Article

Synthesis, Characterization, and Biological Evaluation of Novel 7-Oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic Acid Derivatives

Dong Cai 1, Tai Li 1, Qian Xie 1, Xiaofei Yu 1, Wei Xu 2, Yu Chen 2, Zhe Jin 1,*, and Chun Hu 1,*,  

1 Key Laboratory of Structure-based Drug Design & Discovery, Ministry of Education, School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang 110016, China; caidong0804@163.com (D.C.); litai159@163.com (T.L.); xqqx1996@163.com (Q.X.); yuxiaofei2017@163.com (X.Y.)  

2 School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, Shenyang 110016, China; shxuwei8720@163.com (W.X.); gzweishengwu@126.com (Y.C.)  

* Correspondence: jinzheln@163.com (Z.J.); chunhu@syphu.edu.cn (C.H.); Tel.: +86-24-43520246 (Z.J. & C.H.)

Received: 13 January 2020; Accepted: 11 March 2020; Published: 13 March 2020

Abstract: A series of novel 7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid derivatives was synthesized in good yields by a multi-step procedure that included the generation of the S-alkylated derivatives from 6-substituted arylmethyl-3-mercapto-1,2,4-triazin-5-ones with ethyl 2-chloroacetoacetate, intramolecular cyclization with microwave irradiation, hydrolysis and amidation. All of the target compounds were fully characterized through 1H-NMR, 13C-NMR and HRMS spectra. The intramolecular cyclization occurred regioselectively at the N2-position of 1,2,4-triazine ring, which was confirmed by compound 3e using single-crystal X-ray diffraction analysis. The antibacterial and antitubercular activities of the target compounds were evaluated. Compared with Ciprofloxacin and Rifampicin, compounds 5d, 5f and 5g containing the terminal amide fragment exhibited broad spectrum antibacterial activity, and carboxylic acid derivatives or its corresponding ethyl esters had less effect on antibacterial properties. The most potent compound 5f also displayed excellent in vitro antitubercular activity against Mycobacterium smegmatis (minimum inhibitory concentration (MIC) = 50 µg/mL) and better growth inhibition activity of leucyl-tRNA synthetase (78.24 ± 4.05% at 15 µg/mL).

Keywords: thiazolo[3,2-b]-1,2,4-triazinone; inhibitor; antibacterial activity; antitubercular activity; leucyl-tRNA synthetase

1. Introduction

Since the first discovery of penicillin by Alexander Fleming in 1928, many new antibacterial drugs have been used clinically to treat bacterial pathogen infection. However, the misuse of antibiotics has led to drug-resistant or widespread bacteria strains [1,2]. Moreover, immunocompromised patients infected with tubercular bacteria frequently also present with other severe bacterial and viral infections. Therefore, there is an urgent need for a novel multi-target agents in clinical treatment which can have an antibacterial effect and can effectively inhibit mycobacteria [3,4].

Fused thiazole derivatives have been widely used in organic and medicinal chemistry, as well as in agricultural science [5]. In a recent study, heterocycle-fused thiazole derivatives have been synthesized and investigated with the aim of finding effective antimicrobial agents [6]. In this respect, many thieno[2,3-d]pyrimidinones have been reported as potent antibacterial and antitubercular agents [7,8],
and these scaffolds are structurally unrelated to any current clinical antibiotics. Besides this, different side chains introduced into the thieno[2,3-d]pyrimidinone scaffold resulted in a large difference in antibacterial activities. Compound A (Figure 1) possessed broad antibacterial and antimycobacterial activity, especially an anti-resistant effect [9,10]. The structure–activity relationships of this series of compounds reported in the literature demonstrated that the presence of a flexible side chain containing a phenyl ring was critical for activity. Furthermore, the presence of a 4-methoxyphenyl group increased in antibacterial effect by 2-fold when compared to the 4-hydroxyphenyl group [9,10]. The compounds containing an amido/imino linker fragment (such as B and C, Figure 1) exhibited significant antibacterial activity against tRNA-(N1G37) methyltransferase (TrmD) isoenzymes. The substituted-amido or imino side chain on thienopyrimidinone ring of compounds B and C exerted a positive influence on the antimicrobial activity [9,10]. In general, the side chains containing substituted phenyl fragments were found to be more active than the corresponding aliphatic and heterocyclic parts. Furthermore, the presence of electron-withdrawing substituents on the phenyl ring increased the potency of test compounds. The compound D showed most promising inhibitory activity against Escherichia coli with an IC₅₀ of 0.91 μM (Figure 1), and the studies revealed that the hydrophobic side chain on the thiophene ring has significant effect on the antimicrobial activity [11].

![Figure 1. Known compounds with antimicrobial activity.](image_url)

Many of these studies provided a ray of hope for the emergence of a new antimicrobial leads, However, the poor bioavailability of these compounds cannot meet the medical requirements. In recent studies, optimization strategies were focused on modification of the thieno[2,3-d]pyrimidinone nucleus and the side chains on the nucleus.

Among the various heterocycle-fused thiazole derivatives, the thiazolo[3,2-b]-1,2,4-triazinone nucleus is a crucial class of N-bridged heterocyclic compounds with a wide range of applications in the field of therapeutic chemistry. These thiazolo[3,2-b]-1,2,4-triazinone derivatives have exhibited a broad spectrum of pharmacological and biological activities, such as anticancer [12], anti-inflammatory [13], antirheumatic [14] and antiacetylcholinesterase activities [15,16], etc. Comparing thieno[2,3-d]pyrimidinone with thiazolo[3,2-b]-1,2,4-triazinone nucleus using molecular overlay method, the overlay similarity value was over 60%.

Inspired by the above-mentioned facts, a series of novel 7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid derivatives was designed based on the bioisosteric replacement of the thieno[2,3-d]pyrimidinone nucleus in potent compounds with a thiazolo[3,2-b]-1,2,4-triazinone nucleus. Comparing the diverse linker fragments of bioactive compounds [9–11], the linker part was replaced with a methylene group (-CH₂-). As shown in Figure 2, various substituent groups were simultaneously introduced to the thiazolo[3,2-b]-1,2,4-triazinone nucleus to explore the structure–activity relationships.
The molecular overlay (Figure 3) between the compound D and target compound 5f revealed a striking overlap between the bulky substituted benzyl amide side chains. Another overlapping feature was the presence of the two structural nuclei, especially the thiophene ring fragments. The structural similarity based on 50% steric field and 50% electrostatic field was accessed in the Discovery Studio 3.5 software (Accelrys Inc., San Diego, CA, USA), and the overlay similarity value was 36.15%. Thus, the structural resemblance between thieno[2,3-d]pyrimidinone and thiazolo[3,2-b]-1,2,4-triazinone indicated that the target compounds based upon the thiazolo[3,2-b]-1,2,4-triazinone nucleus are likely to exhibit more potent antimicrobial activities. The antibacterial and antitubercular activities of the target compounds were evaluated.

2. Results and Discussion

2.1. Chemistry

The known approaches for the synthesis of 7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid derivatives can be divided into two main paths: the construction of the thiazole ring from 1,2,4-triazin-5-one derivatives [15,17] and construction of the 1,2,4-triazine ring from 2-aminothiazole derivatives [18–20]. There are several limitations to the second approach such as a scarcity of raw materials, low yields, and several side reactions with less selectivity of the process. Herein, a novel efficient approach was described with the aim of obtaining the target compounds in good yield and with a simple isolation procedure (Scheme 1). The synthetic approach was based on the first approach above [15]. The intermediates 6-substituted-3-mercaptop-1,2,4-triazin-5-ones 1a–1e were prepared using known methods [15,21]. Sulfur atoms have a larger size and lower electronegativity than those of
oxygen atoms, which makes the outer electron shell more prone to donate electrons, and results in most likely nucleophilic attack [22]. The intermediates 1a–1e underwent selectively rapid S-alkylation reaction, in which any possible N-, O-, or C-alkylation of the same substrate is much slower and hence negligible [23,24]. The intermediates 3a–3e were synthesized by Williamson ether synthesis followed by an intramolecular cyclization reaction in PPA (polyphosphoric acid) with microwave irradiation. Next, the intermediates 3a–3e underwent hydrolysis reaction in KOH methanolic solution and then were acidized to form the carboxylic acids 4a–4e, which were subsequently amidated with various substituted methylimines to create the target compounds 5a–5j. 1H-NMR and 13C-NMR spectra for the synthesized compounds are available in the Supplementary Materials.

![Scheme 1](image)

**Scheme 1.** The synthetic route of the target compounds. Reagents and conditions: (a) ethyl 2-chloroacetoacetate, 10% KOH, DMF, 25 °C, 30 min; (b) polyphosphoric acid (PPA), 120 °C, 30 min, microwave; (c) 10% NaOH, CH3OH, 25 °C, 2 h; (d) substituted primary amine, HOBt, EDCI, triethylamine, DCM, 25 °C, 16 h.

Subsequently, the intermediate β-keto esters 2a–2e underwent intramolecular cyclization followed by dehydration to the intermediates 3a–3e. Both polyphosphoric acid (PPA) and concentrated H2SO4 were tested for their effectiveness as cyclization agents; it was found that PPA gave good results, whereas sulfuric acid made the reaction mixture a dark solution and gave several kinds of unidentified products (analyzed by TLC). This may be due to the active carbonyl group of the ethyl 2-chloroacetoacetate, resulting in the occurrence of side reactions. The reactions were studied in both classical and microwave-assisted conditions. In classical conditions, the products 3a–3e were obtained in yields of 66.28%–75.26% after 60 min, while with microwave irradiation, the same products were isolated in 80.12%–87.66% yields after only 20 min (Table 1). In the cyclization with PPA, the critical factors were the reaction time and temperature. When the temperatures were above 130 °C using classical heating methods, after heating for 60 min or more, a burnt smell was dispersed. The β-keto esters 2a–2e cannot be completely converted to the products below 110 °C.

**Table 1.** Optimization for the synthesis of compounds 3a–3e.

| Reaction Condition | Conventional Heating | MW-Irradiation |
|--------------------|----------------------|----------------|
|                    | Timemin | Yield *% | Time/min | Yield *% |
| 3a                 | 60      | 68.86    | 20       | 81.24  |
| 3b                 | 60      | 70.41    | 20       | 85.12  |
| 3c                 | 60      | 71.31    | 20       | 83.78  |
| 3d                 | 60      | 66.28    | 20       | 80.12  |
| 3e                 | 60      | 75.26    | 20       | 87.66  |

* isolated yield.
The β-keto esters 2a–2e were cyclized in PPA, which yielded the intermediates 3a–3e. It was reported in the literature [25] that the intramolecular condensation reaction gave the compounds 6a–6e rather than the compounds 3a–3e (Scheme 1). According to an X-ray structural analysis of compound 3e, the direction of regioselective cyclization at N2 of the triazine ring was directly confirmed (Figure 4 and Table 2). All of the bond lengths and bond angles of compound 3e were in the normal range. The bicyclic fragments were coplanar. The benzene ring C (11)…C (16) was almost perpendicular to the intramolecular bicyclic thiazolo[3,2-b]-1,2,4-triazine fragment with a θ angle of 89.584(90)°. In the meantime, the ester groups were nearly coplanar with the corresponding bicyclic fragment, and the torsion angles of S(1)-C(4)-C(3)-O(2) and S(1)-C(4)-C(3)-O(1) with the bicyclic were 3.278(550)° and 1.742(2185)°, respectively.

![Figure 4. ORTEP diagram of compound 3e at 30% Probability.](image)

**Table 2.** X-ray crystallographic data for compound 3e.

| Parameter | Value |
|-----------|-------|
| CCDC number | 1435646 |
| Formula | (C\textsubscript{17}H\textsubscript{16}N\textsubscript{3}O\textsubscript{4}S)\textsubscript{4}·CH\textsubscript{3}OH |
| Formula weight | 1468.62 |
| Crystal color, shape | Colorless, block |
| Crystal system | Triclinic |
| Space group | P-1 |
| a/Å | 11.7136 (8) |
| b/Å | 12.5137 (9) |
| c/Å | 13.8247 (9) |
| α (°) | 104.2510 (10) |
| β (°) | 98.4010 (10) |
| γ (°) | 110.7180 (10) |
| Volume/Å\textsuperscript{3} | 1775.2 (2) |
| Temperature/K | 296 (2) |
| Z | 1 |
| Density (calculated)/g·cm\textsuperscript{-3} | 1.378 |
| F (000) | 769 |
| Reflections collected/unique | 10269/6238 [R(int) = 0.0197] |
| Goodness-of-fit of F\textsuperscript{2} | 1.137 |
| Final R indices [I > 2σ(I)] | R1 = 0.0547, wR2 = 0.1655 |
| R indices (all data) | R1 = 0.0821, wR2 = 0.1780 |
| Largest diff. peak and hole/e.Å\textsuperscript{-3} | 0.448 and −0.401 |

### 2.2. Biological Assays

All of the synthesized compounds were evaluated in vitro using a 96-well microtiter plate and a serial dilution method to obtain their minimum inhibitory concentration (MIC) values against two Gram-positive bacterial strains (*Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis*)), two Gram-negative bacterial strains (*Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*)), and *Mycobacterium smegmatis* (*M. smegmatis*) [26,27]. The MIC values of these compounds are presented in Table 3.
Table 3. Antibacterial and antitubercular activity of the synthesized compounds. MIC: minimum inhibitory concentration.

| Entry | Antibacterial Activity MIC (µg/mL) | Antitubercular Activity MIC (µg/mL) |
|-------|-----------------------------------|-------------------------------------|
|       | S. aureus | B. subtilis | E. coli | P. aeruginosa | M. smegmatis |
| 3a    | >800     | >800     | 400     | >800        | >800        |
| 3b    | 100      | >800     | 400     | 800         | >800        |
| 3c    | 400      | 400      | 200     | 200         | >800        |
| 3d    | >800     | 800      | 400     | >800        | >800        |
| 3e    | >800     | >800     | >800    | >800        | >800        |
| 4a    | 200      | >800     | 100     | 800         | >800        |
| 4b    | 400      | 400      | 100     | 400         | >800        |
| 4c    | 200      | 400      | 50      | 200         | >800        |
| 4d    | >800     | >800     | 400     | >800        | >800        |
| 4e    | >800     | >800     | 800     | >800        | >800        |
| 5a    | 100      | 50       | 100     | 400         | >800        |
| 5b    | 200      | 100      | 200     | 100         | >800        |
| 5c    | 50       | 100      | 400     | >800        | >800        |
| 5d    | 50       | 200      | 50      | 100         | >800        |
| 5e    | 100      | 50       | 400     | 400         | >800        |
| 5f    | 200      | 200      | 100     | 200         | 50          |
| 5g    | 100      | 50       | 100     | 50          | >800        |
| 5h    | 100      | 200      | 200     | 400         | 400         |
| 5i    | 200      | 400      | 200     | 100         | >800        |
| 5j    | 200      | 200      | 400     | 400         | >800        |
| Ciprofloxacin | 25      | 100      | 25      | 50          | NT          |
| Rifampicin    | NT *     | NT       | NT      | NT          | 25          |

* NT = Not tested.

According to the investigation of antibacterial screening data, most of the compounds exhibited low to moderate activity against Gram-positive bacteria and Gram-negative bacteria. The antibacterial activity listed in Table 3 demonstrated that, in general, the carboxylic acid derivatives 4a–4e and their corresponding ethyl esters 3a–3e had less effect on antibacterial properties, and compounds 3a–3e containing ester moieties caused considerable losses in the inhibitory potency. Besides, the activities were only slightly affected by substituents in the 2- or 4-position of the phenyl ring of compounds 3a–3c and 4a–4c, which was probably attributed to a freely rotatable benzyl fragment. Furthermore, the compounds 3d and 4d with a p-substituted trifluoromethyl group at the phenyl ring had a lower antibacterial activity than the compounds 3b, 3c, 4b and 4c) with p-substituent halogen atoms (e.g., Cl, F). The compounds (3e and 4e) were almost inactive or had low activity against all bacteria, presumably because of the presence of the electron-donating groups (e.g., CH₃O).

Differently substituted amide chains were introduced at the carboxyl group of the thiazolo[3,2-b]-1,2,4-triazinone nucleus. Consequently, the antibacterial activity of compounds 5a–5j was enhanced dramatically as compared with that of compounds 4a–4e. It is noteworthy that the different substituents in the benzyl group on the triazine ring exert an evident influence on the antibacterial activity. The presence of the electron-withdrawing group in the phenyl ring in general...
increases the antibacterial activity of compounds 5a–5h compared to compounds 5i–5j containing electron-donating groups. In the meantime, the comparison of the compounds 5a–5j with the different terminal amide fragment indicated that there was no significant difference in activity.

Obviously, only one compound 5f exhibited significant antitubercular activity against \textit{M. smegmatis}, compared with the reference compound of Rifampicin.

### 2.3. Target Identification by Pharmacophore

Automated pharmacophoric profiling is a readily available and effective target fishing method which is conducted by using the ligand profiler protocol, which can be used to explore the most likely targets of a query chemical structure or determine the off-targets of an existing drug [26,27]. To provide more evidence for the possible binding targets of the synthesized compounds, the compound 5f was selected to evaluate the antibacterial and antitubercular properties on a molecular level. The FitValue was used as an important judgment of the overlap degree between the query compound and pharmacophore models [28].

According to the screening results (Table 4), compound 5f was able to map some effective antimicrobial pharmacophore models, including methyltransferases, aminoacyl-tRNA synthetases (AARSs), and enoyl-[acyl-carrier-protein] reductase (NADH). The results indicated that compound 5f might have good antimicrobial activity, especially inhibiting bacterial thymidylate synthase and AARSs. In practice, the bacterial thymidylate synthase is a highly conserved protein in bacteria and humans, and it has proven to be unsuitable as a target for selective antibacterial inhibitors [29]. The AARSs family are key components in protein synthesis; among them, leucyl-tRNA synthetase (LeuRS) has been validated as a promising target for antimicrobial drug therapy. Based on the above results, compound 5f could be regarded as a potential LeuRS inhibitor candidate for further research.

| PDB Id | FitValue | Pharmacophore/Metadata/Target-Class C | Target |
|--------|----------|-------------------------------------|--------|
| 1TSL   | 0.774082 | Methyltransferases                   | Bacterial thymidylate synthase |
| 1OBH   | 0.347426 | Aminoacyl-tRNA synthetases (AARSs)  | Leucyl-tRNA synthetase          |
| 2HGZ   | 0.310616 | Aminoacyl-tRNA synthetases (AARSs)  | p-benzoyl-l-phenylalanyl-tRNA synthetase |
| 1I2Z   | 0.0866648| Enoyl-[acyl-carrier-protein] reductase [NADH] | Bacterial enoyl acyl carrier protein reductase (FabI) |
| 1LX6   | 0.0729051| Enoyl-[acyl-carrier-protein] reductase [NADH] | Bacterial enoyl-ACP reductase (FabI) |
| 1MFP   | 0.058642 | Enoyl-[acyl-carrier-protein] reductase [NADH] | Bacterial Enoyl-ACP reductases FabI and FabK |
| 2B35   | 7.90584 $\times 10^{-5}$ | Enoyl-[acyl-carrier-protein] reductase [NADH] | Mycobacterium tuberculosis enoyl reductase (InhA) |

### 2.4. Leucyl-tRNA Synthetase Activity

The enzyme inhibition data against LeuRS from \textit{M. smegmatis} for the selected compounds are given in Table 5. It was found that compound 5f with a 4-fluorophenyl group at R₁ was more potent than its carboxylic acid precursor 4b. Compound 5f exhibited a high inhibitory effect on the \textit{M. smegmatis} LeuRS with the percent inhibition of 78.24 ± 4.05% at 15 µg/mL, which is a six-fold improvement as compared to compound 4b (with a percentage inhibition of 12.89 ± 2.31% at 15 µg/mL). The obtained results showed that novel 7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid derivatives seem to be effective \textit{M. smegmatis} LeuRS inhibitors and worthy of further exploitation.
Table 5. Inhibitory effects of selected compounds on *M. smegmatis* LeuRS activity at 15 µg/mL.

| Compound | Percent Inhibition (%) |
|----------|------------------------|
| 4b       | 12.89 ± 2.31           |
| 5f       | 78.24 ± 4.05           |

3. Materials and Methods

3.1. General Information

All solvents and chemicals were purchased from common commercial suppliers and were used without further purification. Melting points were taken in open capillary tubes and are reported uncorrected. IR spectra were recorded on a FT-IR 920 spectrophotometer (Tianjin Tuopu Instrument Co., Ltd., Tianjin, China) using KBr pellets. The $^1$H- and $^{13}$C-NMR spectra were obtained with TMS as internal standard using a 400/54 Premium Shielded NMR Magnet System (Agilent Technologies, Santa Clara, CA, USA). Mass spectra were determined on an Agilent 6200 Series TOF and 6500 Series Q-TOF LC/MS System B.05.01. (B5125, Agilent Technologies). The microwave assisted reactions were carried out in a synthetic microwave apparatus (Shanghai Biaohe Instrument Co., Ltd., Shanghai, China). X-ray single-crystal structure determinations were carried out on a Bruker SMART APEX II CCD diffractometer (Bruker AXS GMBH, Karlsruhe, Germany).

The original figures of IR, $^1$H-NMR, $^{13}$C-NMR, MS, and HRMS of all the target compounds and the key intermediates are available online.

3.2. Synthesis

3.2.1. General Procedure for the Synthesis of the β-Keto Esters (2a–2e)

A 10% aqueous solution of KOH (5.6 mL) was added dropwise under stirring to form a suspension of compound 1 (10 mmol) in DMF (10 mL). Then, ethyl 2-chloroacetoacetate (1.4 mL, 10 mmol) was added and the mixture was stirred at 25 °C for 30 min. Then, the mixture was poured into ice water to obtain compound 2, which was filtered and washed with water, and then subsequently recrystallized from ethyl acetate.

**Ethyl 2-((6-(2-chlorobenzyl)-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl)thio)-3-oxobutanoate (2a):** White solid; Yield: 73.12%; mp: 129.4–130.1 °C; IR (KBr): $\nu$ 3260, 2986, 2950, 1743, 1653, 1575, 1482, 1422, 1309, 1184, 1018, 750, 686 cm$^{-1}$; $^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.79 (s, 1H), 7.46 (dt, $J = 7.6, 2.9$ Hz, 1H), 7.39–7.24 (m, 3H), 5.16 (s, 1H), 4.21 (qd, $J = 7.1, 3.0$ Hz, 2H), 4.09–3.94 (m, 2H), 1.79 (s, 3H), 1.24 (t, $J = 7.1$ Hz, 3H); HRMS (m/z): calcd. for C$_{16}$H$_{17}$ClN$_3$O$_4$S (M$^+$ + H$^+$) 382.06283, found 382.06076.

**Ethyl 2-((6-(4-chlorobenzyl)-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl)thio)-3-oxobutanoate (2b):** White solid; Yield: 75.34%; mp: 126.8–128.7 °C; IR (KBr): $\nu$ 3392, 3100, 2982, 2933, 1724, 1654, 1580, 1501, 1407, 1316, 1273, 1203, 806 cm$^{-1}$; $^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.85 (s, 1H), 7.42–7.32 (m, 2H), 7.33–7.17 (m, 2H), 5.15 (s, 1H), 4.22 (tdd, $J = 7.2, 5.5, 2.5$ Hz, 2H), 3.93–3.75 (m, 2H), 1.89 (s, 3H), 1.26 (d, $J = 7.1$ Hz, 3H); HRMS (m/z): calcd. for C$_{16}$H$_{17}$ClN$_3$O$_4$S (M$^+$ + H$^+$) 382.06283, found 382.06076.

**Ethyl 2-((6-(4-fluorobenzyl)-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl)thio)-3-oxobutanoate (2c):** White solid; Yield: 72.69%; mp: 125.4–125.7 °C; IR (KBr): $\nu$ 3388, 3297, 2930, 1724, 1659, 1578, 1505, 1410, 1316, 1273, 1203, 806 cm$^{-1}$; $^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.88 (d, $J = 33.9$ Hz, 1H), 7.42–7.32 (m, 2H), 7.33–7.17 (m, 2H), 5.15 (s, 1H), 4.22 (tdd, $J = 7.2, 5.5, 2.5$ Hz, 2H), 3.93–3.75 (m, 2H), 1.89 (s, 3H), 1.26 (d, $J = 7.1$ Hz, 3H); HRMS (m/z): calcd. for C$_{16}$H$_{17}$ClFN$_3$O$_4$S (M$^+$ + H$^+$) 366.09238, found 366.09278.

**Ethyl 3-oxo-2-((5-oxo-6-(4-(trifluoromethyl)benzyl)-2,5-dihydro-1,2,4-triazin-3-yl)thio)butanoate (2d):** White solid; Yield: 76.52%; mp: 128.1–128.7 °C; IR (KBr): $\nu$ 3121, 2997, 1741, 1633, 1580, 1501, 1434, 1368, 1263, 1170, 1028, 856 cm$^{-1}$; $^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.84 (s, 1H), 7.44–7.36 (m, 2H), 7.31 (d, $J = 7.1$ Hz, 3H), 4.22 (t, $J = 7.2$ Hz, 2H), 3.93–3.75 (m, 2H), 1.89 (s, 3H), 1.26 (d, $J = 7.1$ Hz, 3H); HRMS (m/z): calcd. for C$_{16}$H$_{17}$ClFN$_3$O$_4$S (M$^+$ + H$^+$) 366.09238, found 366.09278.
3.2.2. General Procedure for the Synthesis of Ethyl 6-Aryl-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylates (3a–3e)

Compound 2 (0.01 mol) was added to PPA (85% P2O5) (10.0 g). The mixture was irradiated in a microwave at 120 °C for 20 min at a power of 400 W. The reaction mixture was then poured into ice water. The resulting solid was filtered, washed with cold water (3 × 20 mL) and dried. The crude product was recrystallized from ethyl acetate to give compound 3.

Ethyl 6-(2-chlorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylate (3a): White solid; Yield: 81.24%; mp: 162.3–163.3 °C; IR (KBr): ν 3059, 2986, 2941, 1697, 1609, 1495, 1474, 1242, 1196, 760 cm−1; 1H-NMR (400 MHz, Chloroform-d) δ 7.41 (ddd, J = 8.7, 5.4, 3.5 Hz, 2H), 7.26 (dd, J = 5.8, 3.5 Hz, 2H), 4.40 (q, J = 7.1 Hz, 2H), 4.33 (s, 2H), 2.60 (s, 3H), 1.40 (t, J = 7.1 Hz, 3H); 13C-NMR (101 MHz, Chloroform-d) δ 163.94, 160.49, 158.92, 154.33, 143.13, 134.72, 133.15, 131.81, 129.45, 128.65, 109.99, 62.43, 35.14, 14.17, 11.86; HRMS (m/z): calcd. for C16H13ClN3O3S (M + H+) 364.05226, found 364.0527.

Ethyl 6-(4-chlorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylate (3b): White solid; Yield: 85.12%; mp: 130.7–132.1 °C; IR (KBr): ν 3030, 2984, 2960, 1715, 1585, 1501, 1463, 1285, 1221, 808 cm−1; 1H-NMR (600 MHz, DMSO-d6) δ 7.35 (s, 4H), 4.33 (q, J = 7.1 Hz, 2H), 4.01 (s, 2H), 2.61 (s, 3H), 1.30 (t, J = 7.1 Hz, 3H); 13C-NMR (101 MHz, Chloroform-d) δ 164.05, 160.45, 158.82, 155.18, 142.98, 133.54, 133.04, 130.93, 128.66, 110.28, 62.50, 36.61, 14.18, 12.08; HRMS (m/z): calcd. for C16H13ClN3O3S (M + H+) 364.05226, found 364.0527.

Ethyl 6-(4-fluorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylate (3c): White solid; Yield: 83.78%; mp: 128.3–129.1 °C; IR (KBr): ν 3066, 2984, 2938, 1715, 1501, 1463, 1285, 1221, 808 cm−1; 1H-NMR (400 MHz, Chloroform-d) δ 7.39 (dd, J = 8.6, 5.4 Hz, 2H), 7.02 (t, J = 8.7 Hz, 2H), 4.42 (q, J = 7.1 Hz, 2H), 4.13 (s, 2H), 2.76 (s, 3H), 1.41 (t, J = 7.1 Hz, 3H); 13C-NMR (101 MHz, Chloroform-d) δ 164.03, 163.21, 160.77, 158.64, 155.42, 142.99, 131.18, 131.10, 115.49, 115.28, 110.20, 62.48, 36.46, 14.17, 12.06; HRMS (m/z): calcd. for C16H13ClNF3O3S (M + H+) 348.08182, found 348.0824.

Ethyl 3-methyl-7-oxo-6-(4-(trifluoromethyl)benzyl)-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylate (3d): White solid; Yield: 80.12%; mp: 94.9–95.2 °C; IR (KBr): ν 2998, 2918, 1720, 1490, 1267, 1161, 816 cm−1; 1H-NMR (400 MHz, Chloroform-d) δ 7.45 (d, J = 8.5 Hz, 2H), 7.18 (d, J = 8.0 Hz, 2H), 4.42 (q, J = 7.1 Hz, 2H), 4.16 (s, 2H), 2.77 (s, 3H), 1.42 (t, J = 7.1 Hz, 3H); 13C-NMR (101 MHz, Chloroform-d) δ 165.36, 158.74, 156.67, 156.37, 154.83, 152.49, 148.11, 146.62, 130.59, 120.73, 109.93, 62.23, 36.25, 13.85, 11.79; HRMS (m/z): calcd. for C14H18F3N4O3S (M + NH4+) 415.0517, found 415.0767.

Ethyl 6-(4-methoxybenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylate (3e): Yellow solid; Yield: 87.66%; mp: 105.3–105.5 °C; IR (KBr): ν 2982, 2918, 1721, 1695, 1569, 1613, 1575, 1505, 1368, 1290, 1242, 1174, 1131, 1052, 1028, 815 cm−1; 1H-NMR (400 MHz, Chloroform-d) δ 7.38–7.30 (m, 2H), 6.91–6.82 (m, 2H), 4.41 (q, J = 7.1 Hz, 2H), 4.10 (s, 2H), 3.81 (s, 3H), 2.77 (s, 3H), 1.42 (d, J = 7.1 Hz, 3H); 13C-NMR (101 MHz, Chloroform-d) δ 163.95, 160.54, 158.94, 158.67, 155.75, 143.08, 130.61, 127.04, 113.94, 109.98, 62.43, 55.24, 36.35, 14.18, 12.09; HRMS (m/z): calcd. for C17H18N3O3S (M + H+) 360.10180, found 360.09732.
3.2.3. General Procedure for the Synthesis of 6-Aryl-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acids (4a–4e)

The ester derivative (3) (4.0 mmol) was hydrolyzed by treatment with a mixture of NaOH 10% (10 mL) and CH₃OH (10 mL) for 2 h at 25 °C. The solution was concentrated to half volume and the product was precipitated by acidification with 10% HCl to pH value of 3.0, filtered, washed with water, then dried.

6-(2-Chlorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid (4a): White solid; Yield: 95.35%; mp: 209.6–210.4 °C; IR (KBr): v 2920, 1629, 1577, 1475, 1440, 1351, 1038, 751 cm⁻¹; ¹H-NMR (600 MHz, DMSO-d₆) δ 7.46 (dd, J = 7.5, 1.8 Hz, 1H), 7.42–7.37 (m, 1H), 7.31–7.26 (m, 2H), 4.13 (s, 2H), 2.46 (s, 3H); ¹³C-NMR (101 MHz, DMSO-d₆) δ 135.81, 131.59, 121.77, 121.32, 119.23, 36.50, 12.05; HRMS (m/z): calcd. for C₁₄H₁₁ClN₂O₃S (M + H⁺) 336.02096, found 336.02300.

6-(4-Chlorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid (4b): White solid; Yield: 93.11%; mp: 197.2–198.2 °C; IR (KBr): v 2959, 2927, 1717, 1649, 1613, 1575, 1484, 1417, 1356, 1242, 1093, 803 cm⁻¹; ¹H-NMR (400 MHz, DMSO-d₆) δ 7.37 (s, 4H), 4.02 (s, 2H), 2.61 (s, 3H); ¹³C-NMR (101 MHz, DMSO-d₆) δ 164.10, 162.66, 159.00, 154.26, 142.19, 135.29, 131.78, 131.61, 131.10, 128.60, 36.51, 12.04; HRMS (m/z): calcd. for C₁₄H₁₁ClN₂O₃ (M + H⁺) 335.0296, found 335.02190.

6-(4-Fluorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid (4c): White solid; Yield: 91.34%; mp: 205.9–206.2 °C; IR (KBr): v 2950, 1697, 1569, 1507, 1470, 1287, 1252, 1015, 809 cm⁻¹; ¹H-NMR (600 MHz, DMSO-d₆) δ 7.36 (dd, J = 8.4, 5.5 Hz, 2H), 7.12 (t, J = 8.8 Hz, 2H), 3.99 (s, 2H), 2.59 (s, 3H); ¹³C-NMR (101 MHz, DMSO-d₆) δ 164.10, 162.76, 162.61, 160.35, 159.02, 154.49, 132.33, 132.30, 131.67, 131.59, 115.52, 115.30, 36.34, 12.07; HRMS (m/z): calcd. for C₁₄H₁₁FClN₂O₃S (M + H⁺) 320.05052, found 320.05222.

3-Methyl-7-oxo-6-(4-(trifluoromethyl)benzyl)thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid (4d): White solid; Yield: 89.88%; mp: 212.6–213.8 °C; IR (KBr): v 3049, 2957, 1711, 1612, 1508, 1476, 1423, 1381, 1354, 1286, 921, 779, 764 cm⁻¹; ¹H-NMR (600 MHz, DMSO-d₆) δ 7.46 (d, J = 8.2 Hz, 2H), 7.29 (d, J = 8.1 Hz, 2H), 4.04 (s, 2H), 2.59 (s, 3H); ¹³C-NMR (101 MHz, DMSO-d₆) δ 164.15, 162.64, 159.01, 154.23, 147.57, 142.45, 135.81, 131.59, 121.77, 121.32, 119.23, 36.50, 12.05; HRMS (m/z): calcd. for C₁₅H₁₁F₃N₂O₃S (M + NH₄⁺) 387.07387, found 387.04752.

6-(4-Methoxybenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid (4e): Yellow solid; Yield: 94.50%; mp: 209.8–210.8 °C; IR (KBr): v 2944, 2835, 2752, 2595, 2503, 1709, 1662, 1618, 1580, 1486, 1434, 1359, 1273, 1244, 1184, 1026, 767 cm⁻¹; ¹H-NMR (400 MHz, DMSO-d₆) δ 14.27 (s, 1H), 7.30–7.21 (m, 2H), 6.91–6.82 (m, 2H), 3.94 (s, 2H), 3.72 (s, 3H), 2.63 (s, 3H); ¹³C-NMR (101 MHz, DMSO-d₆) δ 164.05, 162.64, 158.98, 158.49, 154.80, 142.91, 130.77, 127.88, 114.12, 110.63, 55.44, 36.29, 12.17; HRMS (m/z): calcd. for C₁₅H₁₄N₃O₄S (M + H⁺) 332.07050, found 332.07303.

3.2.4. General Procedure for the Synthesis of the Target Compounds 5a–5j

Substituted primary amine (1.0 mmol) was added to a solution of compound 4 (1.0 mmol), HOBt (1-hydroxybenzotriazole, 0.15 g, 1.1 mmol), triethylamine (0.34 g, 3.3 mmol), and EDCI (N’-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride, 0.21 g, 1.1 mmol) in DCM (dichloromethane, 30 mL). The solution was then stirred at 25 °C for 16 h. The reaction was diluted with H₂O (30 mL), and the organic layer was separated and sequentially washed with 1 mol·L⁻¹ HCl (10 mL), saturated NaHCO₃ aqueous solution (10 mL), and brine (10 mL), dried (Na₂SO₄). The solvent was filtered and evaporated, resulting in the crude product which was purified by chromatography (CH₂Cl₂-CH₃OH, V:V = 50:1).

6-(2-Chlorobenzyl)-N-(furan-2-ylmethyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5a): White solid; Yield: 72.60%; mp: 138.5–139.0 °C; IR (KBr): v 3331, 3055, 2926, 1655, 1609, 1570, 1487,
N,6-Bis(2-chlorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5b): White solid; Yield: 72.20%; mp: 148.1–149.4 °C; IR (KBr): δ 3271, 3073, 2940, 1663, 1614, 1575, 1504, 1440, 1352, 1293, 113, 44, 35, 21, 12; HRMS (m/z): calcd. for C_{19}H_{16}ClN_{2}O_{2}S (M + H^+) 415.0636, found 415.0649.

6-(2-Chlorobenzyl)-N-(2,4-dichlorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5c): White solid; Yield: 71.40%; mp: 198.5–212.4 °C; IR (KBr): δ 3292, 3062, 2928, 1638, 1616, 1570, 1541, 1487, 1441, 1387, 756 cm\(^{-1};\) H-NMR (400 MHz, Chloroform-d) δ 7.46–7.32 (m, 4H), 7.30–7.20 (m, 3H), 6.48 (d, J = 6.1 Hz, 1H), 4.65 (d, J = 5.9 Hz, 2H), 4.30 (s, 2H), 2.54 (s, 3H); \(^{13}\)C-NMR (101 MHz, Chloroform-d) δ 162.90, 159.75, 158.96, 154.20, 153.15, 153.07, 152.65, 151.78, 151.50, 129.54, 129.47, 128.68, 127.54, 126.86, 112.96, 41.88, 35.15, 12.10; HRMS (m/z): calcd. for C_{21}H_{16}ClN_{2}O_{2}S (M + H^+) 493.0059, found 493.0835.

6-(2-Chlorobenzyl)-N-(4-fluorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5d): White solid; Yield: 69.90%; mp: 153.5–154.4 °C; IR (KBr): δ 3292, 3062, 2928, 1638, 1616, 1570, 1434, 1385, 765 cm\(^{-1};\) H-NMR (400 MHz, Chloroform-d) δ 7.42–7.24 (m, 6H), 7.08–7.03 (m, 2H), 6.34 (s, 1H), 4.57 (d, J = 5.6 Hz, 2H), 4.29 (s, 2H), 2.55 (s, 3H); \(^{13}\)C-NMR (101 MHz, Chloroform-d) δ 162.90, 159.93, 159.18, 154.02, 139.13, 134.61, 133.32, 132.38, 130.53, 130.33, 129.73, 129.65, 129.47, 128.69, 115.61, 115.40, 113.53, 43.52, 35.10, 12.13; HRMS (m/z): calcd. for C_{21}H_{16}ClFN_{2}O_{2}S (M + H^+) 443.07448, found 443.07726.

6-(2-Chlorobenzyl)-3-methyl-N-(4-methylbenzyl)-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5e): White solid; Yield: 72.00%; mp: 166.6–167.3 °C; IR (KBr): δ 3292, 3062, 2928, 1638, 1504, 1440, 1384, 764 cm\(^{-1};\) H-NMR (400 MHz, Chloroform-d) δ 7.39 (dd, J = 11.2, 5.5, 3.5 Hz, 2H), 7.30–7.15 (m, 6H), 6.09 (s, 1H), 4.56 (d, J = 5.3 Hz, 2H), 4.30 (s, 2H), 2.55 (s, 3H); \(^{13}\)C-NMR (101 MHz, Chloroform-d) δ 162.85, 159.64, 159.06, 154.07, 139.02, 137.69, 134.68, 134.03, 133.13, 131.76, 129.48, 129.46, 128.66, 127.93, 126.84, 113.25, 44.13, 35.11, 21.10, 12.09; HRMS (m/z): calcd. for C_{21}H_{16}ClN_{2}O_{2}S (M + H^+) 439.09955, found 439.10299.

6-(4-Chlorobenzyl)-N-(4-fluorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5f): White solid; Yield: 70.20%; mp: 210.8–211.2 °C; IR (KBr): δ 3292, 3062, 2939, 1652, 1607, 1570, 1542, 1508, 1373, 1352, 836, 807 cm\(^{-1};\) H-NMR (400 MHz, Chloroform-d) δ 7.39–7.25 (m, 6H), 7.07 (t, J = 8.6 Hz, 2H), 6.18 (s, 1H), 4.59 (d, J = 5.7 Hz, 2H), 4.10 (s, 2H), 2.72 (s, 3H); \(^{13}\)C-NMR (101 MHz, Chloroform-d) δ 162.83, 159.62, 158.86, 155.06, 139.42, 133.46, 133.10, 130.88, 129.78, 129.70, 128.69, 115.91, 115.69, 112.72, 43.65, 36.11, 12.33; HRMS (m/z): calcd. for C_{21}H_{17}ClFN_{2}O_{2}S (M + H^+) 443.07448, found 443.07610.

6-(4-Fluorobenzyl)-N-(4-fluorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5g): White solid; Yield: 73.30%; mp: 155.4–155.8 °C; IR (KBr): δ 3292, 3062, 2932, 1636, 1508, 1479, 1418, 1346, 812 cm\(^{-1};\) H-NMR (400 MHz, Chloroform-d) δ 7.39 (dd, J = 1.8, 0.8 Hz, 1H), 7.38–7.32 (m, 2H), 7.05–6.94 (m, 2H), 6.42–6.28 (m, 3H), 4.61 (d, J = 5.4 Hz, 2H), 4.10 (s, 2H), 2.71 (s, 3H); \(^{13}\)C-NMR (101 MHz, Chloroform-d) δ 163.15, 163.05, 160.71, 159.68, 159.08, 155.09, 150.27, 142.38, 139.06, 131.11, 131.03, 130.72, 130.69, 115.44, 115.22, 113.46, 110.52, 108.15, 37.04, 36.44, 12.31; HRMS (m/z): calcd. for C_{19}H_{18}FN_{2}O_{2}S (M + H^+) 399.09271, found 399.09391.
N-(2-Chlorobenzyl)-6-(4-fluorobenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5h): White solid; Yield: 69.40%; mp: 161.7–162.9 °C; IR (KBr): ν 3331, 3057, 2937, 1639, 1616, 1572, 1510, 1477, 1441, 1348, 804, 752 cm⁻¹; ¹H-NMR (400 MHz, Chloroform-δ) δ 7.50–7.27 (m, 6H), 7.01 (t, J = 8.7 Hz, 2H), 6.27 (s, 1H), 4.71 (d, J = 5.9 Hz, 2H), 4.11 (d, 2H), 2.71 (s, 3H); ¹³C-NMR (101 MHz, Chloroform-δ) δ 163.16, 163.07, 160.71, 159.81, 159.01, 155.07, 134.62, 133.52, 131.09, 131.01, 130.72, 130.69, 130.21, 129.59, 129.22, 127.12, 115.45, 115.24, 113.70, 42.28, 36.42, 12.29; HRMS (m/z): calcd. for C₂₁H₁₇CIFN₄O₂S (M + H⁺) 443.07448, found 443.07588.

N-(Furan-2-ylmethyl)-6-(4-methoxybenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5i): White solid; Yield: 75.00%; mp: 142.6–143.6 °C; IR (KBr): ν 3215, 2924, 2842, 1646, 1569, 1509, 1475, 1393, 814 cm⁻¹; ¹H-NMR (400 MHz, Chloroform-δ) δ 7.41 (dd, J = 1.8, 0.8 Hz, 1H), 7.35–7.27 (m, 2H), 6.88–6.80 (m, 2H), 6.41–6.31 (m, 2H), 6.26 (s, 1H), 4.62 (d, J = 5.2 Hz, 2H), 4.07 (s, 2H), 3.80 (s, 3H), 2.72 (s, 3H); ¹³C-NMR (101 MHz, Chloroform-δ) δ 162.95, 159.74, 159.17, 158.65, 155.44, 150.25, 142.42, 139.16, 130.54, 126.98, 113.90, 113.14, 110.53, 108.18, 55.23, 37.05, 36.33, 12.33; HRMS (m/z): calcd. for C₂₀H₂₀N₄O₃S (M + H⁺) 411.11270, found 411.11199.

N-(2-Chlorobenzyl)-6-(4-methoxybenzyl)-3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxamide (5j): White solid; Yield: 71.50%; mp: 156.4–172.2 °C; IR (KBr): ν 3258, 3156, 3160, 1570, 1512, 1350, 1300, 1036, 752 cm⁻¹; ¹H-NMR (400 MHz, Chloroform-δ) δ 7.49–7.38 (m, 2H), 7.34–7.24 (m, 4H), 6.88–6.80 (m, 2H), 6.52 (s, 1H), 4.70 (d, J = 5.3 Hz, 2H), 4.06 (s, 2H), 3.79 (s, 3H), 2.71 (s, 3H); ¹³C-NMR (101 MHz, Chloroform-δ) δ 162.92, 159.71, 158.99, 158.67, 155.58, 138.96, 134.44, 133.70, 130.67, 130.58, 129.73, 129.53, 127.30, 127.01, 113.93, 112.98, 55.25, 42.46, 36.34, 12.30; HRMS (m/z): calcd. for C₂₀H₂₀ClFN₄O₃S (M + H⁺) 455.09446, found 455.07662.

3.3. Crystal Structure Determination

The crystals suitable for X-ray single-crystal structure study were grown by slow evaporation from methanol solution for compound 3e. Data collections were performed on the Bruker AXS Smart APEX II CCD X-diffractometer equipped with graphite monochromated Mo Kα radiation (λ = 0.71073 Å) at 296 ± 2K. The crystal structure was determined by direct methods and refined by full-matrix least squares fitting on F² by SHELXS–97 [30]. All non-hydrogen atoms were refined anisotropically.

The crystallographic data have been deposited at the Cambridge Crystallographic Data Centre. These data can be obtained free of charge at http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +44 1223 336033; E-mail: deposit@ccdc.cam.ac.uk).

3.4. Determination of MIC for Bacterial Strains

The newly prepared compounds were screened for their antibacterial and antitubercular activity against S. aureus, B. subtilis, E. coli, P. aeruginosa, and M. smegmatis. The strains used in antibacterial tests were obtained from the National Center for Medical Culture Collection, China. A standard inoculum (1.5 × 10⁸ c.f.u/mL 0.5 McFarland standards) was introduced onto the surface of sterile agar plates and spread with a sterile glass spreader. The samples were dissolved in methanol at a concentration of 2 mg/mL. The MIC value was determined using a series dilution method. The diluted solutions in Mueller Hinton Broth were dispensed into the wells of a microtiter plate, and then an aliquot of 5 × 10⁵ c.f.u/mL of bacterial suspension was added to each well. The value of MIC was determined after 24 h of incubation at 37 °C.

3.5. Pharmacological Target Profiling

Pharmacophore screening was carried out using the Ligand Profiler protocol in Discovery Studio 3.5 software. The diverse conformations of the active compound were generated by using the Prepare Ligand protocol. The generated conformations were regarded as a query to map with the pharmacophoric features of the selected database by a flexible 3D searching method.
The pharmacophore model contained different antibacterial and antitubercular targets. Other operations were performed using default parameters.

3.6. Aminoacylation Assay

The LeuRS gene from *M. smegmatis* was cloned and expressed using vector pET28a(+) in *Escherichia coli* BL21 (DE3). The amount of *M. smegmatis* LeuRS required to catalyze the production of AMP from 1 µmol of ATP in 1 min at 37 °C was defined as one activity unit (1u). The expression product was purified according to the literature [31].

The aminoacylation assay was performed according to the literature [32,33]. All test compounds were dissolved in CH$_3$OH. The reaction mixture normally consisted of 50 mM HEPES-KOH (pH 8.0), 30 mM MgCl$_2$, 1 mM DTT, 30 mM KCl, 13 µM L-[¹⁴C]leucine (306 mCi/mmol), 15 µM *E. coli* tRNA, 0.2 pM *M. smegmatis* LeuRS, and 4 mM ATP, 0.02% BSA (wt/vol). The appropriate concentrations of test compounds were added to the reaction mixture, and then the aminoacylation reactions were incubated at 37 °C for 7 min after the addition of 4 mM ATP, and aliquots were quenched by the addition of 10% TCA (wt/vol). The inhibitory activity of the aminoacylation of *M. smegmatis* LeuRS was obtained by liquid scintillation counting.

4. Conclusions

In summary, a series of new 7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid derivatives was synthesized starting from the 6-substituted arylmethyl-3-mercapto-1,2,4-triazin-5-ones and ethyl 2-chloroacetoacetate. The intermediate β-keto ethers 2a–2e underwent intramolecular cyclization in PPA on heating to give 3-methyl-7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazines 3a–3e. The higher nucleophilicity at the N2 position of 1,2,4-triazine ring led to the regioselectivity of the cyclization step, and the selective annulation was confirmed by the single-crystal X-ray crystallographic structure of compound 3e. Further hydrolysis of these esters gave the corresponding acids, which were then amidated with various substituted arylmethylamines to obtain the amide derivatives. According to the results of biological evaluation, carboxylic acid derivatives or their corresponding ethyl esters have less of an effect on antibacterial properties, whereas compounds containing the terminal amide fragment exhibit better antibacterial and antitubercular activity. Particularly, compound 5f displayed a significant antitubercular effect with MIC values of 50 µg/mL against *M. smegmatis* and better inhibition efficiency of *M. smegmatis* LeuRS (percent inhibition of 78.24 ± 4.05% at 15 µg/mL). As a result, it was found that the novel 7-oxo-7H-thiazolo[3,2-b]-1,2,4-triazine-2-carboxylic acid derivatives containing the terminal amide fragment could be studied as LeuRS inhibitors for further biological research.

**Supplementary Materials:** $^1$H-NMR and $^{13}$C-NMR spectra for the synthesized compounds are available online.

**Author Contributions:** D.C., T.L., Q.X., and X.Y. contributed to the synthesis of the compounds. D.C. contributed to the characterization of the compounds and drafting of the manuscript. W.X. and Y.C. performed the design and the biological experiments. Z.J. and C.H. proposed the studies, and contributed to the analysis and discussion of the results and revision of the manuscript. All authors gave final approval for publication. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Science Foundation of China (NSFC) for the grant No. 21072130 and No. 21342006, and the Program for Innovative Research Team of the Ministry of Education of China (Grant No. IRT_14R36).

**Acknowledgments:** The authors would like to thank Crystal Impact GbR Ltd. Co., OlexSys Ltd. and Cambridge Crystallographic Data Centre (CCDC) for kindly providing us with a free evaluation of their software packages, Diamond, Olex$^2$, Platon and Mercury.

**Conflicts of Interest:** The authors declare no conflict of interest.
Abbreviations

1H-NMR proton nuclear magnetic resonance spectra
13C-NMR carbon nuclear magnetic resonance spectra
AMP adenosine monophosphate
ATP adenosine triphosphate
CCDC Cambridge Crystallographic Data Centre
DCM dichloromethane
DMF N,N-Dimethylformamide
DTT dithiothreitol
EDCI N′-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOBt 1-hydroxybenzotriazole
HRMS high-resolution mass spectra
IC50 half maximal inhibitory concentration
IR infrared spectra
KOH potassium hydroxide
MIC minimum inhibitory concentration
MS mass spectra
ORTEP Oak ridge thermal-ellipsoid plot program
PDB protein data bank
PPA polyphosphoric acid
SHELXS the crystallographic programs for crystal structure solution and refinement written by
TCA trichloroacetic acid
TLC thin layer chromatography
TMS tetramethylsilane

References

1. Stein, K.; Farmer, J.; Singhal, S.; Marra, F.; Sutherland, S.; Quiñonez, C. The use and misuse of antibiotics in
dentistry: A scoping review. *J. Am. Dent. Assoc.* 2018, 149, 869–884. [CrossRef]
2. Chokshi, A.; Sifri, Z.; Cennimo, D.; Horng, H. Global contributors to antibiotic resistance. *J. Glob. Infect. Dis.*
2019, 11, 36–42.
3. Vandevelde, N.M.; Tulkens, P.M.; Van Bambeke, F. Modulating antibiotic activity towards respiratory
bacterial pathogens by co-medications: A multi-target approach. *Drug Discov. Today* 2016, 21, 1114–1129.
[CrossRef] [PubMed]
4. de Oliveira Viana, J.; Ishiki, H.M.; Scotti, M.T.; Scotti, L. Multi-target antitubercular drugs. *Curr. Top.
Med. Chem.* 2018, 18, 750–758. [CrossRef] [PubMed]
5. Mishra, R.; Sharma, P.K.; Verma, P.K.; Tomer, I.; Mathur, G.; Dhakad, P.K. Biological potential of thiazole
derivatives of synthetic origin. *J. Heterocycl. Chem.* 2017, 54, 2103–2116. [CrossRef]
6. Kashyap, A.; Adhikari, N.; Das, A.; Shakya, A.; Ghosh, S.K.; Singh, U.P.; Bhat, H.R. Review on synthetic
chemistry and antibacterial importance of thiazole derivatives. *Curr. Drug Disc. Technol.* 2018, 15, 214–228.
[CrossRef]
7. Mishra, R.; Sachan, N.; Kumar, N.; Mishra, I.; Chand, P. Thiophene scaffold as prospective antimicrobial
agent: A Review. *J. Heterocycl. Chem.* 2018, 55, 2019–2034. [CrossRef]
8. Ali, E.M.H.; Abdel-Maksoud, M.S.; Oh, C.H. Thieno[2,3-d]pyrimidine as a promising scaffold in medicinal
chemistry: Recent advances. *Bios. Med. Chem.* 2019, 27, 1159–1194. [CrossRef]
9. Dewal, M.B.; Wani, A.S.; Vidaillac, C.; Oupický, D.; Rybak, M.J.; Firestine, S.M. Thieno[2,3-d]pyrimidine
derivatives as antibacterial agents. *Eur. J. Med. Chem.* 2012, 51, 145–153. [CrossRef]
10. Chambhare, R.V.; Khadse, B.G.; Bobde, A.S.; Bahekhar, R.H. Synthesis and preliminary evaluation of
some N-[5-(2-furanyl)-2-methyl-4-oxo-4H-thieno[2,3-d]pyrimidin-3-yl]-carboxamide and 3-substituted-
5-(2-furanyl)-2-methyl-3H-thieno[2,3-d]pyrimidin-4-ones as antimicrobial agents. *Eur. J. Med. Chem.*
2003, 38, 89–100. [CrossRef]
11. Hill, P.J.; Abibi, A.; Albert, R.; Andrews, B.; Gagnon, M.M.; Gao, N.; Grebe, T.; Hajec, L.I.; Huang, J.; Livchak, S.; et al. Selective inhibitors of bacterial t-RNA-(N1G37) Methyltransferase (TrmD) that demonstrate novel ordering of the lid domain. *J. Med. Chem.* 2013, 56, 7278–7288. [CrossRef] [PubMed]

12. Ibrahim, E.A.; Dine, S.; Soliman, F.; Labouta, I.M. Potential antineoplastics I: Substituted 3,5-dioxo-and 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines. *J. Pharm. Sci.* 1979, 68, 243–245. [CrossRef] [PubMed]

13. Forlani, L.; Tocke, A.L.; Del Vecchio, E.; Lakhdar, S.; Goumont, R.; Terrier, F. Assessing the nitrogen and carbon nucleophilicities of 2-aminothiazoles through coupling with superelectrophilic 4,6-dinitrobenzofuroxan. *J. Org. Chem.* 2006, 71, 5597–5603. [CrossRef] [PubMed]

14. Musser, J.H.; KrefT, A.F. 5-Lipoxygenase: Properties, pharmacology, and the quinolinyl (bridged) aryl class of inhibitors. *J. Med. Chem.* 1992, 35, 2501–2524. [CrossRef] [PubMed]

15. Liu, S.; Shang, R.; Shi, L.; Wan, D.C.C.; Lin, H. Synthesis and biological evaluation of 7H-thiazolo[3,2-b]-1,2,4-triazin-7-one derivatives as dual binding site acetylcholinesterase inhibitors. *Eur. J. Med. Chem.* 2014, 81, 237–244. [CrossRef]

16. Zhi, H.; Zhang, C.; Cheng, Z.; Jin, Z.; Huang, E.; Li, S.; Lin, H.; Wan, D.C.; Hu, C. 6-Acetyl-5H-thiazolo[3,2-a]pyrimidine derivatives as the novel acetylcholinesterase inhibitors: Design, synthesis, and biological activity. *Med. Chem.* 2013, 9, 703–709. [CrossRef]

17. Jin, Z.; Yang, L.; Liu, S.J.; Wang, J.; Li, S.; Lin, H.Q.; Wan, D.C.C.; Hu, C. Synthesis and biological evaluation of 3,6-diaryl-7H-thiazolo[3,2-b][1,2,4] triazin-7-one derivatives as acetylcholinesterase inhibitors. *Arch. Pharm. Res.* 2010, 33, 1641–1649. [CrossRef]

18. Heilman, W.P.; Heilman, R.D.; Scozzie, J.A.; Wayner, R.J.; Gullo, J.M.; Ariyan, Z.S. Synthesis and antihypertensive activity of novel 3-hydrazino-5-phenyl-1,2,4-triazines. *J. Med. Chem.* 1979, 22, 671–677. [CrossRef]

19. Heravi, M.M.; Aghapoor, K.; Nooshabadi, M.A. Regioselective acid-catalyzed cyclization reaction: Unique synthesis of condensed thiazoles and selenazole. *Synth. Commun.* 1998, 28, 233–237. [CrossRef]

20. Stanovnik, B.; Tišler, M.; Katritzky, A.R.; Denisko, O.V. The tautomerism of heterocycles: Substituent tautomerism of six-membered ring heterocycles. *Adv. Heterocycl. Chem.* 2006, 91, 1–134.

21. Liu, S.; Shang, R.; Shi, L.; Zhou, R.; He, J.; Wan, D.C.C. Design, Synthesis, and evaluation of 7H-thiazolo[3,2-b]-1,2,4-triazin-7-one derivatives as dual binding site acetylcholinesterase inhibitors. *Chem. Biol. Drug Des.* 2014, 84, 169–174. [CrossRef] [PubMed]

22. Wang, Y.; Li, Y.; Jiang, X. Sulfur-center-involved photocatalyzed reactions. *Chem.-Asian J.* 2018, 13, 2208–2242. [CrossRef] [PubMed]

23. Paronikyan, E.G.; Dashtyan, S.S.; Minasyan, N.S.; Stepanyan, G.M.; Babaev, E.V. Synthesis of 10-alkylsulfanyl-substituted pyrido-[4′,3′-4,5] thieno [3,2-d] pyrimidines annulated with pyran, cyclohexane, and cyclopentane rings. *Chem.-Heterocycl. Com.* 2016, 52, 337–345. [CrossRef]

24. Dave, C.G.; Patel, K.J. Pyridopyrimidines. X. Synthesis of 3-substituted 2-thioxo-5,7-dimethylpyrido [2,3-d] pyrimidin-4 (3H)-ones and their S-alkylation under phase transfer conditions. *J. Heterocycl. Chem.* 2001, 38, 457–461. [CrossRef]

25. Kirthikeyan, M.S. Synthesis and antimicrobial studies of thiazolotriazinones. *Eur. J. Med. Chem.* 2010, 45, 5039–5043. [CrossRef] [PubMed]

26. Kinnings, S.L.; Jackson, R.M. ReverseScreen3D: A structure-based ligand matching method to identify protein targets. *J. Chem. Inf. Model.* 2011, 51, 624–634. [CrossRef]

27. Jiang, J.; Zhou, H.; Jiang, Q.; Sun, L.; Deng, P. Novel transforming growth factor-β receptor 1 antagonists through a pharmacophore-based virtual screening approach. *Molecules* 2018, 23, 2824. [CrossRef]

28. Meslamani, J.; Li, J.; Sutter, J.; Stevens, A.; Bertrand, H.-O.; Rognan, D. Protein–ligand-based pharmacoopores: Generation and utility assessment in computational ligand profiling. *J. Chem. Inf. Model.* 2012, 52, 943–955. [CrossRef]

29. Stout, T.J.; Tondi, D.; Rinaldi, M.; Barlocco, D.; Pecorari, P.; Santi, D.V.; Kuntz, I.D.; Stroud, R.M.; Shoichet, B.K.; Costi, M.P. Structure-based design of inhibitors specific for bacterial Thymidylate Synthase. *Biochemistry* 1999, 38, 1607–1617. [CrossRef]

30. Sheldrick, G.M. A short history of SHELX. *Acta Crystallogr. Sect. A: Found. Crystallogr.* 2007, 64, 112–122. [CrossRef]

31. Zhang, R.; Chen, Y.; Liu, H.; Wei, X.U. Cloning and expression of Leucyl-tRNA Synthetase from *Mycobacterium phlei*. *Chin. J. Pharm.* 2016, 47, 1374–1378.
32. Li, X.; Hernandez, V.; Rock, F.L.; Choi, W.; Mak, Y.S.; Mohan, M.; Mao, W.; Zhou, Y.; Easom, E.E.; Plattner, J.J. Discovery of a potent and specific M. tuberculosis Leucyl-tRNA Synthetase inhibitor: (S)-3-(Aminomethyl)-4-chloro-7-(2-hydroxyethoxy) benzo [c][1,2] oxaborol-1 (3H)-ol (GSK656). *J. Med. Chem.* 2017, *60*, 8011–8026. [CrossRef] [PubMed]

33. Palencia, A.; Li, X.; Bu, W.; Choi, W.; Ding, C.Z.; Easom, E.E.; Feng, L.; Hernandez, V.; Houston, P.; Liu, L. Discovery of novel oral protein synthesis inhibitors of Mycobacterium tuberculosis that target leucyl-tRNA synthetase. *Antimicrob. Agents Chemother.* 2016, *60*, 6271–6280. [CrossRef] [PubMed]

**Sample Availability:** All synthesized compounds are available from the authors.

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).