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# Design, Synthesis, and Antibacterial Activity of Novel Pleuromutilin Derivatives Bearing an Amino Thiazolyl Ring

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A series of novel pleuromutilin derivatives containing the amino thiazolyl ring were designed, synthesized, and evaluated for their antibacterial activities *in vitro* against Gram-positive clinical bacteria. All the target compounds showed better aqueous solubility compared with the lead compound (**10**). Most compounds displayed strong antibacterial activities against both susceptible and resistant bacteria, particularly for the compound (**12f**) which showed extraordinary antibacterial properties superior to amoxicillin and tiamulin. Molecular docking studies revealed that the amino thiazolyl ring, the side chains of the pleuromutilin derivatives, can be adopted in the binding pocket of the 50S ribosomal subunit near the mutilin core. Therefore, our novel findings may provide new insights into the design of novel pleuromutilin derivatives and lay the basis for further studies on these promising antibiotics for human clinical use.

**Keywords:** 2-Aminothiazole / Antibacterial activity / Aqueous solubility / Gram-positive pathogens / Molecular docking / Pleuromutilin derivatives

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## Introduction

Antibacterial resistance of hospital-acquired Gram-positive bacterial pathogens, especially methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant *Streptococcus pneumonia* (PRSP), has become an increasingly serious medical issue which has drawn a great concern in community settings and intensive care in the hospitals [1, 2]. Therefore, developing novel classes of antibiotics against resistant bacteria has grown to be a desperate mission for medicinal chemists.

The natural product pleuromutilin **1** (Fig. 1), isolated from two basidiomycete species (e.g., *Pleurotus mutilus* and *Pleurotus passeckerianus*), can inhibit the growth of a variety of Gram-positive bacteria by selectively inhibiting bacterial protein synthesis through interaction with the 50S subunits of prokaryotic ribosomes [3] and has no cross-resistance to marketed antibacterial agents. Therefore, the design and development of more potent derivatives of pleuromutilin will be crucial for the clinical applications of pleuromutilin-based antibacterial agents. The first practical pleuromutilin derivative, tiamulin **2**, was developed by Sandoz in 1974. It is a prophylactic and therapeutic agent for swine dysentery, and has shown remarkable inhibiting potency (MIC $<1$ μg/mL) against anaerobic bacteria, intestinal spirochetes, and *Mycoplasma* spp. [4, 5]. But it can be rapidly metabolized *in vivo* by cytochrome P450-mediated hydroxylation of the tricyclic nucleus, and then eliminated [6]. The next potent pleuromutilin derivative valnemulin **3** was used as an effective medicine in treatment of enzootic pneumonia of pigs and originally approved in 1999 by the European Union [7, 8]. The antibiotic **3**, also once used in some rare human cases, was probably too toxic to be approved [9]. Afterwards, azamulin **4** was designed to overcome the toxicity of

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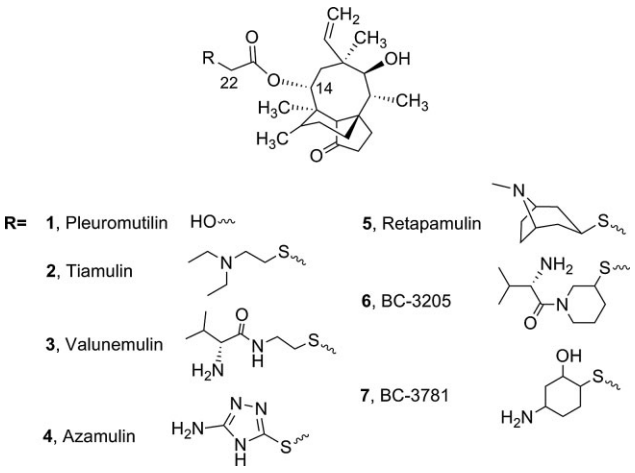



**Figure 1.** Chemical structures of pleuromutilin and derivatives thereof.

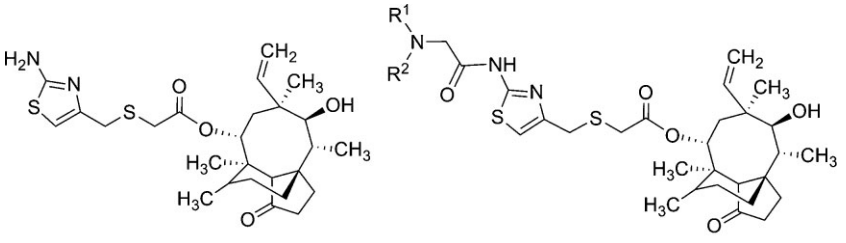
valnemulin and it successfully entered Phase I clinical evaluation. Unfortunately no more researches were conducted due to its low solubility and a short half-life *in vivo* [10]. Retapamulin **5** was so far the first drug developed for human use but only for topical treatment of skin infections [11]. In recent years, some other derivatives like BC-3205 (**6**) and BC-3781 (**7**) had already been under Phase I clinical studies, especially **7** which became the second pleuromutilin derivative that successfully entered Phase II clinical evaluation [12, 13]. These results suggest that pleuromutilin derivatives have the prospect of being developed as new drugs for human use with improvement in pharmacology and ability to counter the development of resistance. However, given the fact that there has been no effective pleuromutilin derivative for human oral use on the market so far, the discovery of new pleuromutilin derivatives with excellent antimicrobial activities against drug resistant pathogens will be of great significance.

The previous structure–activity relationships (SARs) studies of pleuromutilin analogues above revealed that the C-22 thioether side chain can tolerate a variety of modifications to improve their antimicrobial activities [10, 14–16]. We also notice that the new types of cephalosporin containing the thiazolyl ring usually display higher bioactivities than the former ones, and a lot of natural products of thiazolyl peptide with thiazolyl ring have been isolated from marine sources in recent years and show excellent bioactivities against resistant bacteria strains [17, 18]. These results indicate that modifying the side chain of pleuromutilin with the aminothiazole ring moiety may improve their antibacterial activities. With these ideas in mind, the new important pleuromutilin derivative **10** was designed and synthesized as our lead compound by coupling the C-22 position of pleuromutilin with 2-amino thiazolyl group. Meanwhile, aimed to obtain potent drug molecules with high aqueous solubility, a total set of seven target compounds **12a–g** (Fig. 2) were also designed and synthesized with different alkanolamine substituted 2-amino acetyl amides as the polar and water soluble group on the thiazolyl ring in the side chain. After unambiguous characterization, these target compounds were evaluated for their water solubility, and their antibacterial activities were investigated with drug resistant pathogens through standard procedure *in vitro*. Herein, we report the synthesis and biological evaluation of these derivatives.

## Results and discussion

### Chemistry

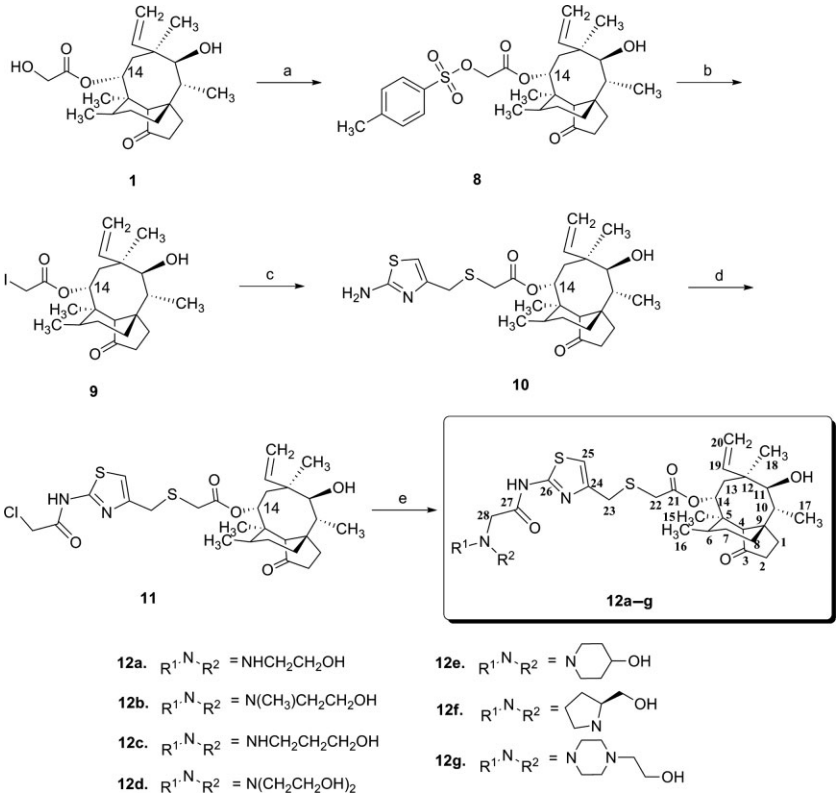
The synthetic routes of lead compound **10** and target compounds **12a–g** are outlined in Scheme 1. The starting material **1** firstly reacted with *p*-toluene sulfonyl chloride (TsCl) in the presence of triethylamine at room temperature for 17 h in $CH_2Cl_2$ to yield 14-*O*-(*p*-toluene sulfonyloxyacetyl) mutilin **8** followed by the displacement with potassium iodide to give the 14-*O*-(2-iodoacetyl) mutilin **9** under reflux for 3 h in acetone. The key intermediate **10** was obtained by the nucleophilic attack of (2-aminothiazol-4-yl)methanethiol on the latter **9** in the presence of 30% NaOH in an ice bath for 2 h in tetrahydrofuran (THF). Furthermore, lead compound **10** was treated with 2-chloroacetyl chloride in the presence of potassium carbonate to give the intermediate **11**. Finally, the resulting intermediate **11** was directly treated with different substituted alkanolamines in the presence of KI and potass-



**10.** The newly designed lead compound **12a—g.** The target compounds

**Figure 2.** The newly designed and synthesized compounds.




**Scheme 1.** Reaction conditions and reagents: (a) TsCl, triethylamine, $CH_2Cl_2$, rt, 17 h, 50%; (b) KI, acetone, reflux, 3 h, 87%; (c) (2-aminothiazol-4-yl)methanethiol, 30% NaOH, THF, 0°C rt, 2 h, 72%; (d) 2-chloroacetyl chloride, $K_2CO_3$, $CH_2Cl_2$, 0°C rt, 1 h, 92%; (e) $R^1R^2NH$, $K_2CO_3$, THF, 40°C, 4–8 h, 53–65%.

ium carbonate to generate the corresponding **12a–g** in yields of 53–65%, respectively. The final products were purified by column chromatography and their structures were confirmed on the basis of their spectral data of IR, $^1$H NMR, MS, $^{13}$C NMR, and elementary analyses. In the $^1$H NMR spectrum, three olefinic protons were observed, a double doublet in the range of 6.50–6.53 ppm and two doublets in the range of 5.35–5.41 and 5.21–5.26 ppm, which indicated the ($CH_2$=CH) protons. Additionally, the $^{13}$C NMR spectra exhibited the characteristic signals for $CH_2$=CH in the range of 139.00–139.10 and 117.05–117.23 ppm, and the characteristic signals for C=O of cyclopentanone, ester or amide in the range of 217.01–217.18 ppm, 169.14–170.32, or 168.43–168.77 ppm, respectively, which confirmed the formation of the compounds **12a–g**.

## Aqueous solubility

The aqueous solubility of the pleuromutilin derivatives was measured using UV at 25 ± 1°C [19] (Table 1). As expected, the aqueous solubility of the synthetic target compounds with a solubility ranging from 1.08 to 1.32 mg/mL was significantly higher than that of the lead compound with an equivalent of 27- to 33-fold solubility of the lead compound.

## Biological evaluation

The target compounds were evaluated for their antibacterial activities against a panel of susceptible and resistant Gram-positive organisms using the minimum inhibitory concentration (MIC). The results of these studies are summarized in Tables 2 and 3. Most target compounds displayed excellent *in vitro* antibacterial activities superior or similar to amoxicillin and tiamulin. Generally, their antibacterial activities against *Staphylococcus* were better than that against *Streptococcus*. Moreover, the key intermediate compound **10** with (2-aminothiazol-4-yl)methyl thioether group at C-14 position was 4–8-fold more potent than amoxicillin and tiamulin against most of the selected Gram-positive pathogens. Especially, compound **12f** containing 4-OH-piperidinyl

**Table 1.** Solubility of the target compounds in water at 25 ± 1°C

| Compd | 10 | 12a | 12b | 12c | 12d | 12e | 12f | 12g |
|---|---|---|---|---|---|---|---|---|
| Solubility (mg/mL) | <0.04 | 1.14 | 1.10 | 1.08 | 1.32 | 1.13 | 1.17 | 1.28 |

**Table 2.** Antibacterial activity (MIC, μg/mL) of pleuromutilin derivatives **12a–g** *in vitro*

| Compd | MIC[a] (μg/mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MSSA | | | MRSA | | | | MSSE | |
| | cpu0587[b] | cpu0211 | cpu1344 | cpu0446 | cpu0887 | cpu1098 | cpu3256 | cpu0347 | cpu2089 |
| Amoxicillin | 2 | 4 | 1 | 4 | 0.125 | 4 | 8 | 8 | 0.125 |
| Tiamulin | 8 | 2 | 2 | 2 | 0.5 | 2 | 2 | 0.5 | 0.5 |
| **10** | 2 | 0.5 | 0.125 | 0.25 | **0.0625** | 0.5 | 0.5 | 0.125 | 0.125 |
| **12a** | 0.125 | 0.25 | 0.25 | 1 | 0.25 | 0.25 | 0.25 | 0.125 | 0.125 |
| **12b** | 0.125 | 0.125 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.5 | 0.125 |
| **12c** | 0.25 | 0.25 | 0.25 | 1 | 0.5 | 0.5 | 0.5 | 0.5 | 0.25 |
| **12d** | 0.125 | 0.125 | 0.125 | 1 | 0.5 | 0.125 | 0.125 | 0.125 | **0.0625** |
| **12e** | **0.0625** | **0.0625** | 0.125 | 0.125 | 0.25 | 0.25 | 0.125 | **0.0625** | 0.25 |
| **12f** | **0.0625** | **0.0625** | **0.0625** | 0.125 | 0.125 | **0.0625** | **0.0625** | **0.0625** | 0.125 |
| **12g** | 0.125 | 0.125 | 0.125 | 0.125 | 0.25 | 0.25 | 0.25 | 0.125 | 0.125 |

[a] Minimal inhibitory concentrations (MIC): lowest concentration of compound that inhibits visible growth of the organism.
[b] MSSA, methicillin-susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*; MSSE, methicillin-susceptible *Staphylococcus epidermidis*; MRSE, methicillin- resistant *S. epidermidis*; PSSP, penicillin-susceptible *Streptococcus pneumonia*; PRSP, penicillin-resistant *S. pneumonia*. All strains were isolated from the clinical bacteria in the Nanjing Gulou hospital and reserved in the Department of Microbiology, College of Life Science and Technology, China Pharmaceutical University. Clinical strains were identified by API bacteria analysis system.

**Table 3.** Antibacterial activity (MIC, μg/mL) of pleuromutilin derivatives **12a–g** *in vitro*

| Compd | MIC (μg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MRSE | | | | PSSP | | PRSP | |
| | cpu2623 | cpu3889 | cpu3569 | cpu2736 | cpu1215 | cpu1653 | cpu2103 | cpu2634 |
| Amoxicillin | 8 | 8 | 8 | 0.125 | 8 | 2 | 2 | 4 |
| Tiamulin | 1 | 1 | 0.25 | 0.0625 | 1 | 2 | 4 | 1 |
| **10** | 0.125 | 0.125 | 0.125 | 0.125 | 1 | 1 | 4 | 0.25 |
| **12a** | 0.125 | 0.25 | 0.5 | 0.5 | 1 | 4 | 1 | 2 |
| **12b** | 0.5 | 0.25 | 0.5 | 0.25 | 2 | 2 | 1 | 2 |
| **12c** | 2 | 0.5 | 1 | 0.5 | 2 | 4 | 2 | 4 |
| **12d** | 0.125 | 0.5 | 0.5 | 0.5 | 1 | 1 | 1 | 1 |
| **12e** | 0.0625 | 0.25 | 0.125 | 0.25 | 1 | 1 | 0.5 | 1 |
| **12f** | **0.0625** | 0.125 | 0.25 | **0.0625** | 0.5 | 1 | 1 | 0.25 |
| **12g** | 0.125 | 0.25 | 0.25 | 0.5 | 2 | 4 | 1 | 2 |

groups exhibited the strongest activity with MIC values of 0.0625–0.25 μg/mL against MRSE, MRSE, MSSE, and MSSA, which was almost twofold more potent than compound **10**, 8–16-fold more potent than amoxicillin (MIC = 0.125–8 μg/mL), and tiamulin (MIC = 0.0625–2 μg/mL) against the same infections pathogens, respectively.

Compared to other pleuromutilin analogues containing different heterocycle from the literature, these novel amino thiazole derivatives in the series exhibited excellent antibacterial activity similar to purine derivatives [15], and better than pyrazole derivatives [20]. Despite the excellent antibacterial effect against both these susceptible and resistant Gram-positive bacteria, they demonstrated few rules of SARs. Among these different substituted alkanolamine derivatives of pleuromutilin, compounds **12a**, **12b**, and **12d** with a terminal aminoethanol residue had higher antibacterial activities against the selected Gram-positive pathogens than those bearing a terminal aminopropanol residue linker, such as **12c**. Secondly, the bioactivities of the compounds **12b**, **12d**, and **12e–g** linked with tertiary amine substituent groups were a little stronger than that of the derivatives **12a** and **12c** linked with secondary amine groups. In sharp contrast, the tertiary amine derivatives of pleuromutilin such as **12f** showed the best activity among the compounds tested. The higher antibacterial activity of **12f** may also stem from good lipid/water partition coefficient and the better binding affinity between the molecular structure and the 50S ribosomal subunit. We also endeavor to further determine

the SARs of pleuromutilin derivatives, which will provide us a new insight into the design of novel antibacterial drugs.

## Molecular docking

To further study the primary antibacterial activities mechanism and the interaction mode of the novel classes of the pleuromutilin derivatives, molecular docking study was performed using software package MOE 2008.10. The X-ray crystal structure of the 50S ribosomal subunit from *Deinococcus radiodurans* in complex with tiamulin (PDB code: 1XBP) was obtained from the Protein Data Bank and applied to build the starting model of the 50S ribosomal subunit.

The docking results are shown in Fig. 3, using compound **12f** as a template which possesses the highest bioactivity to susceptible and resistant bacteria, respectively. As shown in Fig. 3A and B, compound **12f** could adopt different binding modes within the active pocket of the 50S ribosomal subunit. All of the side chains were of the pharmacophores, and the spatial orientations of the pharmacophores direct to the active pocket. The thiazolyl heterocycle moiety at the C-22 side chain position of compound **12f** bound with residue A2045 via $\pi$–$\pi$ stacking interaction. Besides, two hydrogen bonds were also found. One formed between G2484 and the oxygen of OH group in the mother ring of **12f** (O···O distance: 2.68 Å). The other formed between G2044 and the oxygen of C=O group in the side chain (N···O distance: 2.67 Å). Thus, compound **12f** was found to be docked into the gorge of the 50S ribosomal subunit perfectly, which explained its high antibacterial effect. All these results indicated that the side chains of the compounds played an important role in adjusting the physico-chemical properties of the whole molecule, and furthermore, the amino thiazolyl ring in general can be

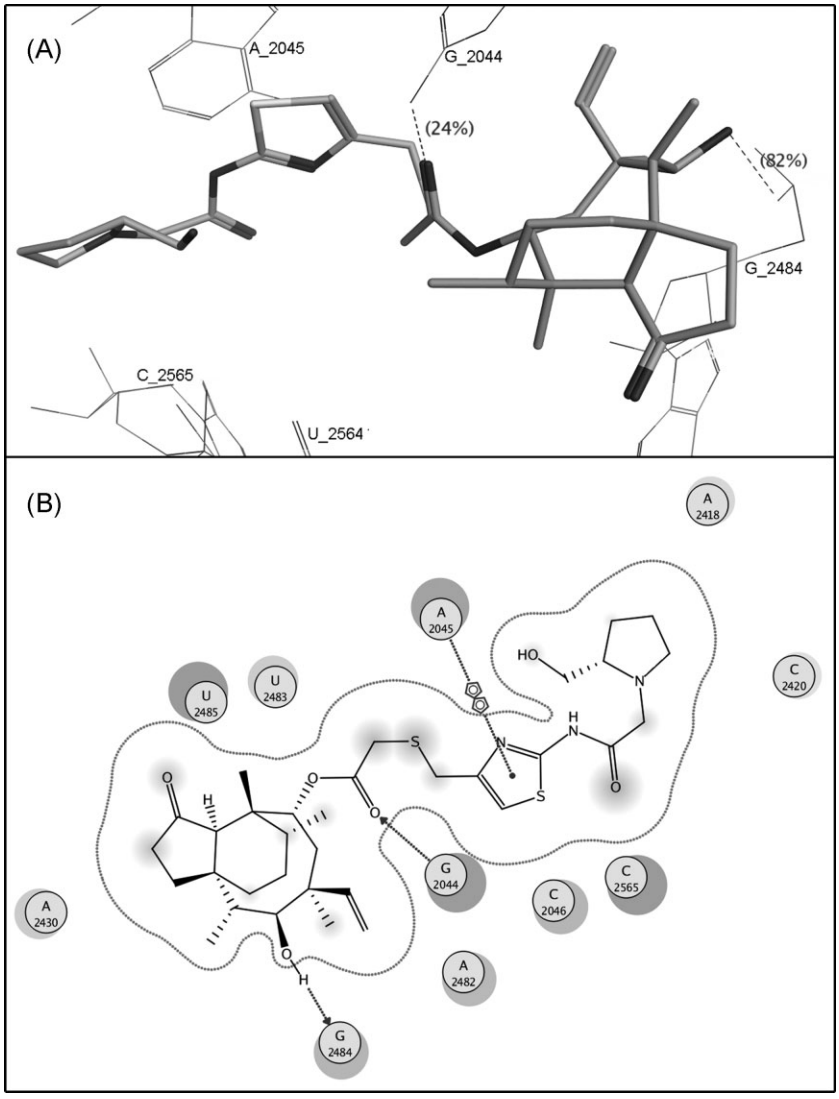



**Figure 3.** The docking conformation of compound **12f** in the active site of the 50S ribosomal subunit from *D. radiodurans* (PDB code: 1XBP) generated with MOE.

adopted near the mutilin core in the binding pocket and used to further improve the binding affinity.

## Conclusion

In summary, new classes of pleuromutilin derivatives have been designed and synthesized with different 2-(substituted amino) acetyl amide groups on the thiazolyl ring in the C-22 side chain, and their antibacterial activities have been evaluated *in vitro* against a number of Gram-positive clinical pathogens from both sensitive and resistant strains. We find that all the target compounds exhibited better aqueous solubility than that of the lead compound and most of the compounds displayed strong antibacterial activities against these susceptible and resistant Gram-positive bacteria, particularly for the compound **12f** which showed excellent antibacterial properties superior to the reference drug amoxicillin and tiamulin. Molecular docking studies revealed that the amino thiazolyl ring, the side chains of pleuromutilin derivatives, can be adopted near the mutilin core in the binding pocket of the 50S ribosomal subunit which may clarify their high antibacterial effect. Therefore, our new findings may provide potentially important information for further development of pleuromutilin derivatives and lay the basis for further studies on the promising antibiotics for human clinical treatment.

## Experimental

### Chemistry

Melting points were determined on a Mel-TEMP II melting point apparatus and uncorrected. Infrared (IR) spectra (KBr) were recorded on a Nicolet Impact 410 instrument (KBr pellet). $^1$H NMR and $^{13}$C NMR spectra were recorded with a Bruker Avance 400 and 100 MHz spectrometer, respectively, at 300 K, using TMS as an internal standard. MS spectra were recorded on a Shimadzu GC-MS 2010 (EI) or a Mariner Mass Spectrum (ESI). Elemental analysis was performed on an Eager 300 instrument. All compounds were routinely checked by TLC and preparative TLC was performed on silica gel GF/UV 254, and the column chromatography were conducted on silica gel (200–300 mesh) and visualized under UV light at 254 and 365 nm. As determined by elemental analysis, the purity of all target compounds is >95%. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. Solutions after reactions and extractions were concentrated using a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Compound **1** was commercially available, and compounds **8** and **9** were prepared according to the literature [21].

#### *14-O-[2-((2-Aminothiazol-4-yl)methylthio)acetyl]mutilin **10***

To a solution of (2-aminothiazol-4-yl)methanethiol (0.33 g, 2.24 mmol) in 2 mL 30% NaOH aqueous solution, compound **9** (1.00 g, 2.04 mmol) in 25 mL THF was slowly dropped. The reaction mixture was stirred in the ice bath environment for 2 h. After the reaction, the solvent was removed under reduced pressure. The crude residue was dissolved in ethyl acetate and washed with water and saturated NaCl solution separately, and then the organic layer was dried with $MgSO_4$, filtered, concentrated, and purified by column chromatography (PE/EtOAc = 5:1–1:1) to yield **10** as a white solid 0.75 g, yield: 72%; m.p.: 67–70°C. Analytical data for **10**: IR (KBr, cm$^{-1}$): 3436, 3353, 2984, 2856, 1716, 1617, 1524, 1280, and 685; $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 6.50 (1H, dd, $J_1 = 11.2$ Hz, $J_2 = 17.6$ Hz, H19), 6.33 (1H, s, H25), 5.77 (1H, d, $J = 8.0$ Hz, H14), 5.37 (1H, d, $J = 11.2$ Hz, H20), 5.23 (1H, d, $J = 17.2$ Hz, H20), 3.67 (2H, s, H23), 3.08 (2H, s, H22), 3.38 (1H, m, H11), 2.38–2.05 (5H, m, H2, H4, H10, H13), 1.83–1.31 (11H, m, H1, H6, H7, H8, H15, H13, 11-OH), 1.19 (3H, m, H, H18), 0.89 (3H, d, $J = 6.8$ Hz, H17), 0.74 (3H, d, $J = 6.8$ Hz, H16); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ 217.12 (C3), 168.82 (C21), 168.18 (C26), 147.13 (C24), 139.10 (C19), 117.27 (C20), 105.89 (C25), 74.82 (C11), 69.16 (C14), 58.20 (C4), 45.46 (C22), 44.76 (C9), 43.9 (C13), 41.73 (C12), 36.78 (C5), 36.00 (C6), 34.48 (C10), 32.80 (C2), 31.77 (C23), 30.42 (C8), 26.84 (C7), 26.39 (C18), 24.85 (C1), 16.90 (C16), 14.99 (C15), 11.58 (C17); ESI-MS (*m*/*z*): 507 [M+H]$^+$; Anal. Calcd. for $C_{26}H_{38}N_2O_4S_2$: C, 61.63; H, 7.56; N, 5.53; Found: C, 61.41; H, 7.81; N, 5.46.

#### *14-O-[2-((2-(2-Chloroacetamido)thiazol-4-yl)-methylthio)acetyl]mutilin **11***

To a solution of compound **10** (1.00 g, 1.98 mmol) and $K_2CO_3$ (0.41 g, 2.96mmol) in 20 mL dry $CH_2Cl_2$, $ClCH_2COCl$ (0.33 g, 2.96 mmol) in 5 mL dry $CH_2Cl_2$ was slowly dropped at 0°C. The reaction mixture was stirred for 1 h. After the reaction, the solution was washed with water three times, and then the organic layer was dried with $NaSO_4$, filtered, concentrated, and purified by column chromatography (PE/EtOAc = 2:1) to yield **11** as a white waxy solid 1.06 g, yield: 92%. Analytical data for **11**: IR (KBr, cm$^{-1}$): 3482, 3204, 3078, 2933, 2864, 1728, 1552, 1455, 1411, 1281, 1115, 1016, 980; $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 6.86 (1H, s, H25), 6.51 (1H, dd, $J_1 = 11.2$ Hz, $J_2 = 17.2$ Hz, H19), 5.79 (1H, d, $J = 8.4$ Hz, H14), 5.39 (1H, d, $J = 11.2$ Hz, H20), 5.24 (1H, d, $J = 17.2$ Hz, H20), 4.29 (2H, s, H28), 3.84 (2H, s, H23), 3.38 (1H, m, H11), 3.05 (2H, s, H22), 2.38–2.05 (5H, m, H2, H4, H10, H13), 1.81–1.32 (11H, m, H1, H6, H7, H8, H15, H13, 11-OH), 1.19 (3H, s, H18), 0.89 (3H, d, $J = 6.8$ Hz, H17), 0.74 (3H, d, $J = 6.8$ Hz, H16); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ 217.11 (C3), 168.55 (C21), 164.01 (C27), 157.31 (C26), 146.34 (C24), 139.11 (C19), 117.28 (C20), 111.94 (C25), 74.61 (C11), 69.29 (C14), 58.16 (C4), 45.44 (C22), 44.79 (C9), 43.89 (C13), 41.94 (C28), 41.72 (C12), 36.74 (C5), 35.99 (C6), 34.46 (C10), 32.77 (C2), 31.35 (C23), 30.40 (C8), 26.83 (C7), 26.38 (C18), 24.84 (C1), 16.90 (C16), 14.93 (C15), 11.57 (C17); ESI-MS (*m*/*z*): 581 [M–H]$^-$; Anal. Calcd. for $C_{28}H_{39}ClN_2O_5S_2$: C, 57.66; H, 6.74; N, 4.80; Found: C, 57.79; H, 6.94; N, 4.66.

#### *General procedure for the synthesis of the target compounds **12a–g***

A solution of compound **11** (1.72 mmol), $R_1R_2NH$ (8.60 mmol), and $K_2CO_3$ (5.16 mmol) in 20 mL acetonitrile was stirred at 40°C for 4–8 h. The solvent was removed under reduced pressure. The crude residue was dissolved in chloroform and washed with saturated NaCl. The organic layer was dried with $MgSO_4$, filtered, concentrated, and purified by column chromatography (EtOAc/MeOH = 20:1–10:1) to yield **12a–g**.

### *14-O-[2-((2-(2-(2-Hydroxyethylamino)acetamido)-thiazol-4-yl)methylthio)acetyl]mutilin* ***12a***

Compound **12a** was synthesized according to the general procedure. White foam solid; yield: 63%. Analytical data for **12a**: IR (KBr, $cm^{-1}$): 3316, 2931, 1728, 1685, 1447, 1413, 1373, 1324, 1153, 1117, 937; $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ 6.78 (1H, s, H25), 6.51 (1H, dd, $J_1 = 11.2$ Hz, $J_2 = 17.6$ Hz, H19), 5.78 (1H, d, $J = 8.4$ Hz, H14), 5.37 (1H, d, $J = 10.8$ Hz, H20), 5.23 (1H, d, $J = 17.2$ Hz, H20), 3.79 (2H, s, H23), 3.44 (2H, m, $CH_2OH$), 3.39 (2H, m, OH, H11), 3.22 (2H, s, H22), 3.05 (2H, s, H28), 2.82 (2H, t, $J = 4.8$ Hz, $NCH_2$), 2.02–2.29 (5H, m, H2, H4, H10, H13), 1.23–1.81 (11H, m, H1, H6, H7, H8, H15, H13, 11-OH), 1.19 (3H, s, H18), 0.89 (3H, d, $J = 6.8$ Hz, H17), 0.74 (3H, d, $J = 6.8$ Hz, H16); $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ 217.02 (C3), 169.14 (C21), 168.58 (C27), 158.15 (C26), 147.07 (C24), 139.00 (C19), 117.05 (C20), 111.11 (C25), 74.32 (C11), 69.13 (C14), 59.21 ($CH_2OH$), 58.12 (C4), 57.97 (C28), 51.37 ($NCH_2$), 45.40 (C22), 44.72 (C9), 43.84 (C13), 41.63 (C12), 36.73 (C5), 35.99 (C6), 34.42 (C10), 32.77 (C2), 31.71 (C23), 30.46 (C8), 26.80 (C7), 26.55 (C18), 24.80 (C1), 16.97 (C16), 14.95 (C15), 11.50 (C17). ESI-MS (*m/z*): 608 $[M+H]^+$; Anal. Calcd. for $C_{30}H_{45}N_3O_6S_2$: C, 59.28; H, 7.46; N, 6.91; Found: C, 59.11; H, 7.74; N, 6.76.

### *14-O-[2-((2-(2-((2-Hydroxyethyl)(methyl)amino)-acetamido)thiazol-4-yl)methylthio)acetyl]mutilin* ***12b***

Compound **12b** was synthesized according to the general procedure. White foam solid; yield: 60%. Analytical data for **12b**: IR (KBr, $cm^{-1}$): 3427, 2934, 1732, 1628, 1451, 1408, 1280, 1165, 1016, 980, 696; $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ 6.83 (1H, s, H25), 6.53 (1H, dd, $J_1 = 11.2$ Hz, $J_2 = 17.2$ Hz, H19), 5.81 (1H, d, $J = 8.0$ Hz, H14), 5.41 (1H, d, $J = 10.8$ Hz, H20), 5.26 (1H, d, $J = 17.2$ Hz, H20), 3.84 (2H, s, H23), 3.45 (2H, m, $CH_2OH$), 3.40 (2H, m, OH, H11), 3.28 (2H, s, H22), 3.06 (2H, s, H28), 2.54 (2H, m, $NCH_2$), 2.04–2.33 (8H, m, H2, H4, H10, H13, $NCH_3$), 1.17–1.81 (14H, m, H1, H6, H7, H8, H15, H13, 11-OH, H18), 0.90 (3H, d, $J = 6.8$ Hz, H17), 0.75 (3H, d, $J = 6.8$ Hz, H16); $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ 217.10 (C3), 170.08 (C21), 168.65 (C27), 158.07 (C26), 146.56 (C24), 139.06 (C19), 117.23 (C20), 111.18 (C25), 74.56 (C11), 69.15 (C14), 60.71 ($NCH_2$), 58.62 ($CH_2OH$), 58.43 (C4), 56.37 (C28), 45.66 ($NCH_3$), 45.48 (C22), 44.82 (C9), 43.86 (C13), 41.65 (C12), 36.75 (C5), 35.99 (C6), 34.44 (C10), 32.76 (C2), 31.65 (C23), 30.44 (C8), 26.83 (C7), 26.51 (C18), 24.83 (C1), 16.97 (C16), 14.95 (C15), 11.52 (C17). ESI-MS (*m/z*): 622 $[M+H]^+$; Anal. Calcd. for $C_{31}H_{47}N_3O_6S_2$: C, 59.87; H, 7.62; N, 6.76; Found: C, 60.05; H, 7.54; N, 6.52.

### *14-O-[2-((2-(2-(2-Hydroxypropylamino)acetamido)-thiazol-4-yl)methylthio)acetyl]mutilin* ***12c***

Compound **12c** was synthesized according to the general procedure. White foam solid; yield: 53%. Analytical data for **12c**: IR (KBr, $cm^{-1}$): 3308, 2934, 1745, 1677, 1532, 1466, 1374, 1456, 1066, 1017; $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ 6.75 (1H, s, H25), 6.50 (1H, dd, $J_1 = 11.2$ Hz, $J_2 = 17.2$ Hz, H19), 5.78 (1H, d, $J = 8.4$ Hz, H14), 5.37 (1H, d, $J = 10.8$ Hz, H20), 5.24 (1H, d, $J = 17.2$ Hz, H20), 3.80 (2H, s, H23), 3.45 (2H, m, $CH_2OH$), 3.37 (2H, m, OH, H11), 3.20 (2H, s, H22), 3.05 (2H, s, H28), 2.76 (2H, t, $J = 4.8$ Hz, $NCH_2$), 2.04–2.31 (5H, m, H2, H4, H10, H13), 1.24–1.83 (13H, m, H1, H6, H7, H8, H15, H13, 11-OH, $NCH_2CH_2$), 1.18 (3H, s, H18), 0.88 (3H, d, $J = 6.8$ Hz, H17), 0.73 (3H, d, $J = 6.8$ Hz, H16); $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ 217.01 (C3), 169.03 (C21), 168.45 (C27), 158.38 (C26), 146.81 (C24), 139.10 (C19), 117.16 (C20), 111.36 (C25), 74.53 (C11), 69.21 (C14), 59.87 ($CH_2OH$), 58.14 (C4), 57.98 (C28), 45.41 (C22), 45.22 ($NCH_2$), 44.74 (C9), 43.86 (C13), 41.65 (C12), 36.75 (C5), 35.98 (C6), 34.43 (C10), 32.79 (C2), 31.70 (C23), 30.46 (C8), 26.80 (C7), 26.52 (C18), 24.82 (C1), 21.15 ($NCH_2CH_2$), 16.94 (C16), 14.96 (C15), 11.54 (C17). ESI-MS (*m/z*): 622 $[M+H]^+$; Anal. Calcd. for $C_{31}H_{47}N_3O_6S_2$: C, 59.87; H, 7.62; N, 6.76; Found: C, 59.61; H, 7.84; N, 6.57.

### *14-O-[2-((2-(2-(Bis(2-hydroxyethyl)amino)-acetamido)thiazol-4-yl)methylthio)acetyl]mutilin* ***12d***

Compound **12d** was synthesized according to the general procedure. White foam solid; yield: 58%. Analytical data for **12d**: IR (KBr, $cm^{-1}$): 3410, 2933, 2879, 2353, 1722, 1533, 1460, 1409, 1280, 1114; $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ 6.71 (1H, s, H25), 6.50 (1H, dd, $J_1 = 11.2$ Hz, $J_2 = 17.6$ Hz, H19), 5.77 (1H, d, $J = 8.4$ Hz, H14), 5.35 (1H, d, $J = 10.8$ Hz, H20), 5.21 (1H, d, $J = 17.2$ Hz, H20), 3.76 (2H, s, H23), 3.46 (4H, m, 2 × $CH_2OH$), 3.36 (3H, m, 2 × OH, H11), 3.20 (2H, s, H22), 3.08 (2H, s, H28), 2.82 (4H, t, $J = 4.8$ Hz, 2 × $NCH_2$), 2.05–2.37 (5H, m, H2, H4, H10, H13), 1.80–1.25 (11H, m, H1, H6, H7, H8, H15, H13, 11-OH), 1.19 (3H, s, H18), 0.89 (3H, d, $J = 6.8$ Hz, H17), 0.73 (3H, d, $J = 6.8$ Hz, H16); $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ 217.18 (C3), 170.32 (C21), 168.77 (C27), 158.55 (C26), 147.21 (C24), 139.13 (C19), 117.18 (C20), 111.02 (C25), 74.45 (C11), 69.36 (C14), 59.37 (2 × $NCH_2$), 58.20 (2 × $CH_2OH$), 58.15 (C4), 57.90 (C28), 45.44 (C22), 44.76 (C9), 43.88 (C13), 41.72 (C12), 36.77 (C5), 35.97 (C6), 34.45 (C10), 32.79 (C2), 31.79 (C23), 30.46 (C8), 26.81 (C7), 26.41 (C18), 24.82 (C1), 16.87 (C16), 14.93 (C15), 11.52 (C17). ESI-MS (*m/z*): 652 $[M+H]^+$; Anal. Calcd. for $C_{32}H_{49}N_3O_7S_2$: C, 58.96; H, 7.58; N, 6.45; Found: C, 59.21; H, 7.33; N, 6.28.

### *14-O-[2-((2-(2-(4-Hydroxypiperidin-1-yl)acetamido)-thiazol-4-yl)methylthio)acetyl]mutilin* ***12e***

Compound **12e** was synthesized according to the general procedure. White foam solid; yield: 64%. Analytical data for **12e**: IR (KBr, $cm^{-1}$): 3431, 2937, 1731, 1454, 1373, 1280, 1146, 1116, 1016; $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ 6.80 (1H, s, H25), 6.51 (1H, dd, $J_1 = 11.2$ Hz, $J_2 = 17.2$ Hz, H19), 5.79 (1H, d, $J = 8.4$ Hz, H14), 5.38 (1H, d, $J = 10.8$ Hz, H20), 5.24 (1H, d, $J = 17.2$ Hz, H20), 4.14 (1H, m, CHOH), 3.83 (2H, m, H23, CH), 3.38 (1H, m, H11), 3.23 (2H, s, H22), 3.06 (2H, s, H28), 2.83 (4H, m, N ($CH_2$–C)$_2$–), 2.00–2.05 (5H, m, H2, H4, H10, H13), 1.19–1.80 (18H, m, H1, H6, H7, H8, H13, H15, H18, 11-OH, N (C–$CH_2$)$_2$), 0.90 (3H, d, $J = 6.8$ Hz, H17), 0.75 (3H, d, $J = 6.8$ Hz, H16); $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ 217.17 (C3), 168.95 (C21), 168.65 (C27), 157.76 (C26), 146.28 (C24), 139.12 (C19), 117.25 (C20), 111.33 (C25), 74.62 (C11), 69.27 (C14), 66.61 (CHOH), 61.94 (C4), 58.21 (C28), 51.64 (N ($CH_2$)$_2$), 45.46 C22), 44.83 (C9), 43.92 (C13), 41.75 (C12), 36.77 (C5), 36.01 (C6), 34.47 (C10), 34.21 (N (C–$CH_2$)$_2$), 32.80 (C2), 31.63 (C23), 30.42 (C8), 26.85 (C7), 26.43 (C18), 24.84 (C1), 16.91 (C16), 14.98 (C15), 11.54 (C17); ESI-MS (*m/z*): 648 $[M+H]^+$; Anal. Calcd. for $C_{33}H_{49}N_3O_6S_2$: C, 61.18; H, 7.62; N, 6.49; Found: C, 59.92; H, 7.83; N, 6.26.

### *(S)-14-O-[2-((2-(2-(2-(Hydroxymethyl)pyrrolidin-1-yl)-acetamido)thiazol-4-yl)methylthio)acetyl]mutilin* ***12f***

Compound **12f** was synthesized according to the general procedure. White foam solid; yield: 65%. Analytical data for **12f**: IR (KBr, $cm^{-1}$): 3435, 2936, 2866, 1732, 1530, 1456, 1414, 1280,

1374, 1115, 1018; $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 6.77 (1H, s, H25), 6.50 (1H, dd, $J_1 = 10.8$ Hz, $J_2 = 17.2$ Hz, H19), 5.78 (1H, d, $J = 8.0$ Hz, H14), 5.38 (1H, d, $J = 10.8$ Hz, H20), 5.23 (1H, d, $J = 17.2$ Hz, H20), 3.81 (2H, d, H23), 3.76 (1H, m, NCH), 3.38 (1H, m, H11), 3.34 (2H, m, C$H_2$OH), 3.19 (3H, m, H22, C–OH), 3.04 (2H, s, H28), 2.49–2.05 (7H, m, H2, H4, H10, H13, $NCH_2$), 1.80–1.14 (18H, m, H1, H6, H7, H8, H15, H13, 11-OH, H18, C$H_2$C$H_2$CH), 0.89 (3H, d, $J = 7.2$ Hz, H17), 0.736 (3H, d, $J = 6.8$ Hz, H16); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ 217.05 (C3), 168.61 (C21), 168.43 (C27), 157.68 (C26), 146.28 (C24), 139.13 (C19), 117.25 (C20), 111.45 (C25), 74.63 (C11), 69.29 (C14), 61.01 (NCH), 59.19 (C4), 58.19 ($CH_2$OH), 57.65 (C28), 52.61 ($NCH_2$), 45.46 (C22), 44.85 (C9), 43.93 (C13), 41.75 (C12), 36.78 (C5), 36.02 (C6), 34.46 (C10), 32.82 (C2), 31.67 (C23), 30.42 (C8), 26.86 (C7), 26.42 (C18), 26.24 (CH$CH_2$), 24.85 (C1), 21.20 ($NCH_2CH_2$), 16.92 (C16), 14.96 (C15), 11.54 (C17); ESI-MS (*m/z*): 648 $[M+H]^+$; Anal. Calcd. for $C_{33}H_{49}N_3O_6S_2$: C, 61.18; H, 7.62; N, 6.49; Found: C, 59.92; H, 7.88; N, 6.22.

### *14-O-[2-((2-(2-(4-(2-Hydroxyethyl)piperazin-1-yl)-acetamido)thiazol-4-yl)methylthio)acetyl]mutilin* **12g**

Compound **12g** was synthesized according to the general procedure. White foam solid; yield: 53%. Analytical data for **12g**: IR (KBr, $cm^{-1}$): 3427, 2937, 2823, 1728, 1456, 1383, 1327, 1280, 1156, 1116. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 6.81 (1H, s, H25), 6.51 (1H, dd, $J_1 = 11.2$ Hz, $J_2 = 17.2$ Hz, H19), 5.79 (1H, d, $J = 8.0$ Hz, H14), 5.39 (1H, d, $J = 11.2$ Hz, H20), 5.24 (1H, d, $J = 17.2$ Hz, H20), 3.83 (2H, s, H23), 3.39 (1H, m, H11), 3.27 (2H, s, H22), 3.05 (2H, s, H28), 3.67 (2H, m, C$H_2$OH), 2.65–2.67 (10H, m, $N(CH_2CH_2)_2NCH_2$), 2.05–2.38 (5H, m, H2, H4, H10, H13), 1.11–1.81 (14H, m, H1, H6, H7, H8, H15, H13, 11-OH, H18), 0.89 (3H, d, $J = 6.8$ Hz, H17), 0.75 (3H, d, $J = 6.4$ Hz, H16); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ 217.12 (C3), 170.15 (C21), 168.67 (C27),158.10 (C26), 146.08 (C24), 139.11 (C19), 117.27 (C20), 111.21 (C25), 74.61 (C11), 69.19 (C14), 65.83 ($HOCH_2$), 64.74 ($NCH_2$), 60.41 ($2 \times NCH_2$), 58.84 (C4), 58.19 ($2 \times NCH_2$), 56.34 (C28), 45.46 (C22), 44.82 (C9), 43.92 (C13), 41.75 (C12), 36.77 (C5), 36.01 (C6), 34.47 (C10), 32.78 (C2), 31.67 (C23), 30.42 (C8), 26.85 (C7), 26.40 (C18), 24.85 (C1), 16.89 (C16), 14.96 (C15), 11.54 (C17); ESI-MS (*m/z*): 677 $[M+H]^+$; Anal. Calcd. for $C_{34}H_{52}N_4O_6S_2$: C, 60.33; H, 7.74; N, 8.28; Found: C, 60.41; H, 7.96; N, 8.05.

## Evaluation of the biological activity

### *Minimum inhibitory concentration testing*

The MICs of the novel compounds against Gram-positive bacteria were tested using amoxicillin and tiamulin as positive control. MIC values were determined by an agar dilution method according to the methods of the National Committee for Clinical Laboratory Standards (NCCLS) [22]. Compounds were dissolved in 50% water in DMSO to prepare a stock solution that had a concentration of 1.28 mg/mL. Serial 2-fold dilutions were prepared from the stock solution with sterile water and then 10-fold diluted with Mueller–Hinton (MH) agar medium to provide concentration ranges of 64–0.0625 μg/mL. The tested organisms were planted in MH broth medium at 35°C for 8 h and were adjusted to the turbidity of the 0.5 McFarland standard. The bacterial suspensions were inoculated onto the drug-supplemented MH agar plates with a multipoint inoculator and incubated at 35°C for 16 h. All experiments were carried out three times.

## Aqueous solubility

Each compound with the accurate weight of 200.0 mg was placed in a 50 mL beaker. Then 10 mL water was added and exposed to ultrasonic oscillation for 5 min and stirred for 24 h in a water bath with a constant temperature of 25°C. It was centrifuged at 10 000 rpm for 10 min, and the supernatant was filtered through a microporous membrane and injected into the HPLC system for determination.

## Molecular docking methodology

All calculations and analysis were conducted with MOE 2008.10 program (Chemical Computing Group, Montreal, Canada). The crystal structure of the 50S ribosomal subunit from *D. radiodurans* in complex with tiamulin (PDB code: 1XBP) used in the docking study were obtained from the Protein Data Bank (www.rcsb.org). Heteroatoms and water molecules in the PDB files were removed at the beginning, and all hydrogen atoms were subsequently added to the 50S ribosomal subunit. Amber99 forcefield was assigned to the ribosomal subunit by MOE. Prior to the docking calculations, an energy minimization using the MMFF94 forcefield was performed on the ribosomal subunit based on the minimization protocol.

Compound **12f** was drawn in MOE and all hydrogen atoms were added subsequently. Then the compounds were protonated using the protonate 3D protocol and energy minimized using the MMFF94 forcefield in MOE. When the ribosomal subunit and compound **12f** were ready for the docking study, compound **12f** was modeled within the ribosomal subunit according to the MOE docking protocol with the Alpha Triangle placement option and the London dG scoring function.

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## References

[1] C. Walsh, G. Wright, *J. Chem. Rev.* **2005**, *105*, 391–394.
[2] D. M. Livermore, *J. Clin. Infect. Dis.* **2003**, *36*, 11–23.
[3] M. Anchel, *J. Biol. Chem.* **1952**, *199*, 133–139.
[4] J. F. Anderson, R. E. Werdin, F. Y. Bates, *J. Vet. Med. Small Anim. Clin.* **1980**, *2*, 293–296.
[5] D. G. Burch, G. T. Jones, T. W. Heard, R. E. Tuck, *J. Vet. Rec.* **1986**, *119*, 108–112.
[6] G. Szucs, V. Tamasi, P. Laczay, K. Monostory, *J. Chem. Biol. Interact.* **2004**, *147*, 151–161.
[7] J. Drews, A. Georgopoulos, G. Laber, E. Schutze, J. Unger, *J. Antimicrob. Agents Chemother.* **1975**, *7*, 507–516.
[8] P. C. T. Inamoto, P. O'Hanlon, N. H. Rogers, *J. Res. Vet. Sci.* **1989**, *46*, 202–211.
[9] C. Heilmann, L. Jensen, J. S. Jensen, K. Lundstrom, D. Windsor, H. Windsor, D. Webster, *J. Infect.* **2001**, *43*, 234–238.
[10] R. Novak, D. M. Shlaes, *Curr. Opin. Invest. Drugs* **2010**, *11*, 182–191.

 

[11] M. R. Jacobs, *J. Future Microbiol.* **2007**, *2*, 591–600.

[12] M. S. Butler, M. A. Cooper, *J. Antibiot. (Tokyo)* **2011**, *64*, 413–425.

[13] W. W. Wicha, Z. Ivezic-Schoenfeld, R. Novak, 50th Interscience Conference on Antimicrobial Agents and Chemotherapy Conference. (Boston, MA), **2010**, Poster F1-2107.

[14] H. Egger, H. Reinshagen, *J. Antibiot. (Tokyo)* **1976**, *29*, 923–927.

[15] Y. Hirokawa, H. Kinoshita, T. Tanaka, T. Nakamura, K. Fujimoto, S. Kashimoto, T. Kojima, S. Kato, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 175–179.

[16] D. M. Springer, A. Bunker, B. Y. Luh, M. E. Sorenson, J. T. Goodrich, J. J. Bronson, K. DenBleyker, T. J. Dougherty, J. Fung-Tomc, *J. Med. Chem.* **2007**, *42*, 109–113.

[17] S. Kehraus, G. M. Konig, A. D. Wright, G. Woerheide, *J. Org. Chem.* **2002** *67*, 4989–4992.

[18] S. B. Singh, J. Occi, H. Jayasuriya, K. Herath, M. Motyl, K. Dorso, C. Gill, E. Hickey, K. M. Overbye, J. F. Barrett, P. Masurekar, *J. Antibiot. (Tokyo)* **2007**, *60*, 565–571.

[19] S. Hess, M. A. Akermann, S. Wnendt, K. Zwingenberger, K. Eger, *Bioorg. Med. Chem.* **2001**, *9*, 1279–1291.

[20] P. Xu, Y. Zhang, Y. Sun, J. Liu, B. Yang, Y. Wang, Y. L. Wang, *Chem. Biol. Drug Des.* **2009**, *73*, 655–660.

[21] K. Riedl, *J. Antibiot.* **1976**, *29*, 132–139.

[22] Clinical and Laboratory Standards Institute, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard M7-A6*, Clinical and Laboratory Standards Institute, Wayne, PA, USA **2003**.

 