducted with reagents as described in the kit manufacturer (Cytoskeleton, Cat.#BK011P) in a 96-well plate. In brief, tubulin was

re-suspended in ice-cold G-PEM buffer (80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP, 20% (v/v) glycerol) and added to wells on a 96-well plate containing the designated concentration of drugs or vehicle. Samples were mixed well, and tubulin assembly was monitored (emission wavelength: 450±20 nm; excitation wavelength: 360±20 nm) at 1 min intervals for 90 min at 37 °C in a SpectraMax 340PC spectrophotometer (Biotek Synergy HT, Winooskin, VT, USA). IC_{50} values were calculated from data at the 20 min time point using GraphPad Prism software. Experiments were repeated three times.

4. Immunostaining of tubulin assembly and DAPI nuclear staining

Immunostaining was carried out to detect microtubule associated tubulin protein after exposure to **4q** and CA-4. The A549 cells were seeded at a density of 1 × 104 per well on a 24-well plate and grown for 24 h. Cells were treated with CA-4 or **4q** for 12 h. Cells in the control group were treated with culture medium. The control and treated cells were fixed with 4% formaldehyde in PBS for 30 min at -20 °C, then washed twice with PBS and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min. Then, the cells were blocked with 3% bovine serum albumin (BSA) in PBS for 30 min. The primary a-tubulin antibody was diluted (1:100) with 2% BSA in PBS and incubated overnight at 4 °C. The cells were washed with PBS to remove unbound primary antibody and then cells were incubated with FITC-conjugated antimouse secondary antibody, diluted (1:100) with 2% BSA in PBS, for 2 h at 37 °C. The cells were washed with PBS to remove unbound secondary antibody, nucleus was stained with 4,6-diamino-2-phenolindol dihydrochloride (DAPI) and then, immunofluorescence was detected using a fluorescence microscope (Olympus, Tokyo, Japan).

5. Cell cycle analysis

A549 cells (8×104 cells) were incubated with various concentrations of **4q**, CA-4 or 0.05% DMSO respectively for indicated time. The cells were collected by centrifugation, washed with PBS and fixed in ice-cold 70% ethanol overnight. The fixed cells were harvested by centrifugation and re-suspended in 500 μl of PBS containing 1 mg/ml RNase. After 30 min incubation at 37 °C, the cells were stained

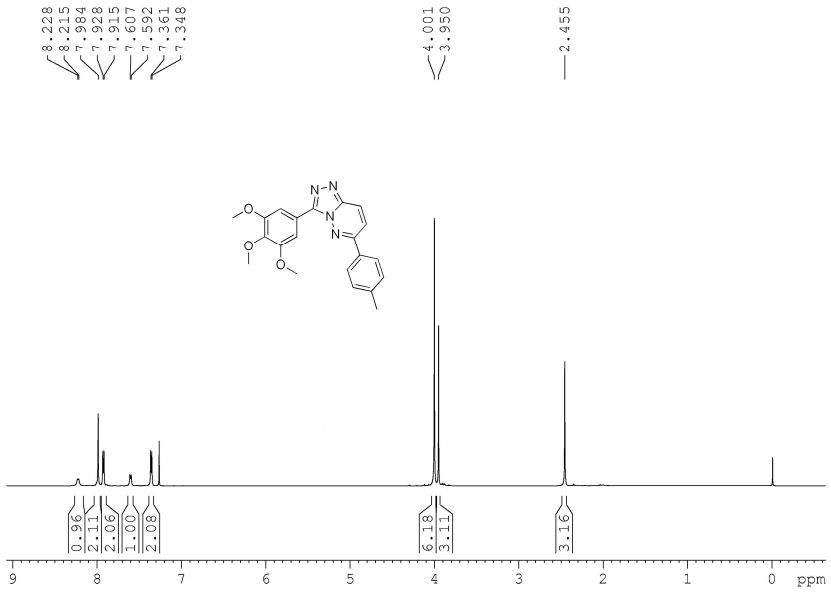
with 50 μg/ml PI at 4 °C in dark for 30 min. Then, the samples were analyzed by FACScan flow cytometry (Becton–Dickinson, Franklin Lakes, NJ, USA). Experiments were repeated at least three times. Data were evaluated using CELL QUEST software (Becton Dickison).

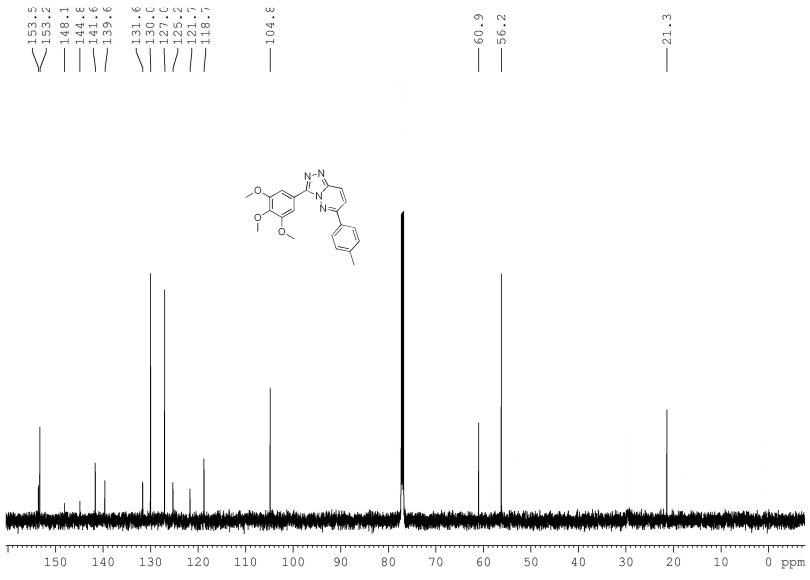
6. Docking study

The initial coordinates for tubulin were taken from the crystal structure of tubulin in complex with colchicine (PDB ID: 1SA0) obtained from the Protein Data Bank. Molecular docking was performed using the Discovery Studio 3.0 software package's CDOCKER protocol with the default settings. The protein was prepared by removing all of the ions and substructures present and then adding hydrogen atoms. In the docking process, the active site was defined along with the colchicine complex. CA-4, **4q** and vinylogous analogues of CA-4 **3** were docked into the active site and the docking simulations were performed using the Discovery studio 3.0 programme.

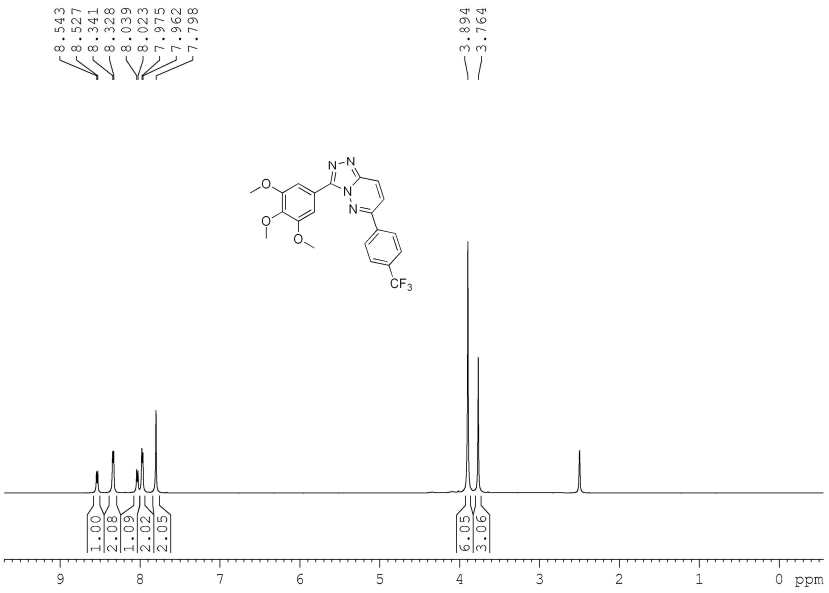
(3). Contents: ^{1}H-NMR and ^{13}C-NMR spectra of all target compounds.

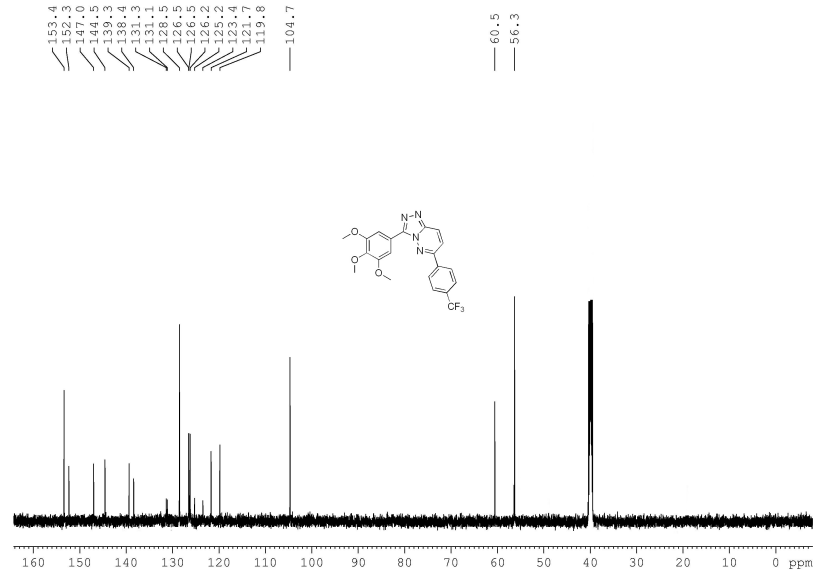
3-(3,4,5-trimethoxyphenyl)-6-(4-methylphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4a**).



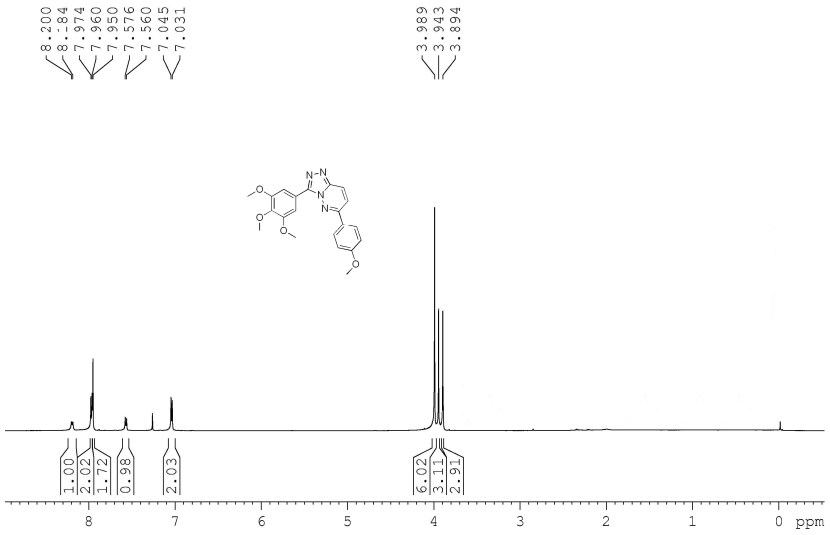


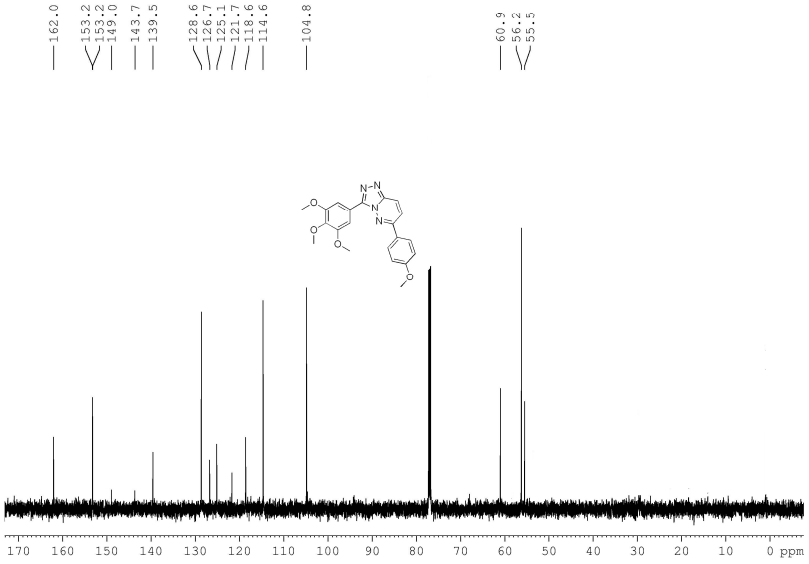
3-(3,4,5-trimethoxyphenyl)-6-(4-trifluoromethylphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4b**).



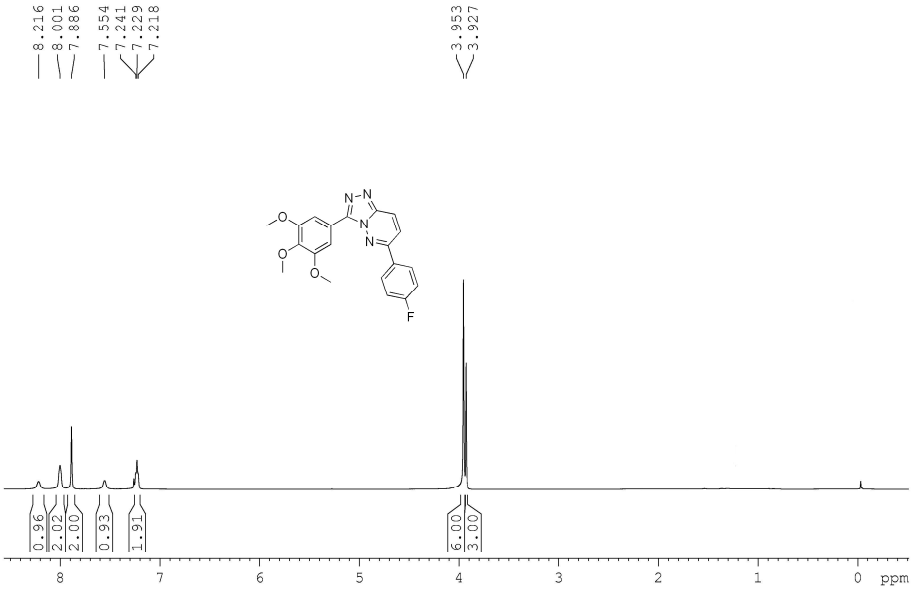


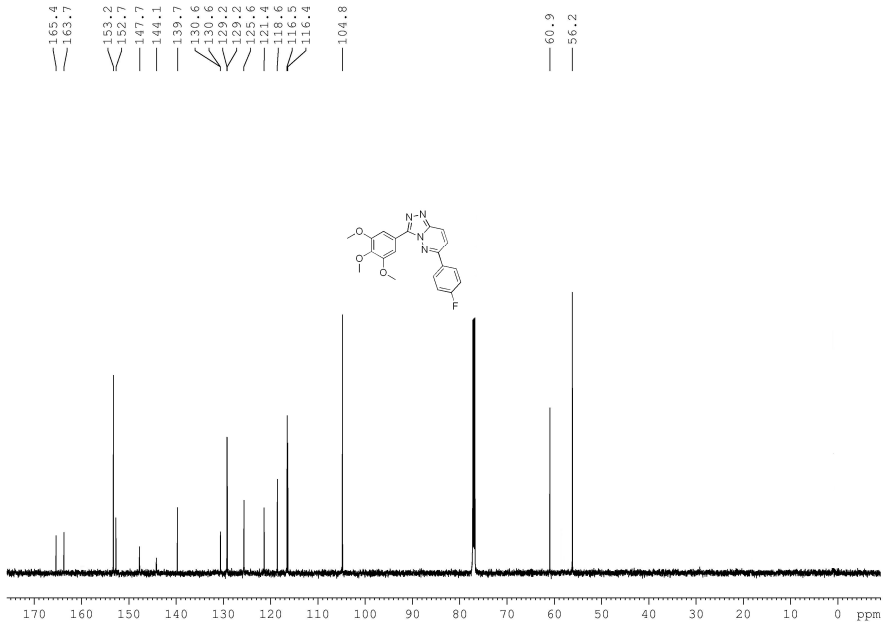
3-(3,4,5-trimethoxyphenyl)-6-(4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4c**).



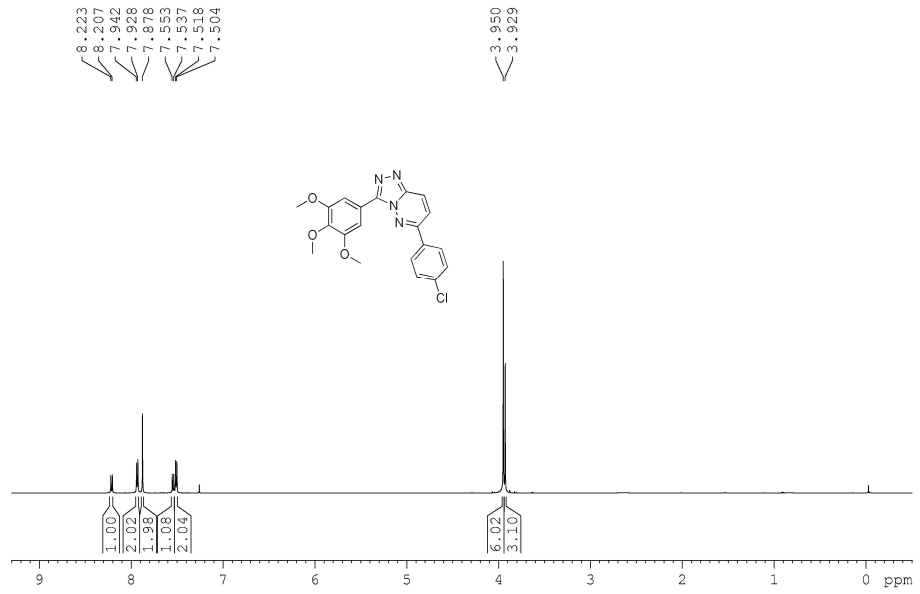


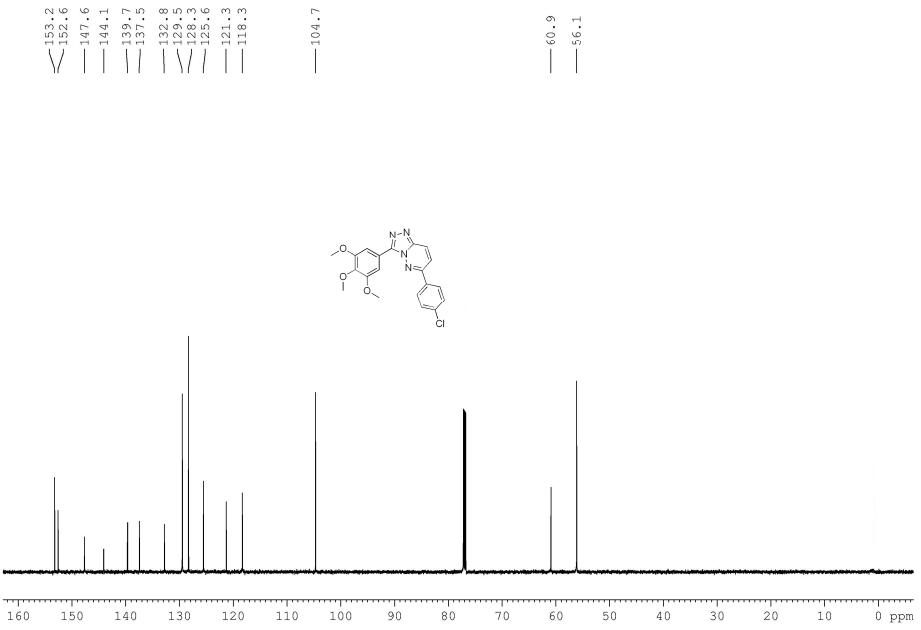
3-(3,4,5-trimethoxyphenyl)-6-(4-fluorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4d**).



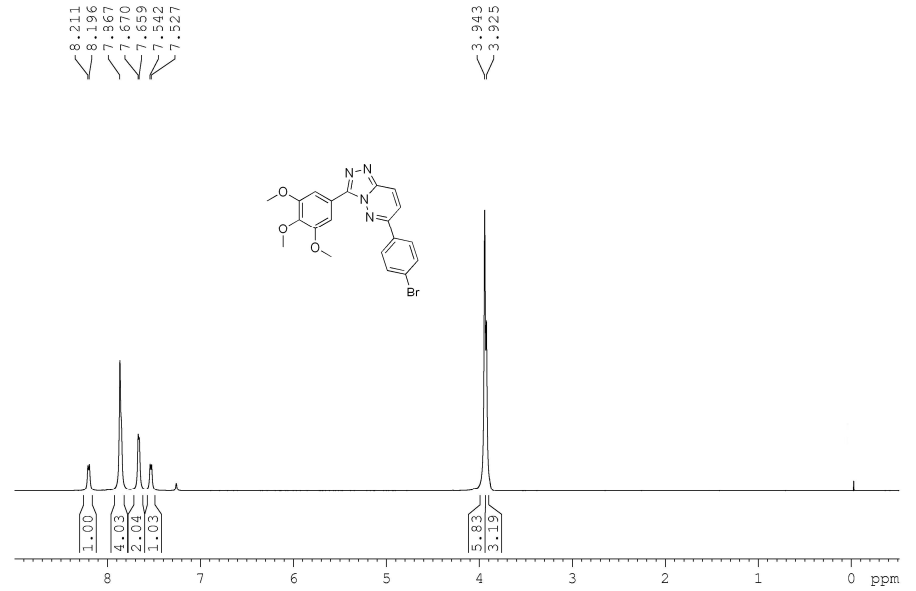


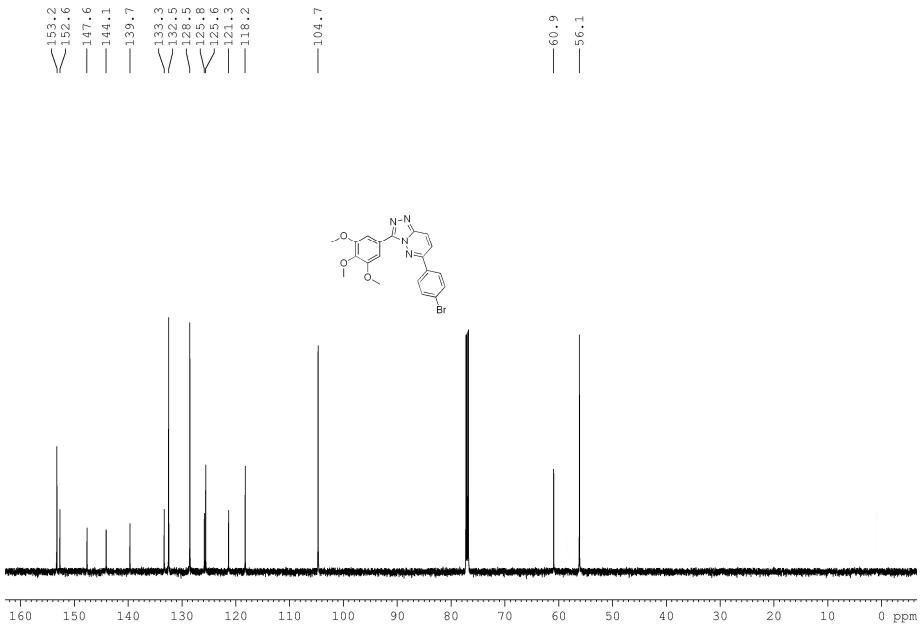
3-(3,4,5-trimethoxyphenyl)-6-(4-chlorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4e**).



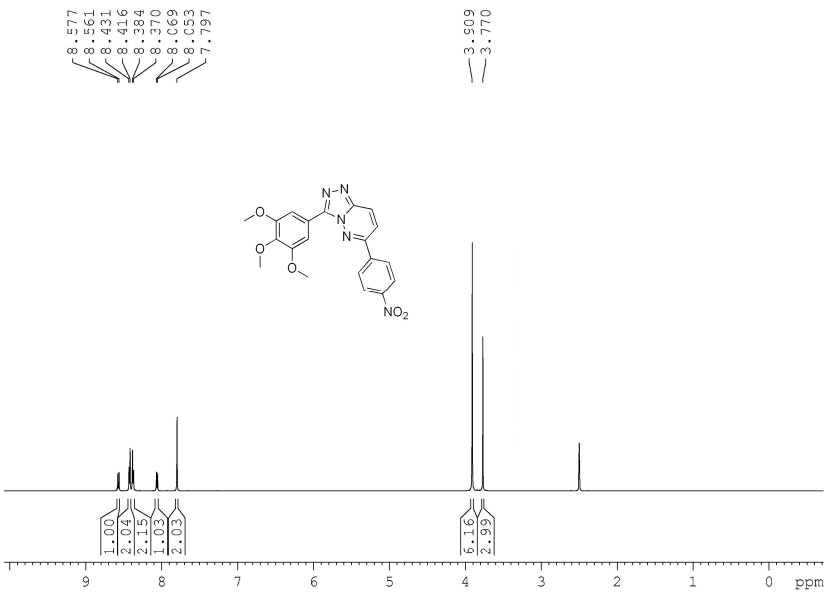


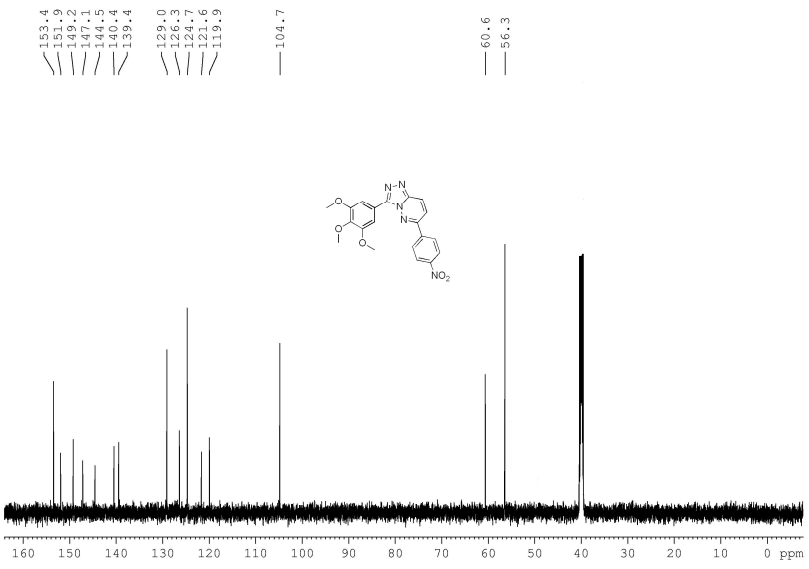
3-(3,4,5-trimethoxyphenyl)-6-(4-bromophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4f**).



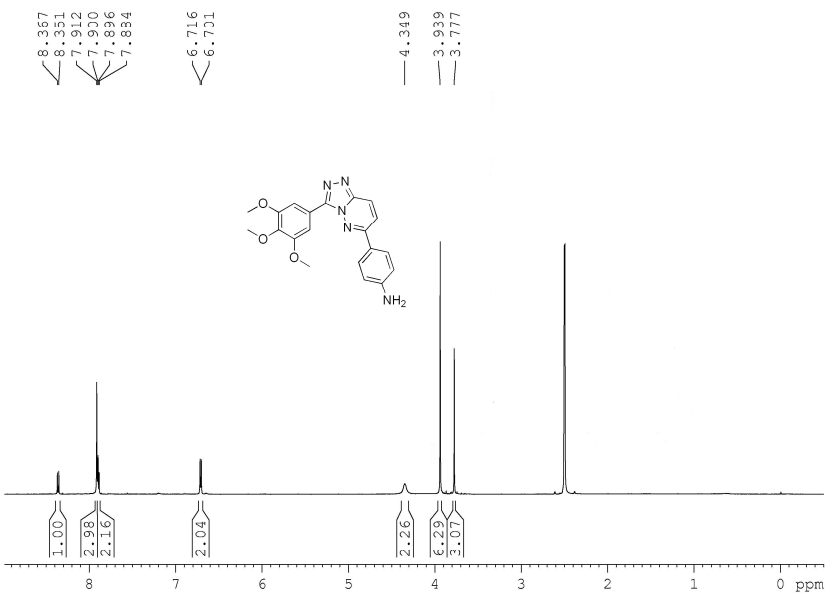


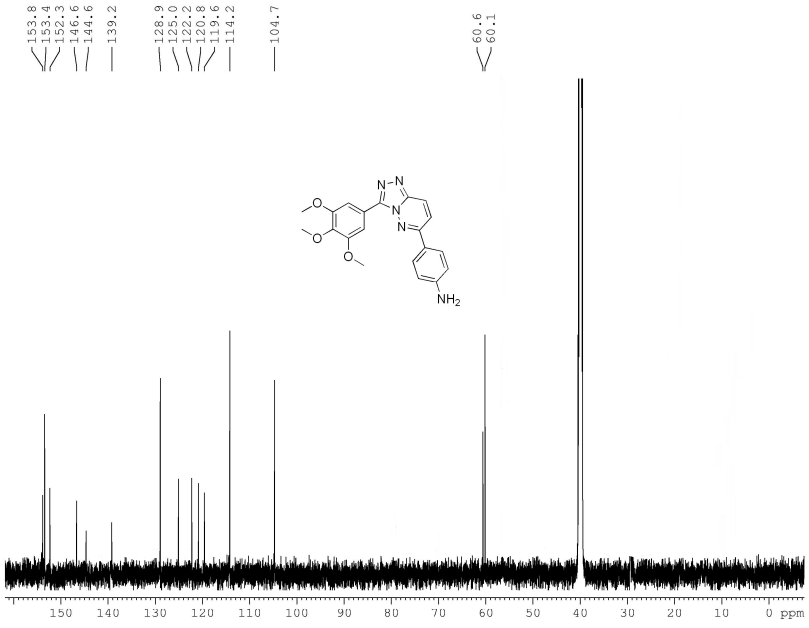
3-(3,4,5-trimethoxyphenyl)-6-(4-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4g**).



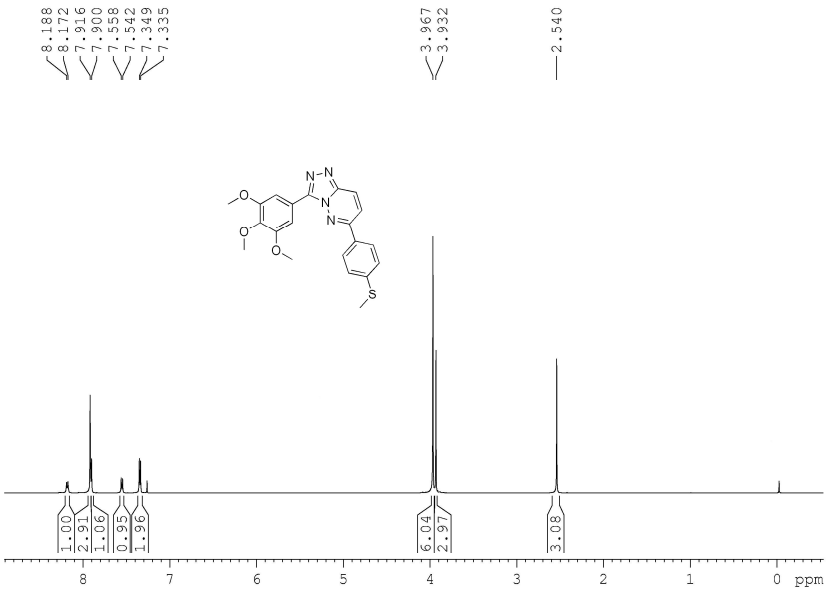


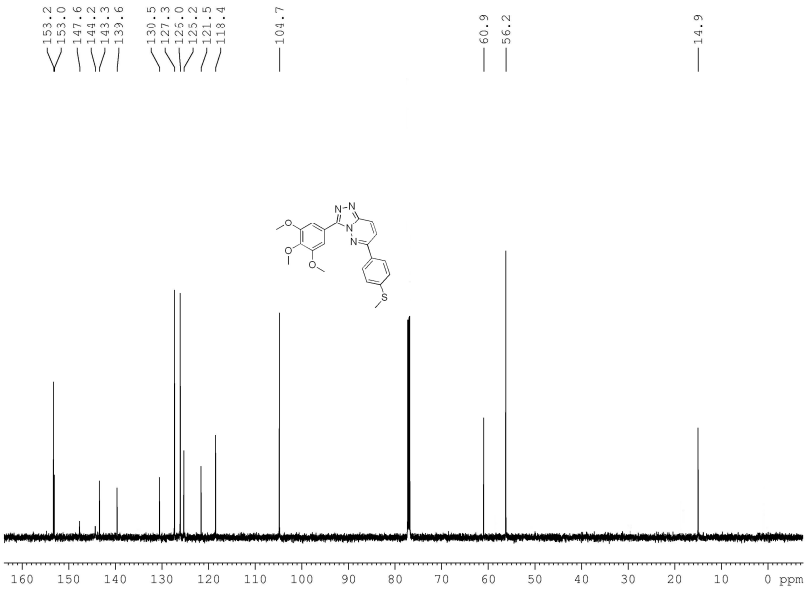
3-(3,4,5-trimethoxyphenyl)-6-(4-aminophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4h**).



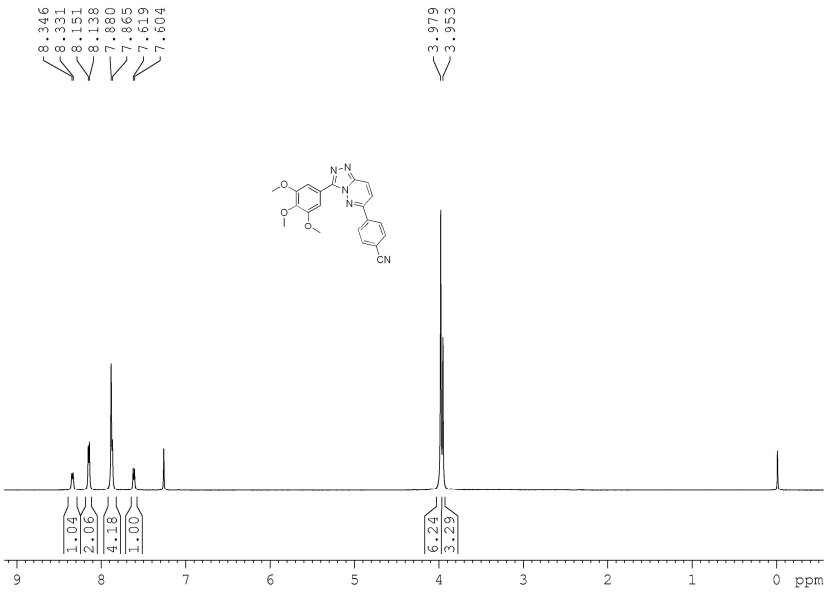


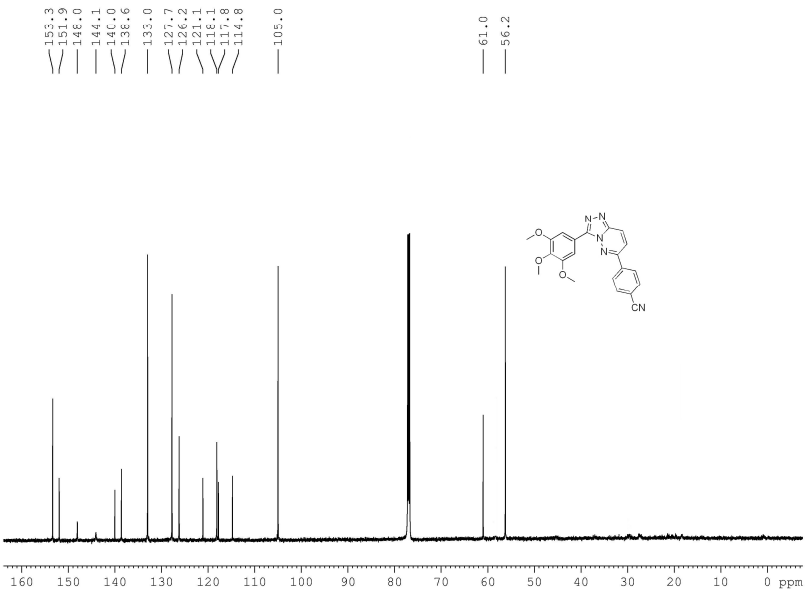
3-(3,4,5-trimethoxyphenyl)-6-(4-methylthiophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4i**).



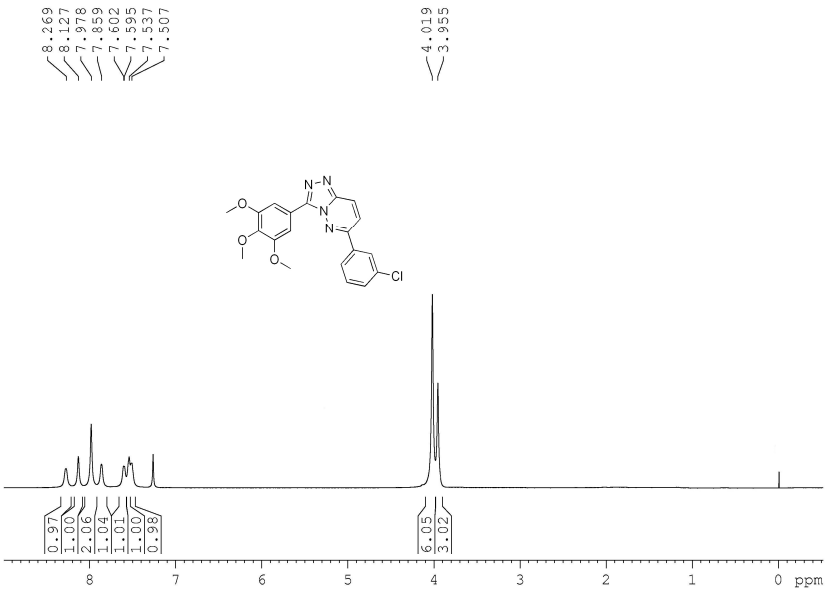


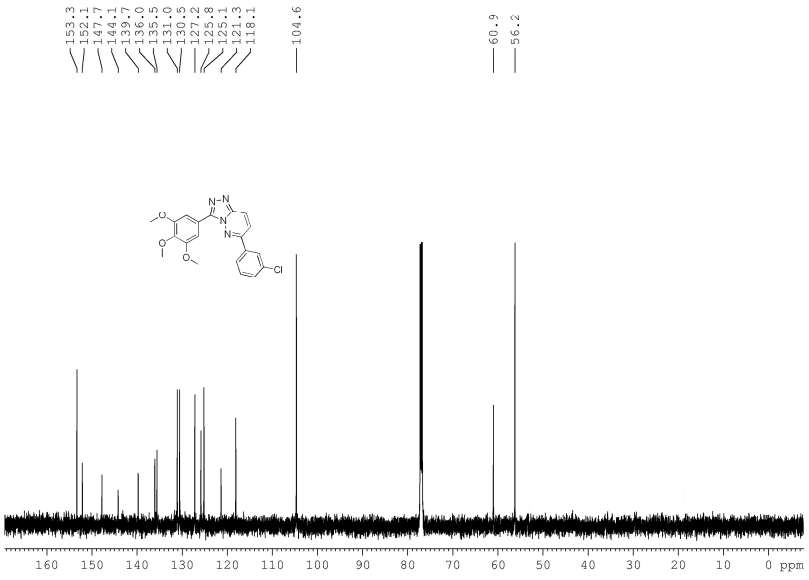
3-(3,4,5-trimethoxyphenyl)-6-(4-cyanophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4j**).



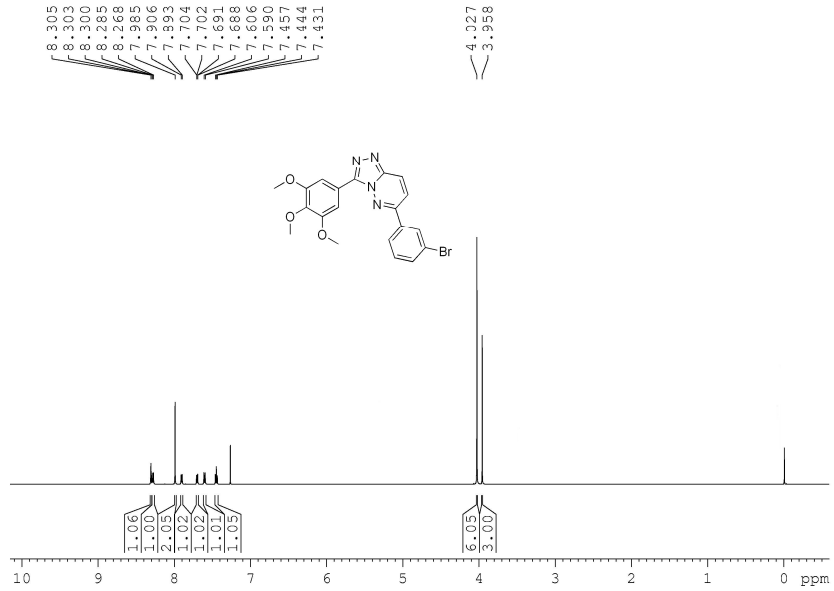


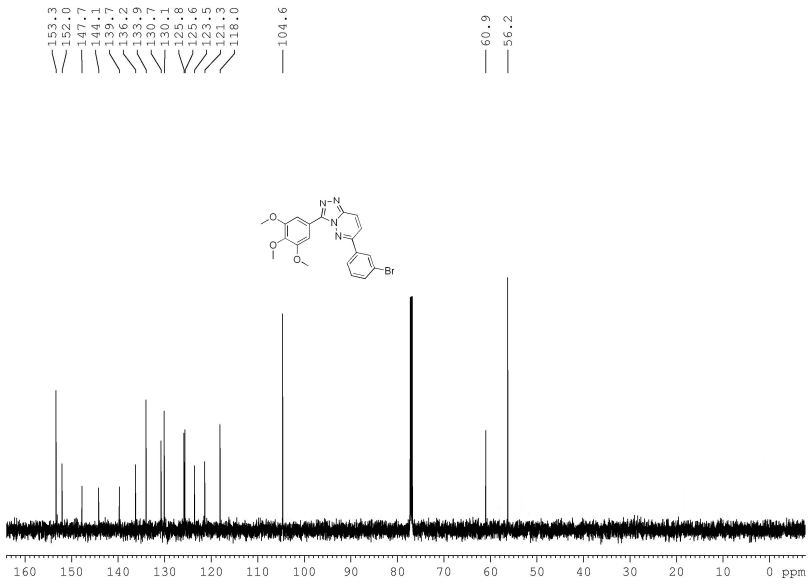
3-(3,4,5-trimethoxyphenyl)-6-(3-chlorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4k**).



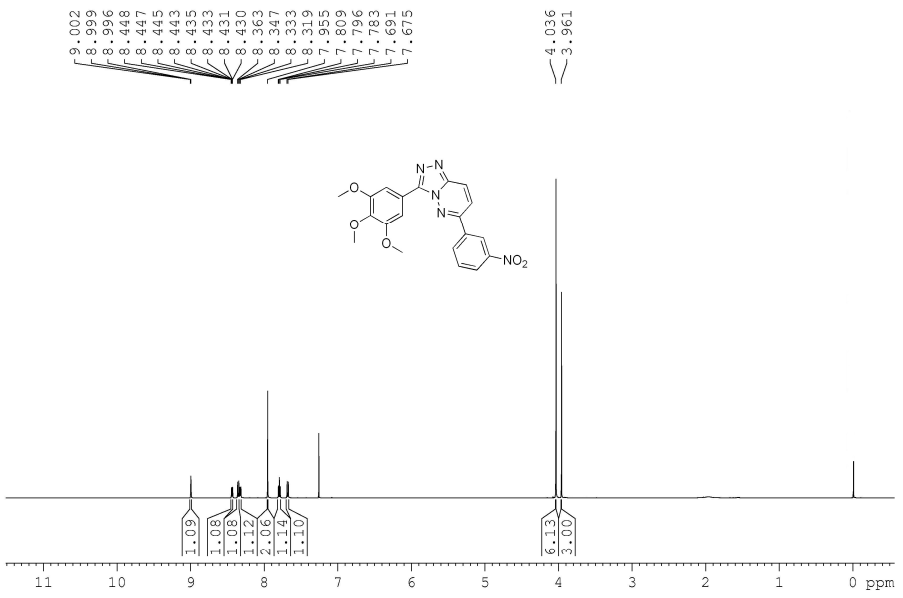


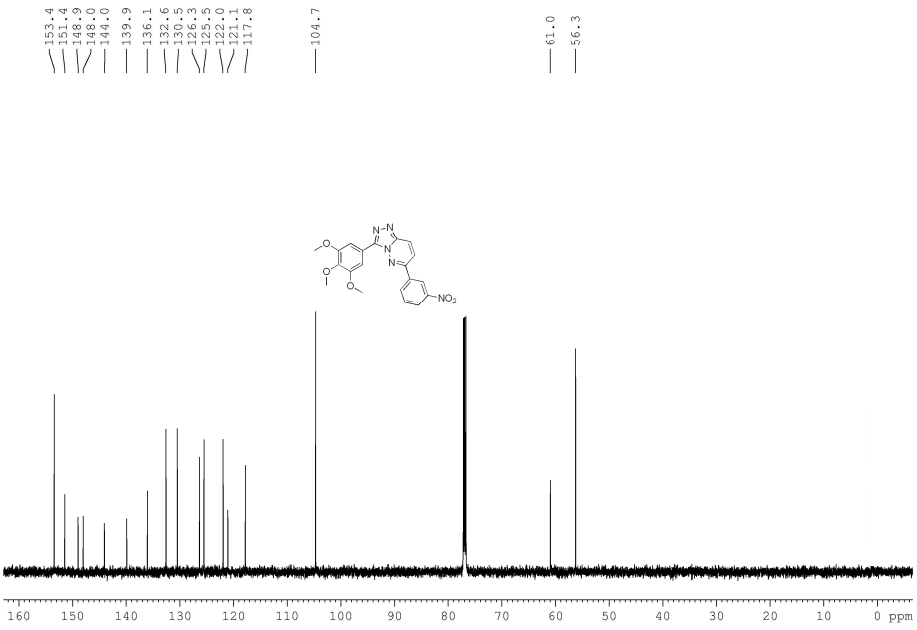
3-(3,4,5-trimethoxyphenyl)-6-(3-bromophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4l**).



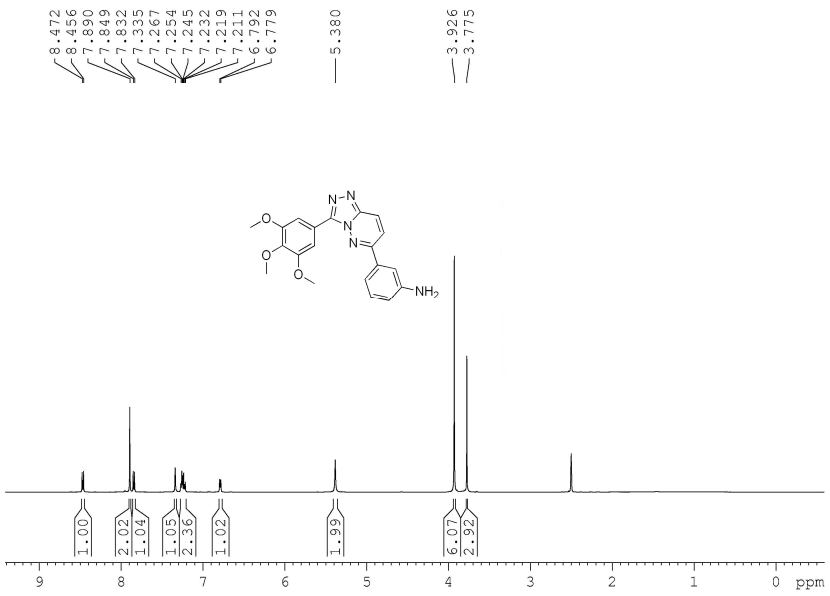


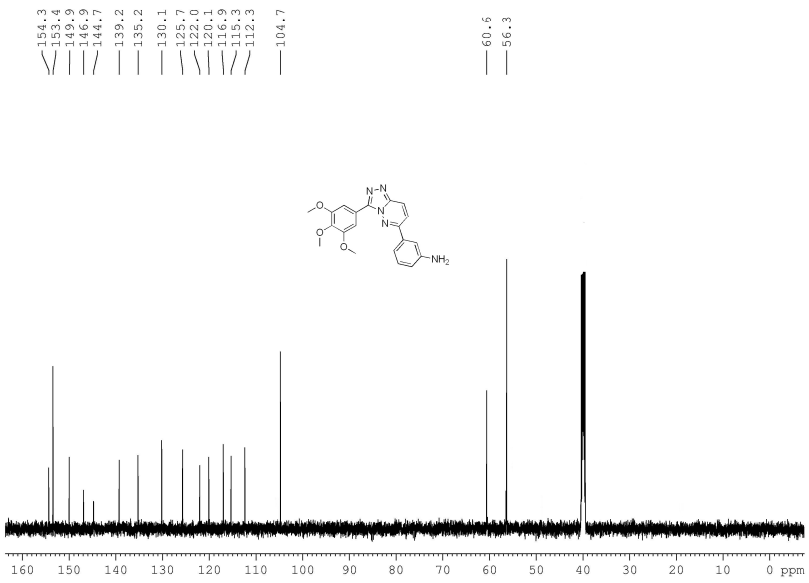
3-(3,4,5-trimethoxyphenyl)-6-(3-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4m**).



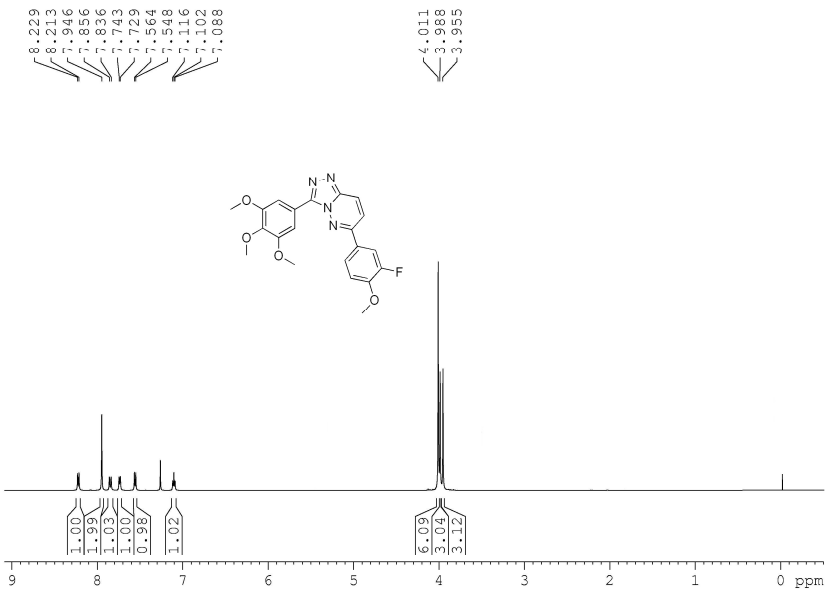


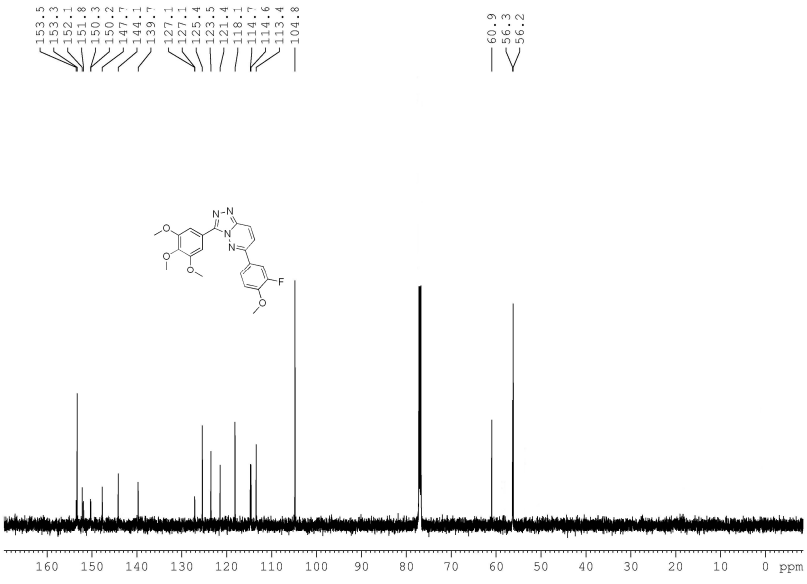
3-(3,4,5-trimethoxyphenyl)-6-(3-aminophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4n**).



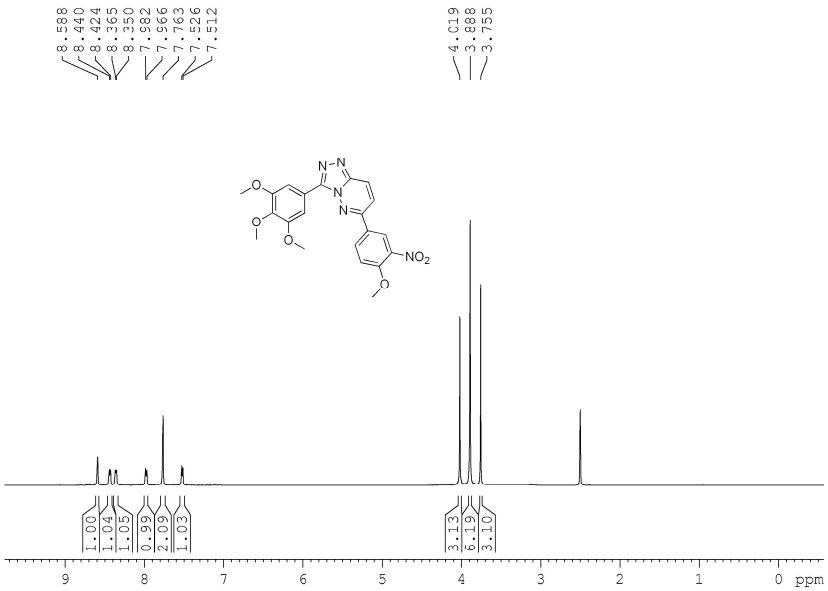


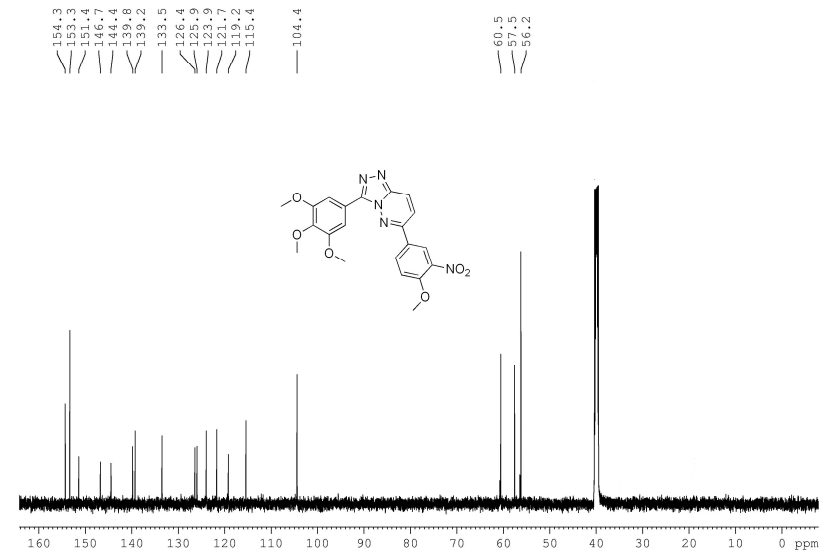
3-(3,4,5-trimethoxyphenyl)-6-(3-fluoro-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4o**).



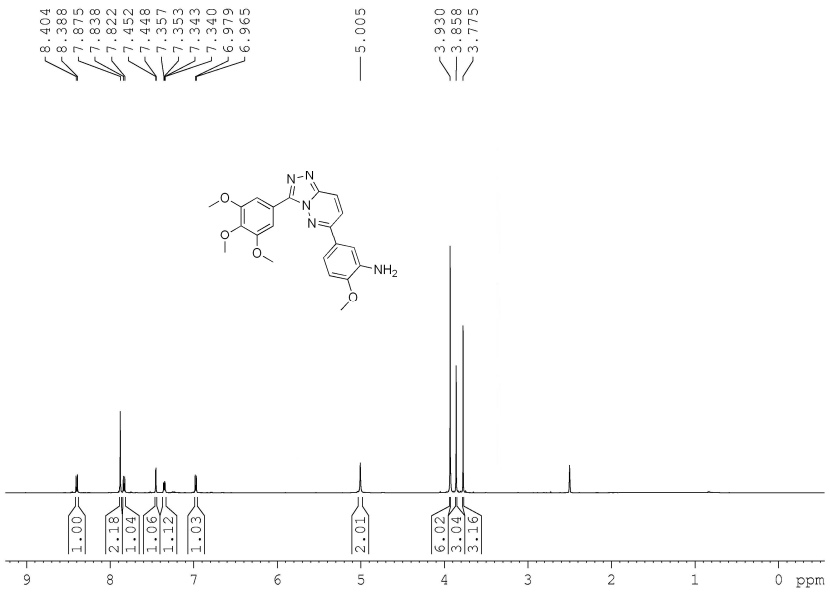


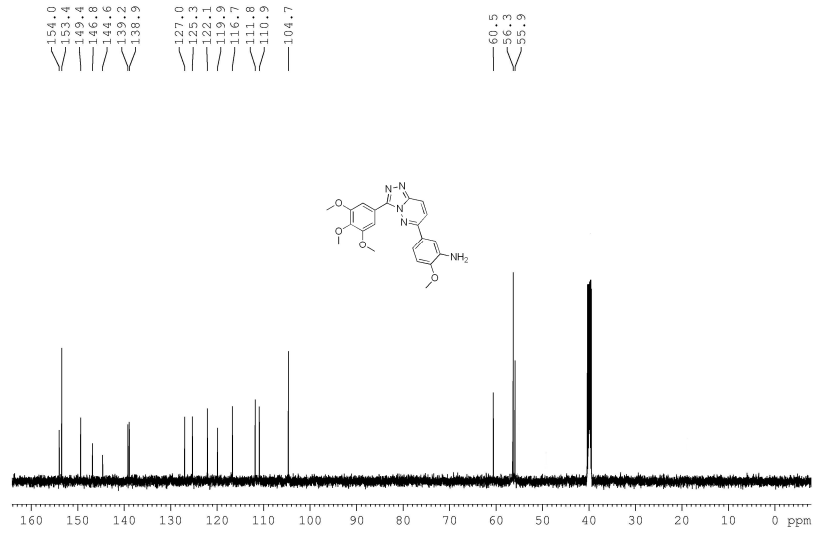
3-(3,4,5-trimethoxyphenyl)-6-(3-nitro-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyrida zine (**4p**).



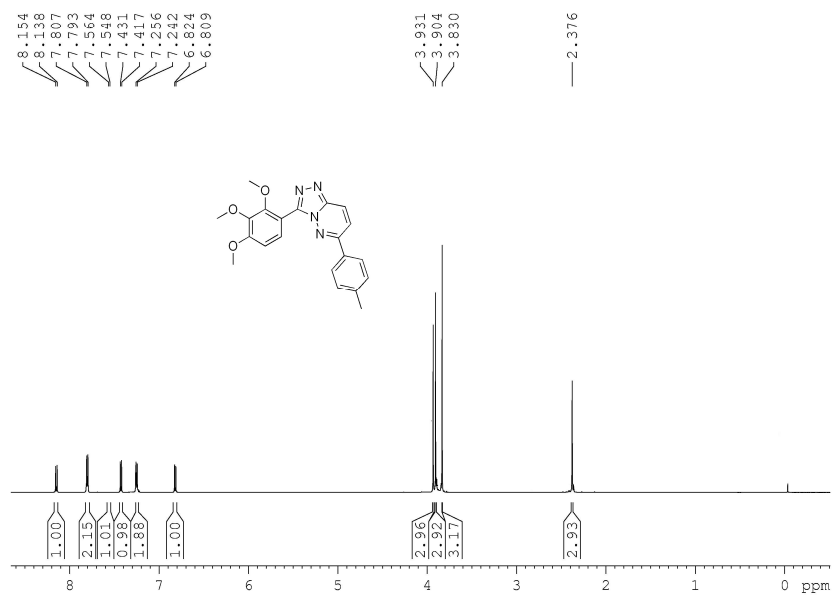


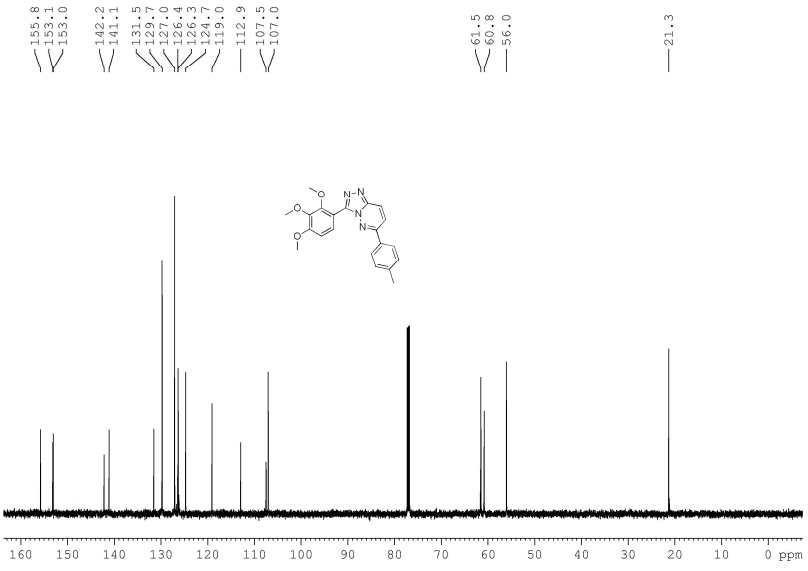
3-(3,4,5-trimethoxyphenyl)-6-(3-amino-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**4q**).



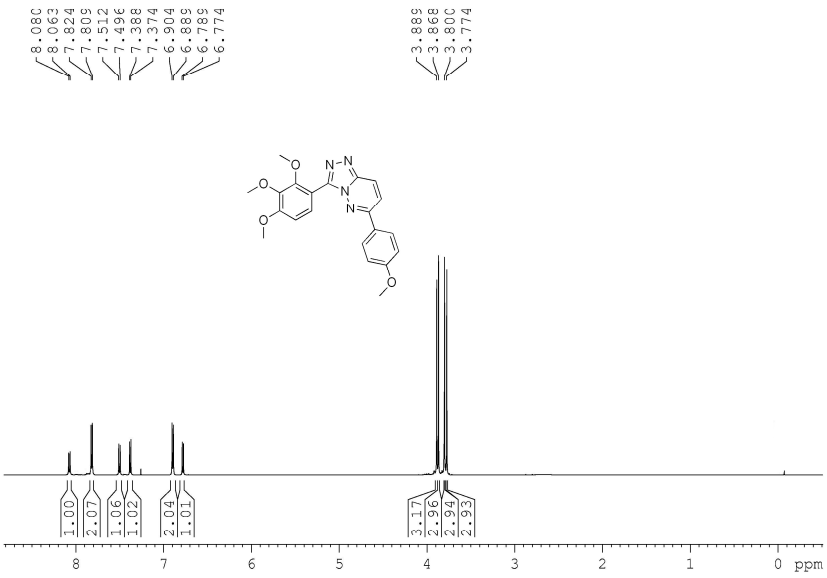


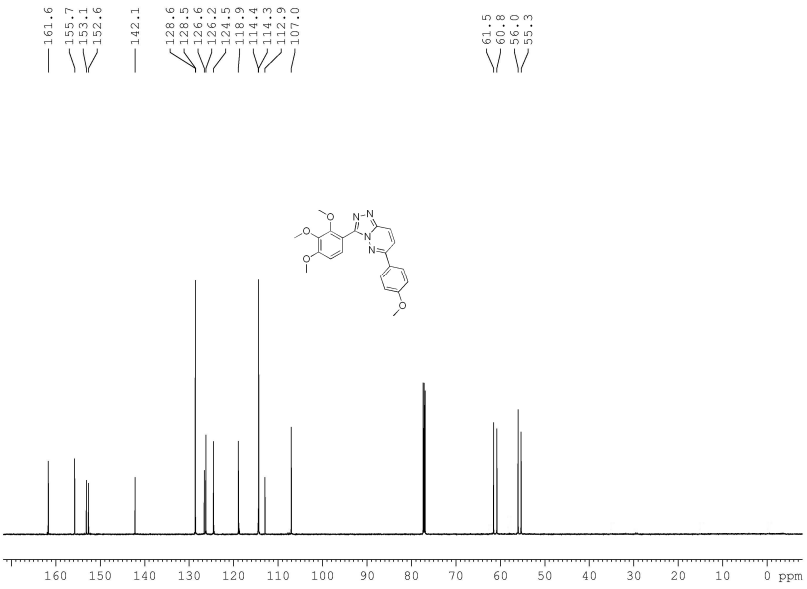
3-(2,3,4-trimethoxyphenyl)-6-(4-methylphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**5a**).



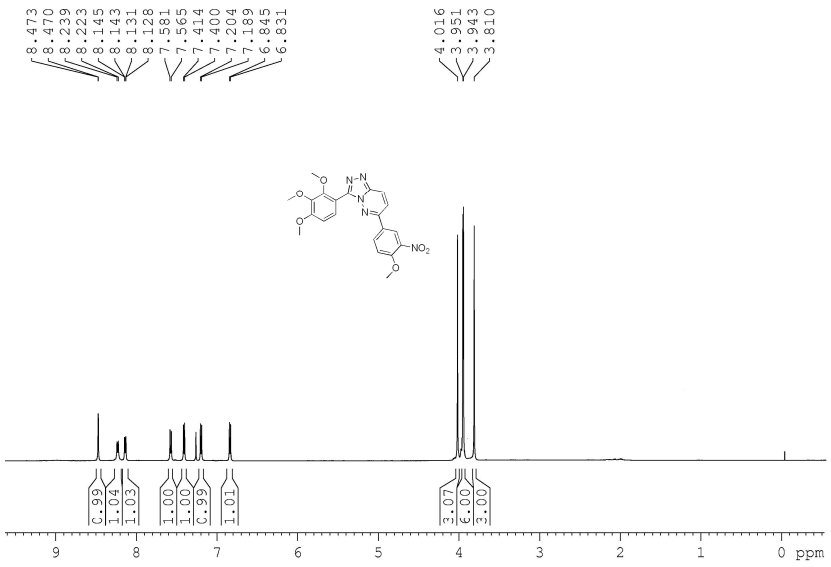


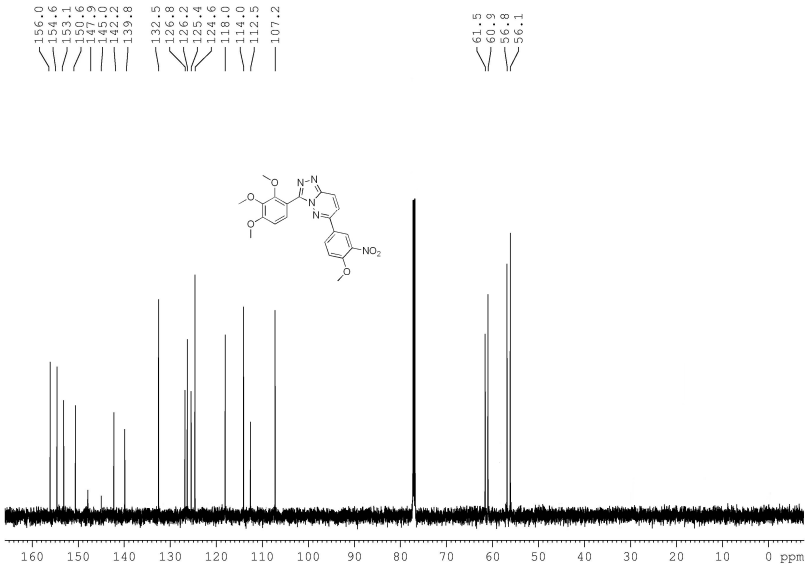
3-(2,3,4-trimethoxyphenyl)-6-(4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**5b**).





3-(2,3,4-trimethoxyphenyl)-6-(3-nitro-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyrida zine (**5c**).





3-(2,3,4-trimethoxyphenyl)-6-(3-amino-4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (**5d**).