

## Synthesis and characterizations of novel thiazolyl-thiadiazole derivatives as telomerase activators

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**Abstract:** Pyridine-3/4-thiocarboxamide derivatives were used as starting materials for the synthesis of the target compounds. The pyridine-3/4-thiocarboxamide derivatives were reacted with ethyl 2-chloroacetoacetate in ethanol to give the thiazole derivatives (**1**, **2**). The two ethyl thiazole-carboxylate derivatives (**1**, **2**) thus obtained were treated with sodium hydroxide solution and ethanol and converted to carboxylic acids (**3**, **4**). The carboxylic acid derivatives (**3**, **4**) were reacted with thiosemicarbazide in phosphoroxo trichloride and aminothiadiazole rings (**5**, **6**) were formed. Thus, two thiazolyl-thiadiazole amine derivatives (**5**, **6**) were obtained. These two derivatives (**5**, **6**) were converted into two chloroacetamidothiadiazole derivatives (**7**, **8**) by reaction with chloroacetylchloride over the amino group in the presence of triethylamine in acetone. After all these steps, the starting materials (**7**, **8**) needed to reach the target compounds were obtained. With the two derivatives (**7**, **8**) obtained in this last step, phenol and thiophenol derivatives were reacted in acetone in the presence of potassium carbonate. The target compounds, thiazolyl-thiadiazole derivatives (**TDA**<sub>1-16</sub>), are completely unique and their structure has been elucidated by elemental analysis, IR, NMR, and MS spectral data. After all these synthesis steps, telomerase activity studies were performed on the target compounds obtained. For this purpose, a PCR ELISA-based TRAP method was used on the heart of zebrafish. According to the enzyme assay results, derivative **TDA**<sub>8</sub> has shown an increase of telomerase enzyme activity.

**Key words:** Thiazole, 1,3,4-thiadiazole, telomerase activity, zebrafish

### 1. Introduction

Thiazole derivatives show physiological activities such as antibacterial, antifungal, antispasmodic, analgesic, anti-inflammatory, anthelmintic, antidiuretic, antituberculosis and anticancer activities.<sup>1-9</sup> The S-C=N group in the thiazole core is the toxophoric unit and is vital to the pharmaceutical applications. In general, structures with this toxophoric group have broad biological activity.<sup>10-12</sup> In recent years it has become popular to investigate the biological activity of five-member heterocyclic compounds containing both nitrogen and sulfur. Some of these compounds, thiadiazole containing two nitrogens and one sulfur, such as 1,2,3-thiadiazole, 1,2,4-thiadiazole, 1,2,5-thiadiazole, and 1,3,4-thiadiazole, have shown antimicrobial, antiinflammatory, antifungal, antibiotic, diuretic, and antidepressant activities. Among these thiadiazole derivatives, studies on the fact that the 1,3,4-thiadiazole core is the most effective are frequently encountered in the literature.<sup>13</sup> Some compounds

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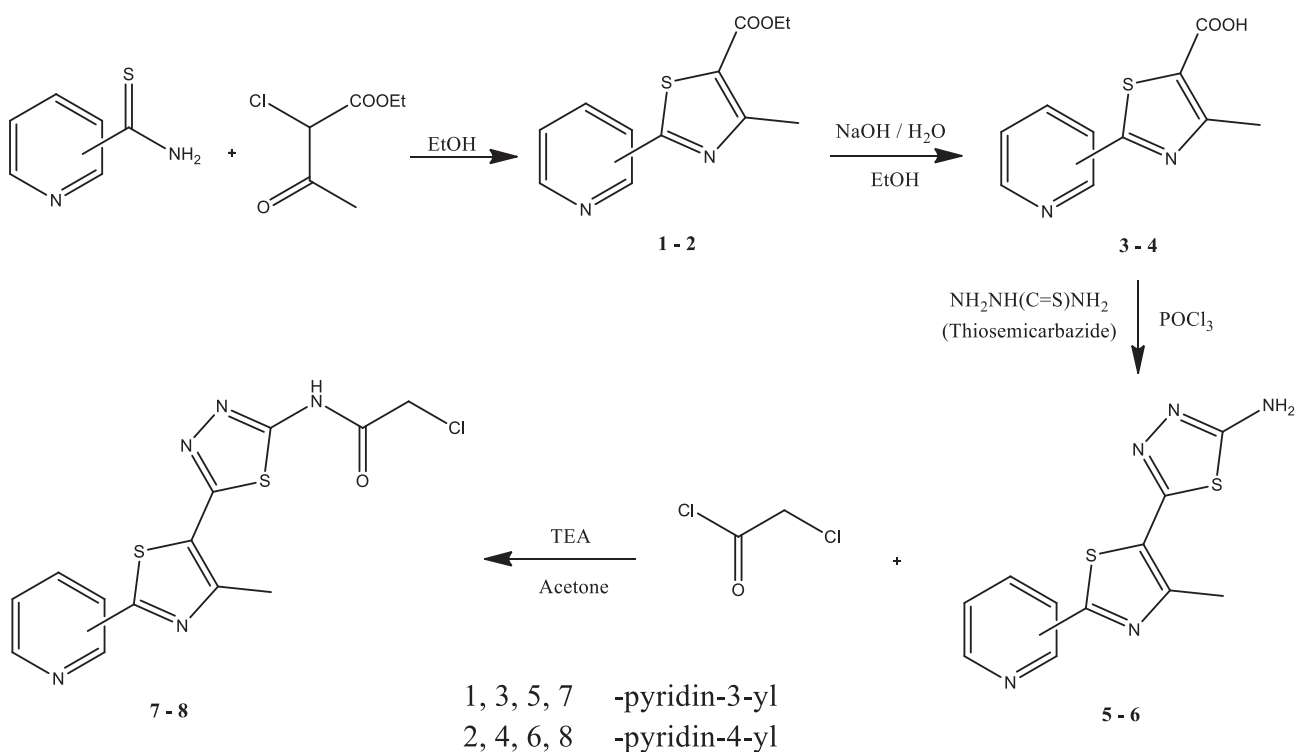
containing thiazole and thiadiazole nuclei have been shown to be effective on colon cancer.<sup>14</sup> It has been reported that antioxidant and anticancer properties of some compounds similarly include thiazole and thiadiazole structures.<sup>15</sup> It is not known in which way these effects are shown. Some thiadiazole derivatives have been found to exhibit high antimicrobial, antifungal, anticancer, anticonvulsant, analgesic, antiinflammatory, anesthetic, and diuretic properties. In addition, they have been reported to be potent enzyme inhibitors of cyclooxygenase, Jun kinase, and carbonic anhydrase, which are important in drug design.<sup>16</sup> As an expert opinion, the inhibition effects of the compounds are generally due to the electronic properties of the substituent at positions 2 and 5 of the compound.<sup>17</sup>

Telomerase is a very important enzyme for the aging process. Telomere shortening can cause aging and death. The loss of telomeric repeats of chromosomes may function as a molecular clock that triggers cellular senescence.<sup>18–20</sup> However, the majority of cancer cells have increased telomerase activity and continuous division of these cells can be attributed to their ability to extend telomeres.<sup>21</sup> Telomerase has been a molecular target for cancer and aging research since its detection.<sup>22</sup> The purpose of this study is to examine the effects of some thiazolyl-thiadiazole derivatives (**TDA<sub>1–16</sub>**) on telomerase activity.

## 2. Results and discussion

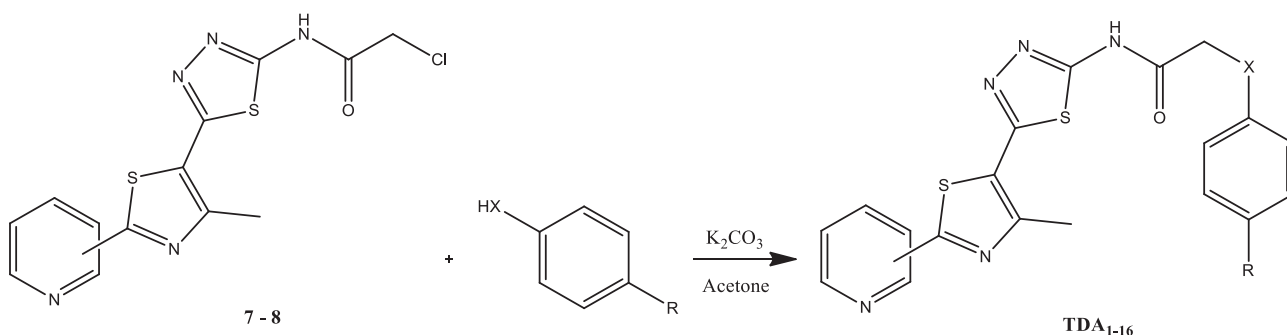
### 2.1. Chemistry

The two compounds (**7**, **8**) to be used as starting materials were synthesized as shown in Figure 1. In the first step, pyridine-thioamide derivatives were converted to pyridinylthiazole derivatives (**1**, **2**) with ethyl 2-



**Figure 1.** Synthesis of starting materials. The conditions of the steps: the first step was refluxed for 5 days, the second step was refluxed for 2 h, the third step was reacted for 3 h in a 90 °C water bath, and the fourth step was stirred at room temperature until the chloroacetyl chloride drop was finished.

chloroacetoacetate. These thiazole compounds (**1**, **2**) obtained were hydrolyzed with sodium hydroxide in the second step. Hydrolysis was easily accomplished but the time was long. In this step, the thiazole esters (**1**, **2**) were converted to carboxylic acid (**3**, **4**). In the third step, these carboxylic acid derivatives (**3**, **4**) were reacted with thiosemicarbazide to form the thiadiazole ring. In this step phosphorus oxychloride was used as an effective agent in withdrawing water from the medium. In this latter step the aminothiadiazole derivatives (**5**, **6**) obtained in the previous step were reacted with chloroacetyl chloride to convert the amino group to the amide group. At this stage, triethylamine was very effective in the progress of the reaction by taking the protons in the medium. At the end of all these steps, two starting materials (**7**, **8**) were obtained. These starting materials (**7**, **8**) were reacted with phenol and thiophenol derivatives as shown in Figure 2 to give the desired compounds (**TDA<sub>1-16</sub>**). The compounds obtained at the end of each step were carefully separated from the medium and recrystallized to be obtained in perfect purity and packed appropriately. Each compound was carefully thin-layer chromatographed and found to be a single spot and subsequently used for spectral analysis. Many spectral studies were carried out to determine the chemical structure of the 16 novel compounds (**TDA<sub>1-16</sub>**). The results obtained with these assays confirmed the structures of the compounds. The melting points, molecular weights, reaction yields, and molecular formulas of all these compounds are given in Table 1.



**Figure 2.** Synthesis of novel thiazolyl-thiadiazole derivatives. Conditions: it was refluxed for 3 h (**Pyridyl** -3 or -4 position; **X**: O, S; and **R**: H,  $CH_3$ ,  $OCH_3$ , Cl).

## 2.2. Computational chemistry

The following values were the binding energies of some amino acids in the 1st region of the telomerase enzyme with the compounds. Investigations were made for four different regions of the telomerase enzyme. However, for the sake of illustration, only the 1st region of the telomerase enzyme is given (Table 2). The smallest negative values indicate the strongest results about binding capacity. The 5 compounds with the smallest values, that is, the strongest bonds, were mathematically selected. The compounds were identified as **TDA<sub>7</sub>**, **TDA<sub>8</sub>**, **TDA<sub>14</sub>**, **TDA<sub>15</sub>**, and **TDA<sub>16</sub>**, respectively, and enzyme studies were performed on these compounds.

## 2.3. Telomerase assay

According to the results of our study, some of the compounds can be used in order to enable telomerase activation. In the application of **TDA<sub>8</sub>**, 2-(4-chlorophenylsulpanyl)-N-(5-(4-methyl-2-pyridin-3-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide, telomerase activity increased to a statistically significant degree (Table 3).

**Table 1.** The chemical properties of the resulting target compounds.

| Compounds               | Pyrid- | -X- | -R                | Formula                                                                        | MW (g/mol) | Mp (°C) | Yield (%) |
|-------------------------|--------|-----|-------------------|--------------------------------------------------------------------------------|------------|---------|-----------|
| <b>TDA<sub>1</sub></b>  | -3-yl  | -O- | -H                | C <sub>19</sub> H <sub>15</sub> N <sub>5</sub> O <sub>2</sub> S <sub>2</sub>   | 409        | 216–217 | 76        |
| <b>TDA<sub>2</sub></b>  | -3-yl  | -O- | -CH <sub>3</sub>  | C <sub>20</sub> H <sub>17</sub> N <sub>5</sub> O <sub>2</sub> S <sub>2</sub>   | 423        | 210–211 | 73        |
| <b>TDA<sub>3</sub></b>  | -3-yl  | -O- | -OCH <sub>3</sub> | C <sub>20</sub> H <sub>17</sub> N <sub>5</sub> O <sub>3</sub> S <sub>2</sub>   | 439        | 209–210 | 77        |
| <b>TDA<sub>4</sub></b>  | -3-yl  | -O- | -Cl               | C <sub>19</sub> H <sub>14</sub> ClN <sub>5</sub> O <sub>2</sub> S <sub>2</sub> | 443        | 207–208 | 79        |
| <b>TDA<sub>5</sub></b>  | -3-yl  | -S- | -H                | C <sub>19</sub> H <sub>15</sub> N <sub>5</sub> OS <sub>3</sub>                 | 425        | 257–258 | 84        |
| <b>TDA<sub>6</sub></b>  | -3-yl  | -S- | -CH <sub>3</sub>  | C <sub>20</sub> H <sub>17</sub> N <sub>5</sub> OS <sub>3</sub>                 | 439        | 258–259 | 83        |
| <b>TDA<sub>7</sub></b>  | -3-yl  | -S- | -OCH <sub>3</sub> | C <sub>20</sub> H <sub>17</sub> N <sub>5</sub> O <sub>2</sub> S <sub>3</sub>   | 455        | 256–257 | 85        |
| <b>TDA<sub>8</sub></b>  | -3-yl  | -S- | -Cl               | C <sub>19</sub> H <sub>14</sub> ClN <sub>5</sub> OS <sub>3</sub>               | 459        | 276–277 | 88        |
| <b>TDA<sub>9</sub></b>  | -4-yl  | -O- | -H                | C <sub>19</sub> H <sub>15</sub> N <sub>5</sub> O <sub>2</sub> S <sub>2</sub>   | 409        | 201–202 | 79        |
| <b>TDA<sub>10</sub></b> | -4-yl  | -O- | -CH <sub>3</sub>  | C <sub>20</sub> H <sub>17</sub> N <sub>5</sub> O <sub>2</sub> S <sub>2</sub>   | 423        | 193–195 | 78        |
| <b>TDA<sub>11</sub></b> | -4-yl  | -O- | -OCH <sub>3</sub> | C <sub>20</sub> H <sub>17</sub> N <sub>5</sub> O <sub>3</sub> S <sub>2</sub>   | 439        | 197–199 | 80        |
| <b>TDA<sub>12</sub></b> | -4-yl  | -O- | -Cl               | C <sub>19</sub> H <sub>14</sub> ClN <sub>5</sub> O <sub>2</sub> S <sub>2</sub> | 443        | 220–221 | 80        |
| <b>TDA<sub>13</sub></b> | -4-yl  | -S- | -H                | C <sub>19</sub> H <sub>15</sub> N <sub>5</sub> OS <sub>3</sub>                 | 425        | 232–237 | 90        |
| <b>TDA<sub>14</sub></b> | -4-yl  | -S- | -CH <sub>3</sub>  | C <sub>20</sub> H <sub>17</sub> N <sub>5</sub> OS <sub>3</sub>                 | 439        | 221–223 | 90        |
| <b>TDA<sub>15</sub></b> | -4-yl  | -S- | -OCH <sub>3</sub> | C <sub>20</sub> H <sub>17</sub> N <sub>5</sub> O <sub>2</sub> S <sub>3</sub>   | 455        | 224–225 | 92        |
| <b>TDA<sub>16</sub></b> | -4-yl  | -S- | -Cl               | C <sub>19</sub> H <sub>14</sub> ClN <sub>5</sub> OS <sub>3</sub>               | 459        | 225–226 | 93        |

**Table 2.** The binding energies for some amino acids for the 1st region of the telomerase enzyme.

| Compounds               | Binding energy (kcal/mol) |       |       |
|-------------------------|---------------------------|-------|-------|
|                         | ALA15                     | ARG12 | GLU54 |
| <b>TDA<sub>1</sub></b>  | -6.74                     | -6.33 | -5.21 |
| <b>TDA<sub>2</sub></b>  | -6.32                     | -5.85 | -6.61 |
| <b>TDA<sub>3</sub></b>  | -6.65                     | -6.27 | -5.50 |
| <b>TDA<sub>4</sub></b>  | -6.57                     | -6.57 | -6.35 |
| <b>TDA<sub>5</sub></b>  | -6.34                     | -5.81 | -5.28 |
| <b>TDA<sub>6</sub></b>  | -5.47                     | -7.11 | -6.04 |
| <b>TDA<sub>7</sub></b>  | -7.58                     | -7.91 | -5.74 |
| <b>TDA<sub>8</sub></b>  | -6.48                     | -7.45 | -     |
| <b>TDA<sub>9</sub></b>  | -6.72                     | -7.29 | -     |
| <b>TDA<sub>10</sub></b> | -6.30                     | -6.30 | -6.03 |
| <b>TDA<sub>11</sub></b> | -6.95                     | -6.95 | -6.31 |
| <b>TDA<sub>12</sub></b> | -6.22                     | -7.09 | -6.28 |
| <b>TDA<sub>13</sub></b> | -7.17                     | -6.79 | -6.67 |
| <b>TDA<sub>14</sub></b> | -7.77                     | -7.77 | -7.14 |
| <b>TDA<sub>15</sub></b> | -8.13                     | -8.13 | -6.62 |
| <b>TDA<sub>16</sub></b> | -6.62                     | -7.35 | -6.98 |

**Table 3.** Relative telomerase activities of the five selected compounds and control.

| Selected compounds      | Relative telomerase activity $\pm$ SE |
|-------------------------|---------------------------------------|
| <b>TDA<sub>7</sub></b>  | 2.53 $\pm$ 0.14                       |
| <b>TDA<sub>8</sub></b>  | 3.21 $\pm$ 0.15*                      |
| <b>TDA<sub>14</sub></b> | 2.6 $\pm$ 0.14                        |
| <b>TDA<sub>15</sub></b> | 2.67 $\pm$ 0.21                       |
| <b>TDA<sub>16</sub></b> | 2.16 $\pm$ 0.14                       |
| Control (vehicle/DMSO)  | 2.36 $\pm$ 0.14                       |

\*Statistically different from vehicle ( $P < 0.05$ ).

#### 2.4. Conclusions

We hypothesized that the presence of pyridine, thiazole, and thiadiazole rings in the structure may show effective results when the target compounds are synthesized. It was frequently seen in the literature that these rings had various biological activities. The synthesized compounds were diversified with phenol and thiophenol derivatives. Five of them were selected by molecular docking method. Enzyme assays were performed for these five compounds. As a result of this study, it was concluded that the compounds activated the telomerase enzyme. Thus, they were referred to as telomerase activators. Clinical studies for telomerase activation are currently carried out in different ways, such as direct telomerase activation through drugs or supplements such as cycloastragenol and as gene therapy. Telomerase gene therapy in adult and old mice delays aging and increases longevity.<sup>23,24</sup> Telomerase activators are important for antiaging and telomerase-dependent disease treatments. According to our results, compound **TDA<sub>8</sub>** has the potential to be used as an antiaging agent after detailed studies. The pyridine in the structure of this compound is attached to the 3-position structure and the substituent is a p-chlorothiophenol residue. While similar compounds are being synthesized, consideration of these points may be important to ensure that the biological activity to be achieved is strong.

### 3. Experimental

The melting points of the target compounds were tested in open capillaries on a WRS-2A Microprocessor melting-point apparatus. Elemental analysis was performed on a LECO CHNS analyzer and the obtained values were acceptable for the calculated values. The IR spectra of the compounds were obtained from a Shimadzu 8400 FT-IR using the KBr disk preparation method. The <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on Bruker Advance III NaNoBay FT-NMR spectrometers (400 MHz for <sup>1</sup>H NMR, 100 MHz for <sup>13</sup>C NMR) using DMSO-d<sub>6</sub> as the solvent. Tetramethylsilane (TMS) was used as the internal standard when the spectra were taken and chemical shift values are given in ppm. The mass spectra were obtained by applying the electron spray method with an Agilent 1100 MSD mass spectrometer. The chemicals, reagents, and solvents supplied by Merck, Aldrich, and Riedel-de Haen were used as if they were not subjected to any treatment.

#### 3.1. General procedure for the synthesis of compounds 1 and 2

Pyridine-3-thiocarboxamide (0.50 mol) and pyridine-4-thiocarboxamide (0.50 mol) were separately dissolved in ethanol (100.0 mL) and then ethyl 2-chloroacetoacetate (0.55 mol) was added to the reaction medium. The mixture was refluxed for 5 days. After the mixture was controlled by thin-layer chromatography, it was poured into water and neutralized with concentrated sodium acetate solution. The precipitated solid was filtered off with water and recrystallized from ethanol (Figure 1).

### 3.2. General procedure for the synthesis of compounds 3 and 4

The ester compounds (0.45 mol) obtained in the previous step were dissolved in ethanol (100.0 mL) and hydrolyzed separately with the sodium hydroxide solution (0.45 mol equiv.) added thereto. The reaction mixture was poured into water and neutralized with acetic acid. The precipitated solid was filtered off with water and recrystallized from ethanol (Figure 1).

### 3.3. General procedure for the synthesis of compounds 5 and 6

The resulting carboxylic acid derivatives (0.40 mol) were reacted separately with phosphorus oxychloride (50.0 mL) and thiosemicarbazide (0.42 mol) in a 90 °C water bath for 3 h. After the reaction was complete, the residue was neutralized with concentrated sodium hydroxide solution. The precipitate was washed with water and filtered. The dried material was recrystallized in ethanol after being checked by thin-layer chromatography (Figure 1).

### 3.4. General procedure for the synthesis of compounds 7 and 8

The aminothiadiazole compounds (0.35 mol) obtained in the previous step were dissolved separately in acetone (80.0 mL) and triethylamine (0.37 mol) was added. This mixture was stirred at room temperature by the dropwise addition of chloroacetyl chloride (0.37 mol). After dripping the mixture was poured into iced water and precipitated. The precipitate was washed with water and filtered. The dried material was recrystallized in ethanol after being checked by thin-layer chromatography (Figure 1).

Two starting materials were synthesized at the end of the 4 steps mentioned here. The compounds obtained so far have been found in the literature. These synthesized compounds were compared and checked with the literature data. Therefore, spectral data of compounds 1–8 are not presented in this study.

### 3.5. General procedure for the synthesis of compounds TDA<sub>1–16</sub>

The synthesized starting materials (0.0030 mol), potassium carbonate (0.0035 mol), and phenol-thiophenol reagents (0.0031 mol) were refluxed in acetone (40.0 mL) for 3 h. At the end of the reaction, the crude product was precipitated by the addition of water. The precipitate was washed with water and filtered. The dried material was recrystallized in ethanol after being checked by thin-layer chromatography (Figure 2). The resulting target compounds were packaged for characterization studies and enzyme studies and stored under appropriate conditions.

#### 3.5.1. 2-Phenoxy-N-(5-(4-methyl-2-pyridin-3-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>1</sub>)

Dark yellow solid; yield 76%; mp 216–217 °C; IR (KBr)  $\nu_{\max}$  (cm<sup>-1</sup>): 3352 (N-H), 3060 (aromatic C-H), 2910 (aliphatic C-H), 1684 (C=O), 1602, 1535, 1481 (C=C, C=N). <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 2.72 (s, thiazolymethyl, 3H), 4.82 (s, methylene, 2H), 6.88 (t, J: 6.5 Hz, phenyl, 1H), 6.91 (d, J: 7.0 Hz, phenyl, 2H), 6.97 (d, J: 6.0 Hz, phenyl, 2H), 7.56 (dd, J: 8.0 Hz, J: 5.0 Hz, pyridyl, 1H), 8.34 (d, J: 8.0 Hz, pyridyl, 1H), 8.70 (d, J: 4.0 Hz, pyridyl, 1H), 9.16 (s, pyridyl, 1H), 13.22 (s, NH). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 15.3, 66.6, 115.0, 120.6, 123.3, 123.6, 127.8, 129.6, 133.0, 148.9, 149.8, 152.3, 157.6, 158.8, 163.9, 167.9, 168.3. Anal. Calcd. for C<sub>19</sub>H<sub>15</sub>N<sub>5</sub>O<sub>2</sub>S<sub>2</sub>: C, 62.10; H, 4.11; N, 7.62; Found: C, 61.97; H, 4.13; N, 7.49. ES-MS  $m/z$  410.20 [M+H]<sup>+</sup> (92).

**3.5.2. 2-(4-Methylphenoxy)-N-(5-(4-methyl-2-pyridin-3-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>2</sub>)**

Dark brown solid; yield 73%; mp 210–211 °C; IR (KBr) $\nu_{\max}$  (cm<sup>-1</sup>): 3349 (N-H), 3065 (aromatic C-H), 2908 (aliphatic C-H), 1685 (C=O), 1605, 1532, 1474 (C=C, C=N). <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 2.25 (s, phenylmethyl, 3H), 2.70 (s, thiazolymethyl, 3H), 4.80 (s, methylene, 2H), 6.90 (d, J: 8.0 Hz, phenyl, 2H), 6.96 (d, J: 8.0 Hz, phenyl, 2H), 7.56 (dd, J: 7.0 Hz, J: 5.0 Hz, pyridyl, 1H), 8.32 (d, J: 8.0 Hz, pyridyl, 1H), 8.69 (s, pyridyl, 1H), 9.15 (s, pyridyl, 1H), 13.20 (s, NH). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 15.3, 21.3, 66.6, 117.1, 120.6, 123.3, 127.8, 131.3, 132.4, 133.0, 148.9, 149.8, 152.3, 158.8, 160.2, 163.9, 167.9, 168.3. Anal. Calcd. for C<sub>20</sub>H<sub>17</sub>N<sub>5</sub>O<sub>2</sub>S<sub>2</sub>: C, 56.72; H, 4.05; N, 16.54; Found: C, 56.25; H, 4.00; N, 16.35. ES-MS  $m/z$  424.10 [M+H]<sup>+</sup> (86).

**3.5.3. 2-(4-Methoxyphenoxy)-N-(5-(4-methyl-2-pyridin-3-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>3</sub>)**

Dark brown solid; yield 77%; mp 209–210 °C; IR (KBr) $\nu_{\max}$  (cm<sup>-1</sup>): 3353 (N-H), 3053 (aromatic C-H), 2912 (aliphatic C-H), 1685 (C=O), 1600, 1535, 1491 (C=C, C=N). <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 2.67 (s, thiazolymethyl, 3H), 3.73 (s, methoxy, 3H), 4.79 (s, methylene, 2H), 6.89 (d, J: 9.0 Hz, phenyl, 2H), 6.92 (d, J: 7.0 Hz, phenyl, 2H), 7.52 (t, J: 6.0 Hz, pyridyl, 1H), 8.29 (d, J: 7.0 Hz, pyridyl, 1H), 8.67 (s, pyridyl, 1H), 9.11 (s, pyridyl, 1H), 13.23 (s, NH). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 17.4, 56.0, 75.2, 120.3, 123.4, 124.3, 128.2, 131.0, 133.7, 136.1, 138.6, 146.9, 151.4, 152.7, 153.6, 158.5, 163.5, 165.6. Anal. Calcd. for C<sub>20</sub>H<sub>17</sub>N<sub>5</sub>O<sub>3</sub>S<sub>2</sub>: C, 54.66; H, 3.90; N, 15.93; Found: C, 54.58; H, 3.98; N, 16.01. ES-MS  $m/z$  440.30 [M+H]<sup>+</sup> (99).

**3.5.4. 2-(4-Chlorophenoxy)-N-(5-(4-methyl-2-pyridin-3-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>4</sub>)**

Dark brown solid; yield 79%; mp 207–208 °C; IR (KBr) $\nu_{\max}$  (cm<sup>-1</sup>): 3356 (N-H), 3058 (aromatic C-H), 2915 (aliphatic C-H), 1683 (C=O), 1605, 1540, 1487 (C=C, C=N). <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 2.69 (s, thiazolymethyl, 3H), 4.80 (s, methylene, 2H), 6.90 (d, J: 8.5 Hz, phenyl, 2H), 7.03 (d, J: 8.0 Hz, phenyl, 2H), 7.54 (t, J: 6.0 Hz, pyridyl, 1H), 8.31 (d, J: 7.5 Hz, pyridyl, 1H), 8.69 (s, pyridyl, 1H), 9.13 (s, pyridyl, 1H), 13.22 (s, NH). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 15.3, 66.6, 115.2, 120.6, 123.3, 127.8, 129.4, 130.5, 133.0, 148.9, 149.8, 152.3, 158.5, 158.8, 163.9, 167.9, 168.3. Anal. Calcd. for C<sub>19</sub>H<sub>14</sub>ClN<sub>5</sub>O<sub>2</sub>S<sub>2</sub>: C, 51.41; H, 3.18; N, 7.99; Found: C, 51.45; H, 3.19; N, 8.01. ES-MS  $m/z$  444.10 [M+H]<sup>+</sup> (94).

**3.5.5. 2-Phenylsulpanyl-N-(5-(4-methyl-2-pyridin-3-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>5</sub>)**

Light yellow solid; yield 84%; mp 257–258 °C; IR (KBr) $\nu_{\max}$  (cm<sup>-1</sup>): 3368 (N-H), 3084 (aromatic C-H), 2908 (aliphatic C-H), 1682 (C=O), 1601, 1543, 1482 (C=C, C=N). <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 2.70 (s, thiazolymethyl, 3H), 4.06 (s, methylene, 2H), 7.24 (tt, J: 6.0 Hz, J: 1.5 Hz, phenyl, 1H), 7.34 (td, J: 6.5 Hz, J: 2.0 Hz, phenyl, 2H), 7.42 (dt, J: 8.0 Hz, J: 1.5 Hz, phenyl, 2H), 7.56 (ddd, J: 8.0 Hz, J: 5.0 Hz, J: 0.5 Hz, pyridyl, 1H), 8.34 (dt, J: 8.5 Hz, J: 2.0 Hz, pyridyl, 1H), 8.70 (dd, J: 4.5 Hz, J: 1.5 Hz, pyridyl, 1H), 9.16 (d, J: 1.5 Hz, pyridyl, 1H), 13.08 (s, NH). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$  (ppm): 15.3, 37.8, 120.6, 123.3, 127.8, 129.1,

129.9, 131.4, 133.0, 134.1, 148.9, 149.8, 152.3, 158.8, 163.9, 164.0, 168.3. Anal. Calcd. for  $C_{19}H_{15}N_5OS_3$ : C, 53.63; H, 3.55; N, 16.46; Found: C, 53.19; H, 3.60; N, 16.52. ES-MS  $m/z$  426.20  $[M+H]^+$  (76).

**3.5.6. 2-(4-Methylphenylsulpanyl)-N-(5-(4-methyl-2-pyridin-3-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>6</sub>)**

Yellow brown solid; yield 83%; mp 258–259 °C; IR (KBr) $\nu_{\max}$  ( $cm^{-1}$ ): 3370 (N-H), 3080 (aromatic C-H), 2906 (aliphatic C-H), 1680 (C=O), 1602, 1538, 1480 (C=C, C=N).  $^1H$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.26 (s, phenylmethyl, 3H), 2.69 (s, thiazolymethyl, 3H), 3.98 (s, methylene, 2H), 7.15 (d, J: 8.0 Hz, phenyl, 2H), 7.32 (d, J: 8.0 Hz, phenyl, 2H), 7.55 (t, J: 6.0 Hz, pyridyl, 1H), 8.33 (d, J: 8.0 Hz, pyridyl, 1H), 8.69 (s, pyridyl, 1H), 9.15 (s, pyridyl, 1H), 13.03 (s, NH).  $^{13}C$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 15.3, 21.3, 37.8, 120.6, 123.3, 127.8, 127.9, 129.8, 133.0, 134.1, 144.3, 148.9, 149.8, 152.3, 158.8, 163.9, 164.0, 168.3. Anal. Calcd. for  $C_{20}H_{17}N_5OS_3$ : C, 54.65; H, 3.90; N, 15.93; Found: C, 54.72; H, 3.92; N, 16.02. ES-MS  $m/z$  440.10  $[M+H]^+$  (100).

**3.5.7. 2-(4-Methoxyphenylsulpanyl)-N-(5-(4-methyl-2-pyridin-3-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>7</sub>)**

Yellow brown solid; yield 85%; mp 256–257 °C; IR (KBr) $\nu_{\max}$  ( $cm^{-1}$ ): 3367 (N-H), 3075 (aromatic C-H), 2911 (aliphatic C-H), 1681 (C=O), 1604, 1532, 1473 (C=C, C=N).  $^1H$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.70 (s, thiazolymethyl, 3H), 3.74 (s, methoxy, 3H), 3.88 (s, methylene, 2H), 6.93 (dt, J: 6.5 Hz, J: 2.0 Hz, phenyl, 2H), 7.40 (dt, J: 9.0 Hz, J: 2.5 Hz, phenyl, 2H), 7.56 (dd, J: 7.5 Hz, J: 5.0 Hz, pyridyl, 1H), 8.34 (dt, J: 8.0 Hz, J: 2.0 Hz, pyridyl, 1H), 8.70 (s, pyridyl, 1H), 9.15 (s, pyridyl, 1H), 12.96 (s, NH).  $^{13}C$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 15.3, 37.8, 55.5, 114.8, 120.6, 123.3, 127.8, 132.2, 133.0, 134.1, 148.9, 149.8, 152.3, 158.8, 160.8, 163.9, 164.0, 168.3. Anal. Calcd. for  $C_{20}H_{17}N_5O_2S_3$ : C, 52.73; H, 3.76; N, 15.37; Found: C, 52.52; H, 3.84; N, 15.39. ES-MS  $m/z$  456.10  $[M+H]^+$  (93).

**3.5.8. 2-(4-Chlorophenylsulpanyl)-N-(5-(4-methyl-2-pyridin-3-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>8</sub>)**

Yellow brown solid; yield 88%; mp 276–277 °C; IR (KBr) $\nu_{\max}$  ( $cm^{-1}$ ): 3363 (N-H), 3068 (aromatic C-H), 2913 (aliphatic C-H), 1686 (C=O), 1601, 1535, 1474 (C=C, C=N).  $^1H$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.73 (s, thiazolymethyl, 3H), 4.08 (s, methylene, 2H), 7.41 (d, J: 8.5 Hz, phenyl, 2H), 7.45 (d, J: 8.0 Hz, phenyl, 2H), 7.57 (s, pyridyl, 1H), 8.36 (d, J: 7.0 Hz, pyridyl, 1H), 8.71 (s, pyridyl, 1H), 9.18 (s, pyridyl, 1H), 13.10 (s, NH).  $^{13}C$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 17.4, 36.7, 120.2, 124.6, 129.1, 129.8, 131.3, 134.9, 136.3, 138.8, 145.6, 150.1, 153.1, 153.3, 158.8, 162.1, 168.1. Anal. Calcd. for  $C_{19}H_{14}ClN_5OS_3$ : C, 49.61; H, 3.07; N, 7.71; Found: C, 49.52; H, 3.11; N, 7.65. ES-MS  $m/z$  460.40  $[M+H]^+$  (84).

**3.5.9. 2-Phenoxy-N-(5-(4-methyl-2-pyridin-4-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>9</sub>)**

Red brown solid; yield 79%; mp 201–202 °C; IR (KBr) $\nu_{\max}$  ( $cm^{-1}$ ): 3351 (N-H), 3054 (aromatic C-H), 2926 (aliphatic C-H), 1683 (C=O), 1600, 1530, 1484 (C=C, C=N).  $^1H$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.76 (s, thiazolymethyl, 3H), 4.83 (s, methylene, 2H), 6.88 (t, J: 6.5 Hz, phenyl, 1H), 6.91 (d, J: 7.5 Hz, phenyl, 2H), 6.97 (d, J: 6.5 Hz, phenyl, 2H), 7.98 (d, J: 5.5 Hz, pyridyl, 2H), 8.77 (d, J: 5.0 Hz, pyridyl, 2H) 13.26 (s, NH).



$^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 17.4, 75.1, 120.4, 124.3, 128.1, 131.0, 133.6, 136.2, 138.8, 151.4, 152.6, 153.6, 158.4, 163.6, 165.5. Anal. Calcd. for  $\text{C}_{19}\text{H}_{15}\text{N}_5\text{O}_2\text{S}_2$ : C, 55.73; H, 3.69; N, 17.10; Found: C, 55.62; H, 3.73; N, 17.31. ES-MS  $m/z$  410.10  $[\text{M}+\text{H}]^+$  (100).

**3.5.10. 2-(4-Methylphenoxy)-N-(5-(4-methyl-2-pyridin-4-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>10</sub>)**

Red brown solid; yield 78%; mp 193–195 °C; IR (KBr) $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3349 (N-H), 3052 (aromatic C-H), 2921 (aliphatic C-H), 1684 (C=O), 1610, 1540, 1480 (C=C, C=N).  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.27 (s, phenylmethyl, 3H), 2.76 (s, thiazolylmethyl, 3H), 4.74 (s, methylene, 2H), 6.90 (d, J: 8.5 Hz, phenyl, 2H), 6.96 (d, J: 8.0 Hz, phenyl, 2H), 7.97 (d, J: 6.0 Hz, pyridyl, 2H), 8.75 (d, J: 5.5 Hz, pyridyl, 2H), 13.30 (s, NH).  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 15.3, 21.3, 66.6, 117.1, 120.7, 131.0, 131.3, 132.4, 133.0, 149.1, 152.3, 158.8, 160.2, 163.9, 165.1, 167.9. Anal. Calcd. for  $\text{C}_{20}\text{H}_{17}\text{N}_5\text{O}_2\text{S}_2$ : C, 56.72; H, 4.05; N, 16.54; Found: C, 56.83; H, 4.02; N, 16.58. ES-MS  $m/z$  424.20  $[\text{M}+\text{H}]^+$  (87).

**3.5.11. 2-(4-Methoxyphenoxy)-N-(5-(4-methyl-2-pyridin-4-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>11</sub>)**

Red brown solid; yield 80%; mp 197–199 °C; IR (KBr) $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3353 (N-H), 3058 (aromatic C-H), 2923 (aliphatic C-H), 1686 (C=O), 1608, 1543, 1473 (C=C, C=N).  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.72 (s, thiazolylmethyl, 3H), 3.73 (s, methoxy, 3H), 4.77 (s, methylene, 2H), 6.89 (d, J: 9.0 Hz, phenyl, 2H), 6.92 (d, J: 7.5 Hz, phenyl, 2H), 7.98 (d, J: 5.5 Hz, pyridyl, 2H), 8.75 (s, pyridyl, 2H), 13.27 (s, NH).  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 15.3, 55.5, 66.6, 114.3, 115.5, 120.7, 131.0, 133.0, 149.1, 152.3, 155.5, 158.5, 158.8, 163.9, 165.1, 167.9. Anal. Calcd. for  $\text{C}_{20}\text{H}_{17}\text{N}_5\text{O}_3\text{S}_2$ : C, 54.66; H, 3.90; N, 15.93; Found: C, 54.81; H, 4.01; N, 16.08. ES-MS  $m/z$  440.30  $[\text{M}+\text{H}]^+$  (100).

**3.5.12. 2-(4-Chlorophenoxy)-N-(5-(4-methyl-2-pyridin-4-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>12</sub>)**

Red brown solid; yield 80%; mp 220–221 °C; IR (KBr) $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3355 (N-H), 3054 (aromatic C-H), 2925 (aliphatic C-H), 1687 (C=O), 1605, 1537, 1476 (C=C, C=N).  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.71 (s, thiazolylmethyl, 3H), 4.79 (s, methylene, 2H), 6.90 (d, J: 8.0 Hz, phenyl, 2H), 7.03 (d, J: 8.0 Hz, phenyl, 2H), 7.93 (d, J: 5.5 Hz, pyridyl, 2H), 8.74 (s, pyridyl, 2H), 13.26 (s, NH).  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 15.3, 66.6, 115.2, 120.7, 129.4, 130.5, 131.0, 133.0, 149.1, 152.3, 158.5, 158.8, 163.9, 165.1, 167.9. Anal. Calcd. for  $\text{C}_{19}\text{H}_{14}\text{ClN}_5\text{O}_2\text{S}_2$ : C, 51.41; H, 3.18; N, 15.78; Found: C, 51.59; H, 3.33; N, 15.52. ES-MS  $m/z$  444.40  $[\text{M}+\text{H}]^+$  (100).

**3.5.13. 2-Phenylsulphanyl-N-(5-(4-methyl-2-pyridin-4-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>13</sub>)**

Brown solid; yield 90%; mp 232–237 °C; IR (KBr) $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3369 (N-H), 3082 (aromatic C-H), 2920 (aliphatic C-H), 1680 (C=O), 1602, 1535, 1481 (C=C, C=N).  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.72 (s, thiazolylmethyl, 3H), 4.07 (s, methylene, 2H), 7.24 (tt, J: 7.0 Hz, J: 1.5 Hz, phenyl, 1H), 7.34 (td, J: 6.5 Hz, J:

2.0 Hz, phenyl, 2H), 7.42 (dt, J: 7.5 Hz, J: 1.0 Hz, phenyl, 2H), 7.93 (dd, J: 4.5 Hz, J: 1.5 Hz, pyridyl, 2H), 8.74 (d, J: 6.0 Hz, pyridyl, 2H), 13.11 (s, NH).  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 15.3, 37.8, 120.7, 129.1, 129.9, 131.0, 131.4, 133.0, 134.1, 149.1, 152.3, 158.8, 163.9, 164.0, 165.1. Anal. Calcd. for  $\text{C}_{19}\text{H}_{15}\text{N}_5\text{OS}_3$ : C, 53.63; H, 3.55; N, 16.46; Found: C, 53.61; H, 3.51; N, 16.57. ES-MS  $m/z$  426.10  $[\text{M}+\text{H}]^+$  (92).

#### 3.5.14. 2-(4-Methylphenylsulphanyl)-N-(5-(4-methyl-2-pyridin-4-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>14</sub>)

Brown solid; yield 90%; mp 221–223 °C; IR (KBr) $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3371 (N-H), 3080 (aromatic C-H), 2918 (aliphatic C-H), 1682 (C=O), 1600, 1536, 1486 (C=C, C=N).  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.28 (s, phenylmethyl, 3H), 2.72 (s, thiazolylmethyl, 3H), 4.00 (s, methylene, 2H), 7.17 (d, J: 8.0 Hz, phenyl, 2H), 7.33 (d, J: 8.0 Hz, phenyl, 2H), 7.93 (d, J: 5.5 Hz, pyridyl, 2H), 8.75 (d, J: 6.0 Hz, pyridyl, 2H), 13.07 (s, NH).  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 17.4, 20.5, 36.7, 120.1, 124.6, 129.5, 129.8, 131.0, 136.4, 138.8, 150.8, 153.0, 153.3, 158.9, 163.7, 168.0. Anal. Calcd. for  $\text{C}_{20}\text{H}_{17}\text{N}_5\text{OS}_3$ : C, 54.65; H, 3.90; N, 15.93; Found: C, 54.61; H, 3.88; N, 15.97. ES-MS  $m/z$  440.30  $[\text{M}+\text{H}]^+$  (87).

#### 3.5.15. 2-(4-Methoxyphenylsulphanyl)-N-(5-(4-methyl-2-pyridin-4-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>15</sub>)

Brown solid; yield 92%; mp 224–225 °C; IR (KBr) $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3370 (N-H), 3078 (aromatic C-H), 2916 (aliphatic C-H), 1684 (C=O), 1603, 1538, 1479 (C=C, C=N).  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.72 (s, thiazolylmethyl, 3H), 3.74 (s, methoxy, 3H), 3.89 (s, methylene, 2H), 6.93 (d, J: 9.0 Hz, phenyl, 2H), 7.40 (d, J: 9.0 Hz, phenyl, 2H), 7.93 (d, J: 6.0 Hz, pyridyl, 2H), 8.74 (d, J: 5.0 Hz, pyridyl, 2H), 13.00 (s, NH).  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 15.3, 37.8, 55.5, 114.8, 120.7, 131.0, 132.2, 133.0, 134.1, 149.1, 152.3, 158.8, 160.8, 163.9, 164.0, 165.1. Anal. Calcd. for  $\text{C}_{20}\text{H}_{17}\text{N}_5\text{O}_2\text{S}_3$ : C, 52.73; H, 3.76; N, 15.37; Found: C, 52.79; H, 3.60; N, 15.88. ES-MS  $m/z$  456.10  $[\text{M}+\text{H}]^+$  (78).

#### 3.5.16. 2-(4-Chlorophenylsulphanyl)-N-(5-(4-methyl-2-pyridin-4-ylthiazol-5-yl)-[1,3,4]-thiadiazol-2-yl)acetamide (TDA<sub>16</sub>)

Brown solid; yield 93%; mp 225–226 °C; IR (KBr) $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3368 (N-H), 3084 (aromatic C-H), 2921 (aliphatic C-H), 1683 (C=O), 1602, 1542, 1477 (C=C, C=N).  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 2.71 (s, thiazolylmethyl, 3H), 4.08 (s, methylene, 2H), 7.41 (d, J: 9.0 Hz, phenyl, 2H), 7.45 (d, J: 9.0 Hz, phenyl, 2H), 7.93 (d, J: 5.5 Hz, pyridyl, 2H), 8.74 (d, J: 5.0 Hz, pyridyl, 2H), 13.12 (s, NH).  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  (ppm): 15.3, 37.8, 120.7, 129.2, 131.0, 131.2, 133.0, 133.2, 135.2, 149.1, 152.3, 158.8, 163.9, 164.0, 165.1. Anal. Calcd. for  $\text{C}_{19}\text{H}_{14}\text{ClN}_5\text{OS}_3$ : C, 49.61; H, 3.07; N, 7.71; Found: C, 49.82; H, 3.12; N, 7.78. ES-MS  $m/z$  460.00  $[\text{M}+\text{H}]^+$  (100).

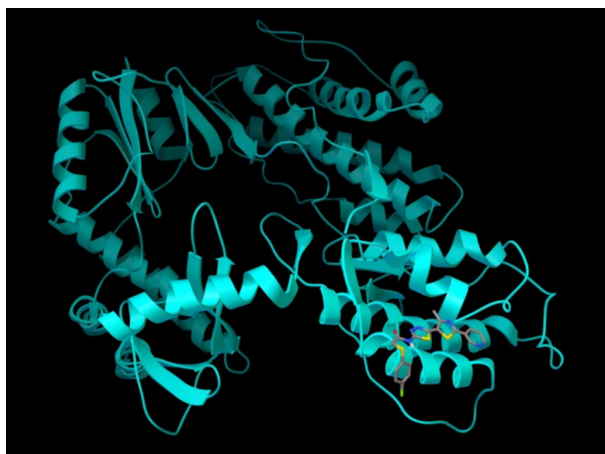
### 3.6. Computational methods

Computational docking can be used to predict bound conformations and free energies of binding for small molecular ligands to macromolecular targets. The AutoDock 4.0 MGLTolls package was used for the docking of the target compounds (TDA<sub>1–16</sub>) to the telomerase.<sup>25,26</sup> Telomerase (PDB Code: 3KYL) and the molecule structure in 3D were optimized by UCSF CHIMERA 1.10.2 before the docking procedure. Telomerase–molecule

interactions were evaluated using grid-based atomic affinity potentials. At the end of the reaction time the final free energy of interaction from the dispersion-repulsion energies, directional hydrogen bonding, and dispersion screened electrostatic states and desolvation were calculated. Atomic solvation parameters and fragmental volumes were assigned to the protein atoms with the source program AutoDock Vina included in the free AutoDock 4.0 program package. In all the docking simulations we used grid maps with  $60 \times 60 \times 60$  points. A total of 25 runs using LGA were performed in each separate case where the substrates to be docked were free to rotate around their center single bonds.

### 3.7. Selection of the target compounds

We investigated the possible telomerase activator effect of five TDA compounds. We selected the five compounds for telomerase assay according to molecular docking results. These five compounds had high binding affinity to telomerase (Figure 3). The five compounds were dissolved in DMSO. We chose a concentration of  $10^{-5}$  M for these molecules according to our previous experiences.



**Figure 3.** Binding of the TDA<sub>8</sub> compound to the first region of the telomerase enzyme.

### 3.8. Maintenance of zebrafish

We used the heart of zebrafish for measuring telomerase enzyme activity. Zebrafish (*Danio rerio*) were maintained at  $24 \pm 2$  °C with a light/dark cycle of 14/10 h and they were fed with dry flake food. The fish were anesthetized with ice before injections and excising of organs. Ten fish were analyzed from each group. Compounds and DMSO were injected as 6  $\mu$ L into the fish. After 180 min, hearts were excised. All zebrafish applications were approved by the Ethics Committee of Mehmet Akif Ersoy University (30.11.2016/236).

### 3.9. Telomerase assay

Telomerase activities were measured with the Roche TeloTAGGG Telomerase PCR ELISA kit. This kit allows highly specific amplification of telomerase-mediated elongation products with PCR and detection by an ELISA protocol. Relative telomerase activity (RTA) values were calculated for mg/mL protein. Protein values were determined by the Lowry method (Table 3).

### 3.10. Statistical analysis

Minitab 13.0 statistical software was used for analysis. The results were estimated with the Mann–Whitney test.

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