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Design, synthesis and biological activities of novel pleuromutilin derivatives with a substituted triazole moiety as potent antibacterial agents

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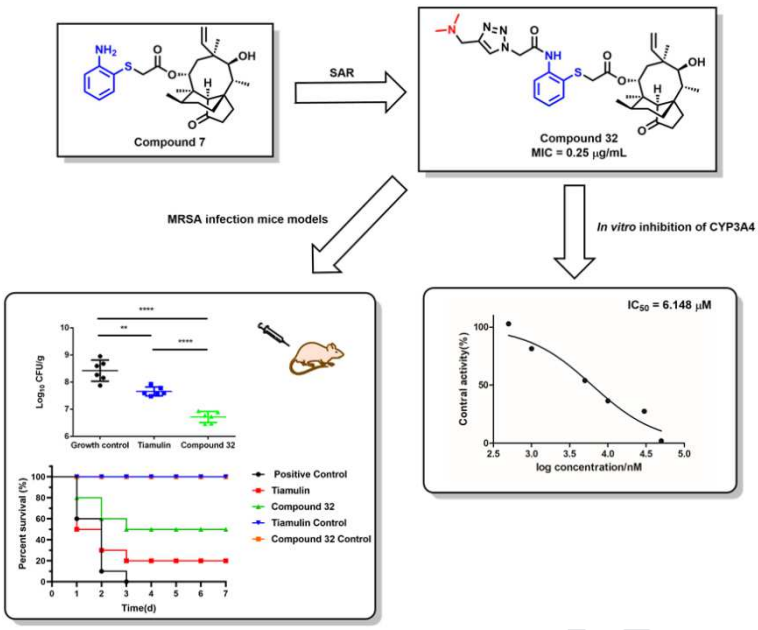

Note: A series of novel pleuromutilin derivatives bearing both aminophenylthiol and 1,2,3-triazole moiety were designed and synthesized. Compound **32** from this series displayed superior *in vivo* efficacy to the reference drug tiamulin against MRSA in both the thigh infection model and the mouse systemic infection model. Compound **32** possessed moderate *in vitro* inhibition of CYP3A4.

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Abstract

A series of novel pleuromutilin derivatives possessing 1,2,3-triazole moieties were synthesized via click reactions under mild conditions. The *in vitro* antibacterial activities of these derivatives against 4 strains of *S. aureus* (MRSA ATCC 43300, ATCC 29213, AD 3, and 144) and 1 strain of *E. coli* (ATCC 25922) were tested by the broth dilution method. The majority of the synthesized derivatives displayed potent antibacterial activities against MRSA (MIC = 0.125 ~ 2 μg/mL). It was also found that most compounds had no significant inhibitory effect on the proliferation of RAW264.7 cells at the concentration of 8 μg/mL. Among these derivatives, compound **32** (~1.71 $\log_{10}$ CFU/g) containing dimethylamine group side chain displayed more effective than tiamulin (~0.77 $\log_{10}$ CFU/g) at the dose of 20 mg/kg in reducing MRSA load in thigh infected mice. Additionally, compound **32** (the survival rate was 50%) also displayed superior *in vivo* efficacy to that of tiamulin (the survival rate was 20%) in the mouse systemic model. Structure-activity relationship (SAR) studies resulted in compound **32** with the most potent *in vitro* and *in vivo* antibacterial activity among the series. Moreover, compound **32** was evaluated in CYP450 inhibition assay and showed moderate *in vitro* inhibition of CYP3A4 (IC_{50} = 6.148 μM).

Keywords: Pleuromutilin; 1,2,3-triazole; MRSA; Synthesis; Antibiotics

1. Introduction

As one of the prominent pathogen with high morbidity and mortality, multidrug-resistant *Staphylococcus aureus* (MRSA) usually cause health-care-associated infections which posed a serious threat to human health around the world [1]. Therefore, there is an urgent need to develop new antibiotics with new mode of action that can be used to treat infections caused by multidrug-resistant *S. aureus* (MRSA). Natural products have always played an important role in dealing with human diseases, especially in the field of infectious diseases. Despite several drawbacks, various natural products or their derivatives have been developed and approved as antibacterial agents, such as penicillin G, tigecycline, vancomycin and aztreonam [2]. The development of new antibacterial agents from natural products or their semisynthetic derivatives is still the most effective way to deal with MRSA infections.

Pleuromutilin (**1**, Figure 1) is a naturally tricyclic diterpenoid produced by the higher fungi Basidiomycetes Pleurotus species *Pleurotus mutilus* and *Pleurotus Passeckerianus*, first isolated in 1951 [3]. Pleuromutilin displays potent antibacterial activity against gram-positive bacteria. It has been identified that pleuromutilin and its derivatives could inhibit the bacterial protein synthesis through binding to the V domain of the peptidyl transferase center (PTC) of the bacterial *50s* ribosomal subunit *23s* RNA [4-6]. Due to the unique mechanism of action, pleuromutilin and its derivatives possess low propensity to select for cross-resistance to currently available antibacterial agents. Thus, pleuromutilins have fascinated many researchers to explore

and develop pleuromutilin derived effective antibacterial agents. The structural modifications of the C14 side chain of pleuromutilin resulted in the discovery of tiamulin (**2**, Figure 1) and valnemulin (**3**, Figure 1**)**, which were approved and used as veterinary antibiotics for poultry and pigs in 1979 and 1999, respectively [5]. In 2007, another pleuromutilin derivative retapamulin (**4**, Figure 1) was approved for human use. As the first pleuromutilin antibiotic for human use, retapamulin is used as a topical antibiotic to treat skin infections [7]. Although pleuromutilin derivatives usually exhibit potent antibacterial activity, most of them were discontinued develop for human use due to their limited bioavailability and CYP450 inhibition [8]. More recently, lefamulin (**5**, Figure 1), as the first systemic pleuromutilin antibiotic, was approved by FDA for intravenous and oral treatment of community-acquired bacterial pneumonia (CABP) on August 19, 2019 [6]. As many pleuromutilin derivatives have poor bioavailability, lefamulin's success could be constituted as a milestone for the pleuromutilin class of antibiotcs.

Figure 1 here

Previous work in our group has led to the synthesis and evaluation of various series of novel pleuromutilin derivatives [9-11]. One of the derivatives containing the 2-aminophenylthiol in the C14 side chain (compound **7**, Figure 1) displayed excellent antibacterial activity *in vitro* and good efficacy *in vivo* against MRSA [11, 12]. Numerous pleuromutilin derivatives have been prepared and evaluated with excellent antibacterial activity achieved by incorporating heteroaromatic substituents containing a sulfide linkage into the C14 side chain [8, 13]. Additionally, 1,2,3-triazole moiety

have been reported that might influence the antibacterial activity of the pleuromutilin derivatives [14]. These research motivated us to develop the pleuromutilin derivatives containing both aminophenylthiol and 1,2,3-triazole moiety.

We now report the synthesis and biological activities of novel pleuromutilin derivatives with 1,2,3-triazole substituents incorporated into compound **7** and the discovery of compound **32** which displayed good antibacterial activities against MRSA both *in vitro* and *in vivo*.

2. Results and Discussion

2.1. Chemistry

A general synthesis strategy based on compound 22- (2-amino-phenylsulfanyl) -22-deoxypleuromutilin (compound **7**) and a variety of piperazine derivatives or secondary amines were used (Scheme 1). Compound **7**, based on which yielded a series of pleuromutilin derivatives with potential antibacterial against MRSA in our previous work [10], was used as a lead compound. Compound **8** was prepared by condensation of acyl chloride group of chloroacetyl chloride with compound **7**. Then, compound **8** was converted into the azide compound **9** through a nucleophilic substitution. This azide compound **9** was reacted with 27 different terminal alkynes compounds **18-25**, **53-71** that were all linked to various secondary amines or piperazine derivatives. A standard click reaction [15] based on the catalysis Cu^{+}, which was produced in situ by Cu^{2+} and sodium ascorbate, was applied for the cycloaddition. Thus, pleuromutilin derivatives compounds **26-33** and **72-90** all containing a 1,2,3-triazol linkage were prepared in good yields (Scheme 1). The

structures of all those 27 pleuromutilin derivatives were confirmed by ^{1}H NMR, ^{13}C NMR and HR-MS(ESI).

2.2. *In vitro* antibacterial activity

All the newly synthesized 1,2,3-triazole linked pleuromutilin derivatives were evaluated for their antibacterial activity against methicillin-resistant *S. aureus* (ATCC 43300), *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922) and two clinical strains of *S. aureus* (AD3 and 144, isolated from Guangdong Province). The MICs and MBCs of all synthesized pleuromutilin derivatives as well as the reference antibacterial drugs, pleuromutilin and tiamulin were determined by the broth micro dilution methods according to the Clinical and Laboratory Standards Institute (CLSI). *S. aureus* (MRSA) ATCC 43300 was used as QC strain, and the quality control range of CLSI quality control bacteria strain was taken as reference. The results of MIC and MBC were shown in Table 1 and Table 2.

Table 1 here

Most of these new pleuromutilin derivatives showed potent antibacterial activities against *S. aureus* (ATCC 43300), *S. aureus* (ATCC 29213), *S. aureus* (AD3) and *S. aureus* (144) *in vitro*. The MIC value of all these 27 compounds against *S. aureus* ranged from 2 to 0.125 μg/mL. The MIC value of all derivatives against *E. coli* (ATCC 25922) are higher than 32 μg/mL. Among these compounds, compounds **26**, **31, 32, 72, 86, 89** and **90** (MIC = 0.125 ~ 0.25 μg/mL) showed more potent antibacterial activity against MRSA in comparison to that of tiamulin (MIC = 0.5 μg/mL). Compound **89** with 1-(3-nitrophenyl)-piperazine and compound **90** with

1-(4-nitrophenyl)-piperazine exhibited the most potent activity with MIC value of 0.125 μg/mL.

Table 2 here

Firstly, compounds **26-33** were designed and prepared by using 1,2,3-triazole to link various nitrogen heterocycles and secondary amine derivatives compound **7**. These 8 compounds all showed potent activity against *S. aureus* (MIC = 0.25 ~ 1 μg/mL).

In order to explore SAR, different electron withdrawing and donating groups were introduced. Then we borrowed from the previous work experience of the laboratory and introduced a series of derivatives of piperazine [9] to obtain compounds **72-90**. Among them, compounds **77-82**, **88-90**, in which the electron-withdrawing group was introduced into the phenylpiperazine of compound **73**, resulting in a decrease in antibacterial activity against MRSA. Nevertheless, compounds **89** and **90** showed better antibacterial effect than compound **73**. This may be explained by the strong electron-withdrawing ability of the nitro group, which can generate local electron-deficient sites in the molecule and interact with proteins and amino acids present in the living system [16], other kinds of substituents or other substituent sites may weaken these effects. However, in the face of the problem that drugs containing nitro groups may cause serious adverse reactions in organisms, we still need to explore in depth in the future development process of pleuromutilin derivatives.

Those ratios of MBC to MIC were all less than or equal to 4, indicating that all synthesized pleuromutilin derivatives had good bactericidal ability [17]. MBC values of all compounds for MRSA ranged 1×MIC from 4×MIC, which illustrated that these

compounds have good bactericidal ability against MRSA. Among these derivatives, compounds **26**, **32**, **89** and **90** exhibited more potent bactericidal effects than tiamulin. Therefore, we carried out an in-depth study on the antibacterial activity of these four compounds.

The time-kill kinetic approach was used to investigate the anti-MRSA activity of compounds **26**, **32**, **89** and **90** *in vitro*. The experimental results were shown in graphic form in **Figure 2** and **Figure SI 28**. When the concentration of compounds **26** (Figure 2a) and **32** (Figure 2b) was 1×MIC, it had a definite inhibitory effect on MRSA; when the concentration was 4×MIC or above, it had a good bactericidal effect on MRSA and killed 99.9% of MRSA. However, compounds **89** (Figure SI 28a) and **90** (Figure SI 28b) can only play a role in killing MRSA at concentrations exceeding 8×MIC. However, from another aspect, after the compound reaches a certain concentration, the bactericidal effect of these pleuromutilin derivatives and tiamulin did not have a positive correlation with the increase of the compound concentration, indicating that these derivatives and tiamulin were time-dependent antibacterial agents. The key to the rational and scientific use of time-dependent antibacterial agents was to optimize the time when bacteria were exposed to the effective concentration of antibiotics, which can be achieved by multiple daily administration in clinical practice. For pathogens with high MIC, even continuous intravenous infusion was needed [18].

Figure 2 here

The postantibacterial effect (PAE) is an important indicator of antibiotic

pharmacodynamics, and it has important guiding significance for the rational use of antibiotics in clinical practice, evaluation of adverse reactions of antibiotics and combined use [19]. Based on the test results that have been obtained, we performed PAE tests on compounds **26** and **32**. The bacterial growth kinetics curve was shown in **Figure 3** and the results of the PAEs were shown in **Table 3**. After espousing 4×MIC for 2h, the PAE of compounds **26** and **32** were 1.84h and 2.74h, respectively. It is worth mentioning that compound **32** showed better postantibacterial effect than tiamulin at the concentrations of 2×MIC or 4×MIC.

Figure 3 here

Table 3 here

In the clinical use of antibacterial drugs, most of the pharmacodynamic indicators referenced are the minimum antibacterial concentration (MIC) and the minimum bactericidal concentration (MBC). However, this method of administration, which is based on these two indicators, ignores the interaction between drugs and bacteria and the potential of the drug to continue to inhibit the growth and reproduction of bacteria [20]. To design the drug delivery regimen reasonably and provide a theoretical basis for clinical adjustment of drug interval [21], we first performed time-kill kinetic tests on four of this batch of compounds, and then PAE was tested on compounds **26** and **32** with stronger bactericidal effects. The above experimental results showed compound **32** may be administered at longer intervals than tiamulin, suggesting that compound **32** may have some potential value for clinical use in the treatment of MRSA infections. Furthermore, a more scientific conclusion could be attained based

on *in vivo*, dose-fractionation, PK/PD studies and target attainment studies as well as PK studies.

2.3. *In vivo* antibacterial activity

2.3.1. Thigh infection model

In order to explore the *in vivo* efficacy of the drug, the mouse thigh infection model with neutropenia was first established to evaluate the therapeutic effect of the drug on the local infection in mice. Due to the longer PAE and the potent bacteriostatic ability of compound **32** on MRSA *in vitro*, compounds **32** was tested *in vivo* at first in this infection model.

First and foremost, a mice model of neutropenia was constructed by intraperitoneal injection of cyclophosphamide into mice, and 0.9% saline was injected intraperitoneally as a negative control group. The mice thigh muscle was injected with about 10^7 CFU/mL of the bacterial solution to establish a thigh infection model caused by MRSA. The experimental results of this part were shown in **Figure 4**.

Figure 4 here

It can be concluded from Figure 4 that 20 mg/kg of compound **32** can significantly reduce the bacterial load (~1.71 $\log_{10}$ CFU/g) in thighs, compared with the no drug control group the difference was statistically significant ($P < 0.001$, n = 6/group). The bacterial clearance rate of tiamulin (~0.77 $\log_{10}$ CFU/g) in thigh muscle of mice was lower than that of compound **32**, but compared with the control group without drugs, tiamulin could still reduce the MRSA load in thighs ($P < 0.01$, n = 6/group). The experimental results revealed that compound **32** displayed potency antibacterial

activity than tiamulin in reducing MRSA load in the mouse thigh infection model. Thus, compound 32 might be used in the treatment of MRSA infection.

2.3.2. MRSA infection model

After determining the potent antibacterial activity of compound **32** in the mice thigh infection model with neutropenia, we further tested its therapeutic effect in mice in the MRSA infection model with no drug treatment as a control group. As shown in Figure 5, compound **32** (30 mg/kg) had a therapeutic effect on a mice model of systemic infection with MRSA. After 7 days' administration, the survival rate of mice in compound **32** group was 50%, which was significantly higher than that of tiamulin group (20%), and the compound **32** did not directly cause animal death at this dose. The result of *in vivo* efficacy demonstrated that compound **32** was a new antibiotic candidate which was worth developing for clinical treatment of MRSA infections.

Figure 5 here

2.4. Cytotoxicity assay

Chemical substances act on the basic physiological processes of cells, which may lead to reduced cell survival, inhibited proliferation and disturbance of physiological functions, and trigger a series of adverse reactions [22]. Therefore, the antibiotics approved for clinical use should have no inhibitory effect on cell proliferation within a certain concentration range.

The cytotoxicity of these pleuromutilin derivatives to RAW 264.7 cells was evaluated by MTT assay. The result of this part showed that most of these compounds including compound **32** did not affect the viability of RAW 264.7 cells at the concentration of 8

μg/mL (**Figure SI 29**), which was an acceptable starting point for further drug discovery efforts.

2.5. Effect on liver microsomal CYP450 enzyme activity

During the process of pleuromutilin structural modification, azamulin (Figure 6a) was screened out and entered the phase I clinical trial. Unfortunately, azamulin had been eliminated during drug development due to its strong and irreversible inhibitory effect on the CYP450 enzyme system (IC_{50} value for CYP3A is 0.03~0.24 μM) [23]. Additionally, pleuromutilin derivatives with thioether side chains had also been reported to have strong inhibitory effects on CYP3A4 [8]. Therefore, we speculated that these synthesized pleuromutilin derivatives might have inhibitory effect on CYP3A4. Thus, compound **32** was selected to evaluate its inhibition potential against CYP3A4. The CYP3A4 inhibition was analyzed by determining IC_{50} value in human liver microsomes through the use of testosterone as the probe substrate. The results of this assay are shown in Figure 6b.

Figure 6 here

The results declared that compound **32** had an intermediate inhibitory effect on CYP3A4 (IC_{50} = 6.148 μM). Compounds with 3 μM < IC_{50} <10 μM are generally considered to be moderate CYP inhibitors, while compounds with IC_{50} > 10 μM are weak CYP inhibitors, compounds with IC_{50} < 3 μM are strong CYP inhibitors [24]. Compared with the strong inhibitory effect of azamulin on CYP450 (IC_{50} = 0.12 μM) [23], compound **32** could only inhibit the activity of CYP enzyme to some extent at a certain concentration. Due to the complexity of living organisms, the results of *in*

vitro experiments may be different from those of *in vivo* experiments, so further clinical studies were still needed for comprehensive evaluation.

3. Conclusions

A series of novel pleuromutilin derivatives were designed and synthesized in few steps. SAR studies have shown that the O atom at the C22 position was replaced by an S atom, which contributes to the improvement of biological activity. The terminal was connected to basic groups such as dimethylamine, diethylamine, tetrahydropyrrole and a phenylpiperazine substituent bearing nitro groups have excellent *in vitro* antibacterial activity against both sensitive and resistant *S. aureus* bacterial strains. Among these prepared derivatives, compounds **26**, **32**, **89** and **90** exhibited the most potent antibacterial activity and that were selected for the time-killing curve determination. The results showed that compounds **26** and **32** manifested a more rapid bactericidal kinetic effect on MRSA. Subsequently, the PAE (post-antibiotic effect) of compounds **26** and **32** were determined. Compound **32** displayed a longer PAE time than tiamulin against MRSA. The *in vivo* antibacterial activity of compound **32** was further studied. The results indicated that compound **32** exhibited a potent *in vivo* antibacterial effect than tiamulin in two MRSA infection mice models. CYP450 inhibition assay demonstrated that compound **32** had a moderate *in vitro* CYP3A4 inhibition. The current research results indicated that compound **32** might serve as a possible lead compound for the development of novel pleuromutilin antibacterial agent.

4. Experimental

4.1. Materials

Pleuromutilin (>90% pure) was purchased from Great Enjoyhood Biochemical Co. Ltd., (Sichuan, China). All analytical grade solvents were purchased from Greagent-bate, and other reagents were purchased from Adamas. Purification of all compounds by column chromatography was carried out using silica gel (200-300 mesh, Branch of Qingdao Haiyang Chemical Co. Ltd., Shandong, China). ^{1}H-NMR and ^{13}C-NMR spectra were recorded at Bruker AV-400 spectrometer. Among them, the chemical shift value (δ) are reported in ppm, and the coupling constant (J) is in Hertz. Tetramethylsilane was used as the internal standard in chloroform-*d* to analyze the compounds. High-resolution mass spectra were conducted using Waters Acquity UPLC-LCT Premier XE with an electro spray ionization (ESI) source.

4.2. Synthesis

The synthetic approaches for the preparation of the intermediates and the synthesis of a series of novel pleuromutilin derivatives based on compound **1** were illustrated in Scheme 1.

Scheme 1 here

Compound **1** (pleuromutilin) was purchased commercially. Compound **6** (22-O-tosylpleuromutilin) and compound **7** (22-(2-amino-phenylsulfanyl)-22-de-oxypleuromutilin) were synthesized according to a reported method [11].

4.2.1. 22- (2-(2-chloroacetamido) phenyl) thioacety-l-yl-22-deoxypleuromutilin (8)

Compound 22-(2-amino-phenylsulfanyl)-22-deoxypleuromutilin (compound **7**) (1 g, 2.06 mmol) was dissolved in toluene (30 mL). Slowly added DIPEA (0.53 g, 4.12 mmol) and chloroacetyl chloride (0.47 g, 3.09 mmol) under ice bath, the remaining mixture was reacted at room temperature for 0.5 h and then refluxed for 2 h. After the reaction was completed, it was quenched with ice water, extracted three times with chloroform, and the organic phases were combined. The organic phase was washed successively with deionized water and saturated brine. The organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain a dark brown oil. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (2:1) as eluent to give a pure product, compound **8** (white solid, 85.1% yield).

4.2.2. 22- (2-(2- azido acetamido) phenyl) thioacety-l-yl-22-deoxypleuromutilin (9)

Compound **8** (1 g, 1.78 mmol) was dissolved in acetone (20 mL), to which a solution of sodium azide (0.58 g, 8.90 mmol) in deionized water (5 mL) was added. The reaction was mixed in a round bottom flask and refluxed for 4 hours. After completion of the reaction, the reaction solution was poured into a separating funnel, extracted with chloroform, and washed twice with a saturated aqueous sodium chloride solution. The organic phase was dried over anhydrous sodium sulfate and concentrated on a rotary evaporator to give the crude product. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (4:1) as eluent to obtain compound **9** (white solid, 73.5% yield).

4.2.3. Synthesis of secondary amine analogs containing propynyl and piperazine derivatives (18~25, 53~71)

Secondary amine derivatives (13.67 mmol) was dissolved in ethyl acetate (30 ml), potassium carbonate (3.78 g, 27.34 mmol) was added, and then 3-bromopropyne (1.63 g, 13.67 mmol) was slowly added dropwise to the reaction system. The reaction was performed at room temperature overnight. After the reaction was completed, deionized water was added. Then the reaction solution was stirred for 0.5 h and poured into a separating funnel, extracted twice with chloroform. The organic phase was washed with saturated aqueous sodium chloride solution, then dried (Na_2SO_4) and concentrated under reduced pressure to obtain a crude product. The crude product was purified by column chromatography to obtain compound **18~25, 53~71**, respectively.

4.2.4. 22-(2-(2-(4-((diethylamino)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl) thioacety-l-yl-22-deoxy pleuromutilin (26)

Compound **9** (1 g, 1.70 mmol) and compound **18** (0.19 g, 1.70 mmol) were added in a mixture solution of *t*-butanol (10 mL) and of water (10 mL), and copper sulfate pentahydrate (0.0033 g, 0.068 mmol) and sodium ascorbate (0.0013 g, 0.068 mmol), and the reaction was stirred at room temperature for 3 h. After the reaction was completed, the reaction solution was poured into a separating funnel, 40 ml of ethyl acetate was added for extraction, and the organic phases were combined. The organic phase was washed twice with a saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give the

crude product. The crude product was purified by silica gel column chromatography using petroleum dichloromethane/methanol (10: 1) as eluent to obtain the product compound **26**.

White powder; yield: 61%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.54 (1 H, s), 8.37 (1 H, s), 8.25 (1 H, d, *J* = 8.3Hz), 7.56 (1 H, d, *J* = 7.8Hz), 7.36 (1 H, d, *J* = 7.8Hz), 7.07 (1 H, t, *J* = 7.6Hz), 6.44 (1 H, dd, *J* = 17.4, 10.9Hz, H19), 5.69 (1 H, d, *J* =8.5Hz, H14), 5.43 (2 H, s, H20), 5.16 (1 H, d, *J* = 17.4Hz, H20), 4.23 (2 H, s), 3.53 – 3.31 (3 H, m, H11,H22), 2.98 (5 H, s, H2), 2.24 (3 H, ddt, *J* = 37.1, 19.5, 8.6Hz, H4,H10,H13), 2.09 – 1.94 (2 H, m, H6,11-OH), 1.78 (1 H, d, *J* =14.7Hz, H8), 1.69 – 1.53 (3 H, m, H1,H7), 1.52 – 1.41 (2 H, m, H13), 1.38 (3 H, s, H15), 1.36 (9 H, s, H8), 1.12 (3 H, s, H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.53 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.85 (C3), 169.70 (C21), 162.81, 139.20 (C19), 138.85, 136.45, 130.88, 130.19, 125.18, 122.49, 121.09, 117.25 (C20), 99.98, 74.55 (C11), 70.78 (C14), 66.04, 57.98 (C4), 53.43, 53.35 (C22), 46.18, 45.39 (C9), 44.58 (C13), 44.04 (C12), 41.75 (C15), 40.14, 36.53 (C6), 35.98 (C10), 34.41 (C2), 30.29 (C8), 28.27, 26.84 (C7), 26.39 (C18), 24.79 (C1), 16.52 (C16), 14.76 (C15), 11.60 (C17), 9.56. HR-MS (ESI): Calcd for $C_{37}H_{54}N_5O_5S$ ($M+H^+$): 680.3846; Found: 680.3839.

4.2.5. 22-(2-(2-(4-((morpholinomethy)methyl)-1H-1,2,3-triazol-1-yl)acetamido) phenyl)thioacety-l-yl-22-deoxy pleuromutilin (27)

White powder; yield: 62%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.42 (1 H, s), 8.28 (1 H, d, *J* = 8.3), 7.78 (1 H, s), 7.54 (1 H, d, *J* = 7.7Hz), 7.35 (1 H, t, *J* = 7.9Hz),

7.06 (1 H, t, *J* = 7.6Hz), 6.42 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.35 (2 H, d, *J* = 3.6Hz, H20), 5.30 (1 H, t, *J* = 5.6Hz), 5.15 (1 H, d, *J* = 17.4Hz, H20), 3.72 (5 H, s), 3.40 (2 H, q, *J* = 16.1Hz, H22), 3.34 – 3.29 (1 H, m, H11), 2.55 (4 H, t, *J* = 4.6Hz), 2.37 – 2.09 (4 H, m, H2,H4,H10,H13), 2.07 – 2.04 (1 H, m, 11-OH), 1.98 (1 H, dd, *J* = 16.1, 8.6Hz), 1.91 (1 H, s), 1.81 – 1.72 (1 H, m), 1.63 (2 H, d, *J* = 9.7Hz, H6,H8), 1.55 – 1.40 (4 H, m, H1,H7,H13), 1.37 (3 H, s, H15), 1.35 – 1.29 (1 H, m, H8), 1.11 (3 H, d, *J* = 4.2Hz, H18), 0.89 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.82 (C3), 169.34 (C3), 163.20, 144.78, 139.10 (C19), 138.86, 136.41, 130.82, 125.17, 124.46, 122.39, 120.96, 117.25 (C20), 77.36, 74.52 (C11), 70.59 (C14), 66.86, 57.97 (C4), 53.61, 53.41, 53.36 (C22), 45.38 (C9), 44.59 (C13), 43.99 (C12), 41.72 (C5), 39.80, 36.53 (C6), 35.96 (C10), 34.40 (C2), 30.29 (C8), 26.82 (C7), 26.51, 26.33 (C18), 24.78 (C1), 16.53 (C16), 14.76 (C15), 11.55 (C17). HR-MS (ESI): Calcd for $C_{37}H_{52}N_5O_6S$ ($M+H^+$): 694.3638; Found: 694.3658.

4.2.6.

22-(2-(2-(4-((4-(2-hydroxyethyl)piperidin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (28)

White powder; yield: 57%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.40 (1 H, s), 8.28 (1 H, d, *J* = 8.3Hz), 7.81 (1 H, s), 7.54 (1 H, s), 7.35 (1 H, t, *J* = 7.9Hz), 7.05 (1 H, t, *J* = 7.6Hz), 6.42 (1 H, dd, *J* = 17.4, 10.9Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.34 (1 H, d, *J* = 2.3Hz, H20), 5.28 (1 H, s), 5.15 (1 H, d, *J* = 17.3Hz, H20), 3.73 (2 H, s), 3.67 (2 H, t, *J* = 6.5Hz), 3.40 (2 H, t, *J* = 15.0Hz, H22), 3.36 – 3.29 (1 H, m,

H11), 2.97 (2 H, d, *J* = 11.3Hz, H4,H10), 2.23 (4 H, ddd, *J* = 27.7, 13.1, 7.4Hz, H2,H13), 2.11 (2 H, d, *J* = 11.5Hz), 2.05 (1 H, d, *J* = 3.7Hz, 11-OH), 1.98 (2 H, dd, *J* = 16.1, 8.6Hz), 1.73 (4 H, dd, *J* = 25.6, 14.3Hz, H6,H8), 1.60 (3 H, dd, *J* = 20.2, 9.7Hz, H1,H7,H13), 1.51 (3 H, dd, *J* = 13.1, 6.7Hz H1,H7,H13), 1.46 – 1.41 (2 H, m), 1.37 (3 H, s, H15), 1.35 – 1.24 (4 H, m, H8,H18), 1.11 (3 H, s, H18), 0.89 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.91 (C3), 169.26 (C21), 163.27, 145.12, 139.09 (C19), 138.85, 136.41, 130.80, 125.12, 124.61, 122.36, 120.93, 117.26 (C20), 99.98, 74.53 (C11), 70.51 (C14), 60.40, 57.99 (C4), 53.59, 53.50, 53.43 (C22), 45.38 (C9), 44.59 (C13), 43.98 (C12), 41.72 (C5), 39.68, 39.26, 36.55 (C6), 35.96 (C10), 34.41 (C2), 32.08, 32.03, 30.30 (C8), 26.81 (C7), 26.32 (C18), 24.78 (C1), 16.55 (C16), 14.78 (C15), 14.19, 11.56 (C17). HR-MS (ESI): Calcd for $C_{40}H_{58}N_5O_6S$ ($M+H^+$): 736.4108; Found: 736.4126.

4.2.7.

22-(2-(2-(4-((3-(hydroxymethyl)piperidin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (29)

White powder; yield: 62%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.38 (1 H, s), 8.26 (1 H, d, *J* = 8.3Hz), 7.80 (1 H, sHz), 7.52 (1 H, d, *J* = 7.8Hz), 7.33 (1 H, t, *J* = 7.9Hz), 7.04 (1 H, t, *J* = 7.6Hz), 6.41 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.66 (1 H, d, *J* = 8.5Hz, H14), 5.34 (2 H, s, H20), 5.28 (2 H, d, *J* = 11.6Hz), 5.13 (1 H, d, *J* = 17.3Hz, H20), 3.72 (2 H, s), 3.57 (1 H, d, *J* = 10.2Hz), 3.52 – 3.44 (2 H, m, H22,H11), 3.39 (2 H, d, *J* = 12.8Hz), 3.34 – 3.29 (1 H, m), 2.92 (1 H, d, *J* = 11.0Hz), 2.83 – 2.72 (1 H, m), 2.23 (5 H, ddd, *J* = 25.7, 15.5, 8.3Hz, H2,H4,H10,H13), 2.05 (2 H, s), 1.97

(1 H, dd, *J* = 16.1, 8.6Hz, 11-OH), 1.75 (3 H, d, *J* = 14.7Hz, H6,H8), 1.66 – 1.57 (4 H, m, H1,H7,H13), 1.45 (3 H, d, *J* = 6.3Hz), 1.36 (3 H, s, H15), 1.27 (2 H, d, *J* = 13.0Hz, H8), 1.10 (3 H, s, H18), 0.88 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.89 (C3), 169.24 (C21), 163.33, 145.05, 139.06 (C19), 138.91, 136.29, 130.74, 125.11, 124.62, 122.44, 120.97, 117.19 (C20), 74.54 (C11), 70.56 (C14), 66.51, 58.00 (C4), 56.89, 54.08, 53.70, 53.42 (C22), 45.39 (C9), 44.60 (C13), 43.99 (C12), 41.73 (C5), 39.64, 38.12, 36.55 (C6), 35.97 (C2), 34.41, 30.31 (C8), 27.11, 26.81 (C7), 26.37 (C18), 24.78 (C1), 24.54, 16.53 (C16), 14.77 (C15), 11.53 (C17). HR-MS (ESI): Calcd for $C_{39}H_{56}N_5O_6S$ ($M+H^+$): 722.3951; Found: 722.3890.

4.2.8. 22-(2-(2-(4-((4-hydroxypiperidin-1-yl))methyl)-1H-1,2,3-triazol-1-yl)acet amido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (30)

White powder; yield: 42%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.41 (1 H, s), 8.26 (1 H, d, *J* = 8.4Hz), 7.80 (1 H, s), 7.52 (1 H, d, *J* = 7.8Hz), 7.34 (1 H, t, *J* = 7.9Hz), 7.05 (1 H, t, *J* = 7.6Hz), 6.40 (1 H, dd, *J* = 17.5, 10.9Hz, H19), 5.65 (1 H, d, *J* = 8.5Hz, H14), 5.34 (2 H, s, H20), 5.28 (1 H, d, *J* = 10.5Hz), 5.13 (1 H, d, *J* = 17.3Hz, H20), 3.71 (3 H, d, *J* = 12.3Hz), 3.41 (2 H, t, *J* = 14.6Hz, H22), 3.30 (1 H, d, *J* = 6.4Hz, H11), 2.84 (2 H, d, *J* = 12.1Hz), 2.31 – 2.22 (4 H, m, H2,H13), 2.21 – 2.15 (2 H, m, H4,H10), 2.04 (1 H, s, 11-OH), 1.97 (2 H, dd, *J* = 16.0, 8.5Hz), 1.91 – 1.85 (2 H, m), 1.75 (1 H, d, *J* = 14.5Hz), 1.64 – 1.55 (5 H, m, H1,H7,H13), 1.51 – 1.39 (3 H, m), 1.36 (3 H, s, H15), 1.33 – 1.24 (1 H, m, H8), 1.10 (3 H, s, H18), 0.88 (3 H, d, *J* = 6.9Hz, H17), 0.51 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz,

Chloroform-*d*) δ 216.94 (C3), 169.29 (C21), 163.29, 145.06, 139.05 (C19), 138.88, 137.71, 136.34, 130.76, 125.16, 124.62, 122.45, 120.99, 117.21 (C20), 111.33, 74.52 (C11), 70.56 (C14), 67.45, 57.99 (C4), 53.40, 53.06 (C22), 50.78, 45.38 (C9), 44.58 (C13), 43.98 (C12), 41.72 (C5), 39.70, 36.54 (C6), 35.96 (C10), 34.41 (C2), 34.21, 30.29 (C8), 26.81 (C7), 26.37 (C18), 24.78 (C1), 16.53 (C16), 14.77 (C15), 11.56 (C17). HR-MS (ESI): Calcd for $C_{38}H_{54}N_5O_6S$ ($M+H^+$): 708.3795; Found: 708.3699.

4.2.9.

22-(2-(2-(4-(pyrrolidin-1-yl-methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl) thioacety-l-yl-22-deoxy pleuromutilin (31)

White powder; yield: 40%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.39 (1 H, s), 8.29 (1 H, dd, *J* = 8.3, 1.3Hz), 7.81 (1 H, s), 7.54 (1 H, dd, *J* = 7.8, 1.6Hz), 7.35 (1 H, td, *J* = 7.9, 1.6Hz), 7.06 (1 H, td, *J* = 7.6, 1.4Hz), 6.43 (1 H, dd, *J* = 17.4, 11.0Hz,H19), 5.67 (1 H, d, *J* = 8.5 Hz, H14), 5.41 – 5.27 (3 H, m, H20), 5.15 (1 H, dd, *J* = 17.5, 1.5Hz, H20), 3.86 (2 H, s), 3.46 – 3.28 (3 H, m, H11, H22), 2.63 (4 H, d, *J* = 6.0Hz), 2.35 – 2.10 (3 H, m, H4,H10), 2.02 – 1.96 (4 H, m, 11-OH,H2,H13), 1.80 (4 H, h, *J* = 3.3Hz, H6,H8,H13), 1.70 – 1.57 (2 H, m, H1), 1.52 – 1.43 (2 H, m, H7), 1.38 (3 H, s, H15), 1.36 – 1.28 (1 H, m, H8), 1.12 (3 H, s, H18), 0.89 (3 H, d, *J* = 6.9Hz, H17), 0.53 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.89 (C3), 169.24 (C21), 163.27, 145.85, 139.08 (C19), 139.08, 138.85, 136.38, 130.77, 125.09, 124.33, 122.40, 122.40, 120.93, 117.21 (C20), 74.52 (C14), 70.49, 57.99, 53.94, 53.44 (C22), 50.49, 45.38 (C9), 44.59 (C13), 43.98 (C12), 41.72 (C5), 39.65, 36.55 (C6), 35.96 (C10), 34.41 (C2), 30.30 (C8), 26.81 (C7), 26.33 (C18),

24.78 (C1), 23.49, 16.53 (C16), 14.77 (C15), 11.55 (C17). HR-MS (ESI): Calcd for $C_{37}H_{52}N_5O_5S$ ($M+H^+$): 679.3689; Found: 679.3705.

4.2.10.

22-(2-(2-(4-((dimethylamino)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thio acety-l-yl-22-deoxy pleuromutilin (32)

White powder; yield: 57%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.36 (1 H, s), 8.26 (1 H, d, *J* =8.2 Hz), 7.76 (1 H, s), 7.51 (1 H, dd, *J* =7.7, 1.6 Hz), 7.32 (1 H, td, *J*= 7.9, 1.5 Hz), 7.06 – 7.00 (1 H, m), 6.39 (1 H, dd, *J* =17.4, 11.0 Hz, H19), 5.64 (1 H, d, *J* =8.5 Hz, H14), 5.37 – 5.24 (4 H, m, H20), 5.12 (1 H, dd, *J* =17.4, 1.6 Hz, H20), 3.64 (2 H, s), 3.39 (1 H, d, *J* =16.2 Hz, H11), 3.35 – 3.29 (2 H, m, H22), 2.28 (6 H, s, H2,H4,H10,H13), 2.27 – 2.10 (3 H, m), 2.06 – 2.02 (1 H, m, 11-OH), 1.95 (1 H, dd, *J* =16.0, 8.6 Hz, H6), 1.74 (1 H, dq, *J* =14.6, 3.2 Hz, H8), 1.65 – 1.54 (2 H, m, H1), 1.50 – 1.38 (2 H, m, H7,H13), 1.35 (3 H, s, H15), 1.30 (1 H, dt, *J* =11.0, 4.0 Hz, H8), 1.09 (3 H, s, H18), 1.08 – 1.04 (1 H, m), 0.87 (3 H, d, *J* =6.9 Hz, H17), 0.50 (3 H, d, *J* =7.1Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.78 (C3), 169.12 (C21), 163.28, 145.78, 139.07 (C21), 138.96, 136.31, 130.71, 125.07, 124.29, 122.43, 120.91, 117.11 (C20), 77.30, 77.09, 76.88, 74.49 (C11), 70.52 (C14), 57.99 (C4), 54.27, 53.44 (C22), 45.38 (C9), 45.13, 44.62 (C13), 44.00 (C12), 41.73 (C5), 39.59, 36.55 (C6), 35.99 (C10), 34.39 (C2), 30.30 (C8), 26.81 (C7), 26.42 (C18), 24.78 (C1), 16.50 (C16), 14.76 (C15), 11.51 (C17). HR-MS (ESI): Calcd for $C_{35}H_{50}N_5O_5S$ ($M+H^+$): 652.3533; Found: 652.3542.

4.2.11.

22-(2-(2-(4-(piperidin-1-yl-methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (33)

White powder; yield: 47%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.35 (1 H, s), 8.28 (1 H, d, *J* = 8.4Hz), 7.78 (1 H, s), 7.53 (1 H, d, *J* = 7.8Hz), 7.34 (1 H, t, *J* = 7.8Hz), 7.04 (1 H, t, *J* = 7.6Hz), 6.41 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.66 (1 H, d, *J* = 8.5Hz, H14), 5.33 (2 H, d, *J* = 2.4Hz, H20), 5.14 (1 H, d, *J* = 17.4Hz, H20), 3.69 (2 H, s), 3.45 – 3.27 (3 H, m, H22,H11), 2.52 – 2.44 (5 H, m, H4,H10), 2.24 (4 H, ddd, *J* = 28.1, 10.6, 5.3Hz, H2,H13), 2.07 – 1.92 (2 H, m, 11-OH,H6), 1.80 – 1.71 (1 H, m), 1.61 – 1.56 (6 H, m, H1,H7,H13), 1.45 (4 H, ddd, *J* = 19.1, 8.6, 4.7Hz), 1.38 – 1.23 (4 H, m, ,H8,H15), 1.11 (4 H, s, H8,H18), 0.88 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.84 (C3), 169.15 (C21), 163.31, 145.43, 139.07 (C19), 138.88, 136.38, 130.76, 125.08, 124.47, 122.37, 120.90, 117.21 (C20), 74.52 (C11), 70.47 (C14), 57.99 (C4), 54.27, 53.93, 53.44, 51.63 (C22), 45.38 (C9), 44.60 (C13), 43.98 (C12), 41.72 (C5), 39.59, 36.55 (C6), 35.96 (C10), 34.40 (C2), 30.31 (C8), 26.81 (C7), 26.33 (C18), 25.89, 25.81, 24.78 (C1), 24.07, 16.53 (C16), 14.77 (C15), 11.53 (C17). HR-MS (ESI): Calcd for $C_{38}H_{54}N_5O_5S$ ($M+H^+$): 692.3846; Found: 692.3719.

4.2.12.

22-(2-(2-(4-(4-methylpiperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (72)

White powder; yield: 51%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.39 (1 H, s), 8.27 (1 H, d, *J* = 8.3Hz), 7.77 (1 H, s), 7.53 (1 H, d, *J* = 7.7Hz), 7.34 (1 H, t, *J* = 7.9Hz), 7.05 (1 H, t, *J* = 7.6Hz), 6.41 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.66 (1 H, d, *J* = 8.5Hz, H14), 5.40 – 5.26 (3 H, m, H20), 5.15 (1 H, d, *J* = 17.4Hz, H20), 3.74 (2 H, s), 3.38 (3 H, dt, *J* = 24.2, 12.1Hz, H11,H22), 2.60 (5 H, s), 2.48 (4 H, s), 2.29 (6 H, s, H2,H4,H10,H13), 2.26 – 2.10 (2 H, m,H8), 2.07 – 1.92 (2 H, m, 11-OH,H6), 1.69 – 1.51 (3 H, m, H1,H7), 1.48 – 1.41 (1 H, m, H13), 1.37 (3 H, s, H15), 1.34 – 1.23 (1 H, m,H8), 1.11 (3 H, s, H18), 0.89 (3 H, d, *J* = 7.0Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.79 (C3), 169.24 (C21), 163.24, 144.96, 139.09 (C19), 138.89, 136.35, 130.75, 125.13, 124.45, 122.42, 120.97, 117.22 (C20), 74.52 (C11), 70.55 (C14), 66.21, 57.98 (C4), 54.89, 53.41, 53.10, 52.69, 45.86, 45.38 (C9), 44.62 (C19), 43.99 (C12), 41.73, 39.71, 36.54 (C6), 35.97 (C10), 34.39 (C2), 30.30 (C8), 26.82 (C7), 26.35 (C18), 24.78 (C1), 16.52 (C16), 14.77 (C15), 14.74, 11.53 (C17). HR-MS (ESI): Calcd for $C_{38}H_{55}N_6O_5S$ ($M+H^+$): 707.3955; Found: 707.3823.

4.2.13.

22-(2-(2-(4-((4-phenylpiperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (73)

White powder; yield: 53%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.41 (1 H, s), 8.29 (1 H, d, *J* = 8.3Hz), 7.82 (1 H, s), 7.54 (1 H, d, *J* = 7.8Hz), 7.36 (1 H, t, *J* = 7.9Hz), 7.25 (1 H, d, *J* = 7.6Hz), 7.08 (1 H, q, *J* = 8.3, 7.6Hz), 6.92 (2 H, d, *J* = 8.2Hz), 6.85 (1 H, t, *J* = 7.3Hz), 6.43 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.67 (1 H, d, *J*

= 8.5Hz, H14), 5.37 – 5.32 (2 H, m, H20), 5.16 (1 H, d, *J* = 17.4Hz, H20), 3.82 (2 H, s), 3.49 – 3.32 (3 H, m, H11,H22), 3.22 (4 H, q, *J* = 6.4, 4.8Hz), 2.73 (5 H, t, *J* = 4.9Hz, H2,H13), 2.24 (3 H, dq, *J* = 27.8, 9.3, 8.0Hz, H4,H10), 2.08 – 1.93 (2 H, m, 11-OH), 1.77 (1 H, dd, *J* = 14.5, 3.4Hz, H6), 1.63 (2 H, td, *J* = 12.3, 11.1, 7.1Hz, H8), 1.53 – 1.41 (4 H, m, H1,H7,H13), 1.38 (3 H, s, H15), 1.32 (1 H, dd, *J* = 14.1, 3.6Hz, H8), 1.12 (3 H, s, H18), 0.90 (3 H, d, *J* = 7.0Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.75 (C3), 169.27 (C21), 163.23, 151.21, 144.89, 144.83, 139.12 (C19), 138.89, 136.37, 133.26, 130.79, 129.08, 126.03, 125.15, 124.54, 122.41, 120.97, 119.74, 117.23 (C20), 116.10, 74.54 (C11), 70.59 (C14), 57.98 (C4), 53.45, 53.23, 52.90 (C22), 49.06, 45.38 (C9), 44.62, 44.01 (C12), 41.74 (C5), 39.75, 36.54 (C6), 35.98 (C10), 34.40 (C2), 30.41, 30.31 (C8), 26.83 (C7), 26.35 (C18), 24.79 (C1), 16.53 (C16), 14.77 (C15), 11.54 (C17). HR-MS (ESI): Calcd for $C_{43}H_{57}N_6O_5S$ ($M+H^+$): 769.4111; Found: 769.4102.

4.2.14.

22-(2-(2-(4-((4-(*o*-tolyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (74)

White powder; yield: 57%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.42 (1 H, s), 8.30 (1 H, d, *J* = 8.3Hz), 7.85 (1 H, s), 7.55 (1 H, d, *J* = 7.8Hz), 7.36 (1 H, t, *J* = 7.9Hz), 7.16 (2 H, t, *J* = 8.2Hz), 7.02 (3 H, ddt, *J* = 21.5, 14.8, 7.5Hz), 6.43 (1 H, dd, *J* = 17.4, 10.9Hz, H19), 5.68 (1 H, d, *J* = 8.5Hz, H14), 5.42 – 5.31 (3 H, m, H20), 5.16 (1 H, d, *J* = 17.4Hz, H20), 3.84 (2 H, s), 3.51 – 3.29 (3 H, m, H11,H22), 2.97 (4 H, t, *J* = 4.8Hz), 2.75 (4 H, s), 2.30 (4 H, s, H2, H13), 2.28 – 2.13 (2 H, m, H4,H10),

2.06 – 1.95 (2 H, m, 11-OH), 1.82 – 1.72 (1 H, m, H7), 1.68 – 1.56 (2 H, m, H1), 1.55 – 1.41 (2 H, m, H13), 1.38 (3 H, s, H15), 1.36 – 1.24 (1 H, m, H8), 1.12 (4 H, s, H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.53 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.77 (C3), 169.28 (C21), 163.22, 151.35, 145.09, 139.13 (C19), 138.79, 136.40, 132.59, 131.03, 130.80, 126.53, 125.14, 124.58, 123.16, 122.41, 120.96, 120.90, 119.02, 117.13 (C20), 116.16, 110.50, 74.55 (C11), 70.58 (C14), 57.99 (C4), 53.53, 53.32 (C22), 51.52, 45.39 (C9), 44.61 (C13), 44.01 (C12), 41.74 (C5), 39.76, 36.55 (C6), 35.98 (C10), 34.40 (C2), 30.31 (C8), 26.83 (C7), 26.34 (C18), 24.80 (C1), 17.86, 16.53 (C16), 14.78 (C15), 11.54 (C17). HR-MS (ESI): Calcd for $C_{44}H_{59}N_6O_5S$ ($M+H^+$): 783.4268; Found: 783.4269.

4.2.15.

22-(2-(2-(4-((4-(*m*-tolyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (75)

White powder; yield: 59%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.41 (1 H, s), 8.30 (1 H, d, *J* = 8.3Hz), 7.83 (1 H, d, *J* = 2.4Hz), 7.55 (1 H, d, *J* = 7.8Hz), 7.36 (1 H, t, *J* = 7.9Hz), 7.11 (2 H, dt, *J* = 31.9, 7.6Hz), 6.77 – 6.65 (3 H, m), 6.43 (1 H, dd, *J* = 17.5, 10.9Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.39 – 5.32 (2 H, m, H20), 5.16 (1 H, d, *J* = 17.4Hz, H20), 3.82 (2 H, d, *J* = 2.4Hz), 3.49 – 3.29 (3 H, m, H11,H22), 3.21 (4 H, t, *J* = 4.7Hz), 2.72 (4 H, t, *J* = 4.8Hz), 2.32 (4 H, s, H2,H13), 2.22 (2 H, dt, *J* = 16.8, 10.1Hz, H4,H10), 2.08 – 1.95 (2 H, m, 11-OH), 1.77 (1 H, d, *J* = 14.5Hz, H6), 1.62 (2 H, t, *J* = 11.0Hz, H8,H13), 1.54 – 1.40 (4 H, m, H1,H7), 1.38 (4 H, s, H8,H15), 1.12 (4 H, d, *J* = 4.9Hz, H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J*

= 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.78 (C3), 169.29 (C21), 163.22, 151.27, 144.89, 139.13 (C19), 138.87, 138.75, 136.59, 136.40, 130.80, 128.92, 125.15, 124.56, 122.39, 120.96, 120.68, 117.26 (C20), 116.96, 113.24, 74.54 (C11), 70.57 (C14), 57.98 (C4), 53.46, 53.23, 52.94 (C22), 49.14, 45.38 (C9), 44.61 (C13), 44.00 (C12), 41.73 (C5), 39.76, 36.54 (C6), 35.97 (C10), 34.41 (C2), 30.30 (C8), 26.83 (C7), 26.33 (C18), 24.79 (C1), 21.76, 16.54 (C16), 14.77 (C15), 13.28, 11.55 (C17). HR-MS (ESI): Calcd for $C_{44}H_{59}N_6O_5S$ ($M+H^+$): 783.4268; Found: 783.4270.

4.2.16.

22-(2-(2-(4-((4-(*p*-tolyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (76)

White powder; yield: 59%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.42 (1 H, s), 8.29 (1 H, d, *J* = 8.3Hz), 7.83 (1 H, s), 7.58 – 7.51 (1 H, m), 7.36 (1 H, t, *J* = 8.2Hz), 7.06 (3 H, dd, *J* = 7.9, 5.4Hz), 6.84 (2 H, d, *J* = 8.2Hz), 6.43 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.40 – 5.32 (2 H, m, H20), 5.16 (1 H, d, *J* = 17.4Hz, H20), 3.82 (2 H, s), 3.43 (2 H, t, *J* = 15.6Hz, H11,H22), 3.33 (1 H, s), 3.17 (4 H, t, *J* = 5.0Hz), 2.73 (4 H, t, *J* = 5.0Hz), 2.27 (5 H, s, H2,H4,H13), 2.25 – 2.15 (1 H, m, H10), 2.08 – 1.93 (2 H, m, 11-OH), 1.93 – 1.83 (2 H, m, H8), 1.82 – 1.72 (1 H, m, H6), 1.63 (2 H, td, *J* = 12.1, 10.7, 6.2Hz, H1), 1.54 – 1.40 (3 H, m, H7,H13), 1.38 (3 H, s, H15), 1.11 (4 H, d, *J* = 4.9Hz, H8,H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.82 (C3), 169.32 (C21), 163.22, 149.11, 144.85, 139.11 (C19), 138.81, 136.38, 130.80, 129.61, 129.31, 125.17, 124.59, 123.75, 122.41, 120.98, 117.21 (C20), 116.47,

74.54 (C11), 72.96, 70.57 (C14), 64.45, 57.98 (C4), 53.46, 53.21, 52.93 (C22), 49.62, 45.38 (C9), 44.60 (C13), 44.00 (C12), 41.73 (C5), 39.77, 36.54 (C6), 35.97 (C10), 34.41 (C2), 30.30 (C8), 26.82 (C7), 26.32 (C18), 24.79 (C1), 20.41, 16.54 (C16), 14.77 (C15), 11.55 (C17). HR-MS (ESI): Calcd for $C_{44}H_{59}N_6O_5S$ ($M+H^+$): 783.4168; Found: 783.4111.

4.2.17.

22-(2-(2-(4-((4-(2-fluorophenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (77)

White powder; yield: 60%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.43 (1 H, s), 8.29 (1 H, d, *J* = 8.3Hz), 7.83 (1 H, s), 7.55 (1 H, d, *J* = 7.9Hz), 7.36 (1 H, t, *J* = 7.9Hz), 7.11 – 6.88 (5 H, m), 6.43 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.36 (2 H, d, *J* = 3.3Hz, H20), 5.16 (1 H, d, *J* = 17.4Hz, H20), 3.83 (2 H, s), 3.50 – 3.28 (3 H, m, H11,H22), 3.13 (4 H, t, *J* = 4.8Hz), 2.76 (4 H, t, *J* = 4.8Hz, H10,H13), 2.35 – 2.10 (3 H, m,H2,H4), 2.08 – 1.88 (2 H, m, 11-OH), 1.85 – 1.72 (2 H, m, H6,H8), 1.62 (2 H, ddd, *J* = 15.9, 11.8, 7.2Hz, H1), 1.54 – 1.40 (3 H, m, H7, H13), 1.38 (4 H, s, H8,H15), 1.11 (4 H, d, *J* = 5.1Hz, H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.72 (C3), 169.26 (C21), 163.21, 156.93, 154.49, 144.87, 140.06, 139.98, 139.12 (C19), 138.87, 136.37, 130.77, 125.14, 124.50, 124.43, 124.40, 122.47, 122.43, 122.39, 120.97, 118.99, 118.96, 117.21 (C20), 116.18, 115.97, 74.55 (C11), 70.58 (C14), 57.99 (C4), 53.47, 53.21, 52.94 (C22), 50.41, 50.38, 45.38 (C9), 44.62 (C13), 44.01 (C12), 41.74 (C5), 39.75, 36.54 (C6), 35.98 (C10), 34.39 (C2), 30.31 (C8), 26.83 (C7),

613 26.32 (C18), 24.79 (C1), 16.51 (C16), 14.77 (C15), 11.51 (C17). HR-MS (ESI): Calcd
614 for $C_{43}H_{56}FN_6O_5S$ ($M+H^+$): 788.4017; Found: 788.4091.

615 **4.2.18.**

616 **22-(2-(2-(4-((4-(3-fluorophenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)aceta**
617 **mido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (78)**

618 White powder; yield: 71%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.43 (1 H, s),
619 8.28 (1 H, d, *J* = 8.3Hz), 7.83 (1 H, s), 7.54 (1 H, d, *J* = 7.8Hz), 7.35 (1 H, t, *J* =
620 7.8Hz), 7.06 (1 H, t, *J* = 7.6Hz), 6.95 (2 H, t, *J* = 8.7Hz), 6.86 (2 H, dd, *J* = 9.1,
621 4.6Hz), 6.43 (1 H, dd, *J* = 17.4, 10.9Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.43 –
622 5.28 (4 H, m, H20), 5.15 (1 H, d, *J* = 17.4Hz, H20), 3.82 (2 H, s), 3.49 – 3.28 (3 H, m,
623 H11,H22), 3.13 (4 H, d, *J* = 5.2Hz), 2.73 (4 H, d, *J* = 5.0Hz), 2.30 – 2.16 (2 H, m,
624 H2), 2.13 – 1.94 (2 H, m, H10), 1.81 – 1.71 (1 H, m, 11-OH), 1.62 (2 H, td, *J* = 12.1,
625 10.6, 6.3Hz, H4,H13), 1.54 – 1.39 (3 H, m, H1,H7), 1.37 (4 H, s, H8,H18), 1.11 (4 H,
626 d, *J* = 4.4Hz, H18), 0.89 (3 H, d, *J* = 6.9Hz,H17), 0.51 (3 H, d, *J* = 7.0Hz, H16). ^{13}C
627 NMR (101 MHz, Chloroform-*d*) δ 216.82 (C3), 169.35 (C21), 165.01, 163.19, 162.59,
628 152.87, 152.78, 144.77, 139.12 (C19), 138.85, 136.43, 130.83, 130.13, 130.03, 125.18,
629 124.58, 122.38, 120.96, 117.24 (C20), 111.16, 111.14, 105.96, 105.74, 102.79, 102.54,
630 74.52 (C11), 70.60 (C14), 57.97 (C4), 53.44, 53.17, 52.69 (C22), 48.52, 45.38 (C9),
631 44.58 (C13), 44.00 (C12), 41.72 (C5), 39.80, 36.53 (C6), 35.96 (C10), 34.41 (C2),
632 30.29 (C8), 26.82 (C7), 26.32 (C18), 24.79 (C1), 16.55 (C16), 14.77 (C15), 11.57
633 (C17). HR-MS (ESI): Calcd for $C_{43}H_{56}FN_6O_5S$ ($M+H^+$): 788.4017; Found:
634 788.4040.

4.2.19.

22-(2-(2-(4-((4-(4-fluorophenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)aceta mido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (79)

White powder; yield: 36%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.43 (1 H, s), 8.28 (1 H, d, *J* = 8.3Hz), 7.83 (1 H, s), 7.54 (1 H, d, *J* = 7.8Hz), 7.35 (1 H, t, *J* = 7.8Hz), 7.06 (1 H, t, *J* = 7.6Hz), 6.95 (2 H, t, *J* = 8.7Hz), 6.86 (2 H, dd, *J* = 9.1, 4.6Hz), 6.43 (1 H, dd, *J* = 17.4, 10.9Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.43 – 5.28 (4 H, m, H20), 5.15 (1 H, d, *J* = 17.4Hz, H20), 3.82 (2 H, s), 3.49 – 3.28 (3 H, m, H11,H22), 3.13 (4 H, d, *J* = 5.2Hz), 2.73 (4 H, d, *J* = 5.0Hz, H2,H13), 2.30 – 2.16 (2 H, m, H4,H10), 2.13 – 1.94 (2 H, m, 11-OH), 1.81 – 1.71 (1 H, m), 1.62 (2 H, td, *J* = 12.1, 10.6, 6.3Hz, H6,H8), 1.54 – 1.39 (3 H, m, H1,H7,H13), 1.37 (4 H, s, H8,H15), 1.11 (4 H, d, *J* = 4.4Hz, H18), 0.89 (3 H, d, *J* = 6.9Hz, H17), 0.51 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.82 (C3), 169.34 (C21), 163.21, 158.36, 155.98, 147.86, 144.72, 139.11 (C19), 138.85, 136.41, 130.82, 129.92, 125.18, 124.63, 122.40, 120.97, 117.91, 117.84, 117.24 (C20), 115.59, 115.37, 74.52 (C11), 70.59 (C14), 57.97 (C4), 53.43, 53.13, 52.86 (C22), 50.03, 45.38 (C9), 44.58 (C13), 44.00 (C12), 41.72 (C5), 39.79, 36.52 (C6), 35.96 (C10), 34.40 (C2), 30.29 (C8), 26.82 (C7), 26.33 (C18), 24.79 (C1), 16.54 (C16), 14.77 (C15), 11.56 (C17). HR-MS (ESI): Calcd for $C_{43}H_{56}FN_6O_5S$ ($M+H^+$): 788.4017; Found: 788.4100.

4.2.20.

22-(2-(2-(4-((4-(2-chlorophenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)aceta mido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (80)

White powder; yield: 34%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.43 (1 H, s), 8.28 (1 H, d, *J* = 8.4Hz), 7.87 (1 H, s), 7.54 (1 H, d, *J* = 7.8Hz), 7.35 (2 H, t, *J* = 7.3Hz), 7.21 (1 H, t, *J* = 7.7Hz), 7.09 – 6.92 (3 H, m), 6.43 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.34 (3 H, dd, *J* = 22.8, 6.6Hz, H20), 5.15 (1 H, d, *J* = 17.4Hz, H20), 3.85 (2 H, s), 3.49 – 3.28 (3 H, m, H11,H22), 3.11 (4 H, s), 2.79 (4 H, s), 2.23 (5 H, ddd, *J* = 23.5, 15.3, 8.2Hz, H2,H4,H10,H13), 2.08 – 1.92 (2 H, m, 11-OH,H8), 1.76 (1 H, d, *J* = 14.5Hz,H6), 1.61 (2 H, t, *J* = 10.4Hz, H1), 1.54 – 1.41 (2 H, m, H7,H13), 1.40 – 1.23 (4 H, m, H8,H15), 1.11 (4 H, s, H18), 0.89 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.84 (C3), 169.31 (C21), 163.24, 149.08, 144.68, 141.69, 139.11 (C19), 138.84, 136.40, 130.80, 130.62, 128.73, 127.57, 125.38, 125.15, 124.76, 123.73, 122.42, 120.97, 120.41, 117.24 (C20), 74.53 (C11), 70.56 (C14), 57.98 (C4), 53.45, 53.45, 53.07 (C22), 50.93, 45.38 (C9), 44.59 (C13), 43.99 (C12), 41.73 (C5), 39.78, 36.54 (C6), 35.97 (C10), 34.41 (C2), 30.30 (C8), 26.82 (C7), 26.33 (C18), 24.79 (C1), 16.54 (C16), 14.78 (C15), 11.57 (C17). HR-MS (ESI): Calcd for $C_{43}H_{56}ClN_6O_5S$ ($M+H^+$): 803.3721; Found: 803.3649.

4.2.21.

22-(2-(2-(4-((4-(3-chlorophenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (81)

White powder; yield: 35%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.42 (1 H, s), 8.29 (1 H, d, *J* = 8.3Hz), 7.82 (1 H, s), 7.55 (1 H, d, *J* = 7.8Hz), 7.36 (1 H, t, *J* = 7.9Hz), 7.15 (1 H, t, *J* = 8.1Hz), 7.07 (1 H, t, *J* = 7.6Hz), 6.86 (1 H, s), 6.83 – 6.74 (2

H, m), 6.43 (1 H, dd, *J* = 17.4, 10.9Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.43 – 5.27 (4 H, m, H20), 5.16 (1 H, d, *J* = 17.4Hz, H20), 3.81 (2 H, s), 3.51 – 3.31 (3 H, m, H11,H22), 3.21 (4 H, t, *J* = 4.7Hz), 2.71 (4 H, t, *J* = 4.9Hz, H10,H13), 2.24 (3 H, dq, *J* = 27.8, 9.4, 8.1Hz, H2,H13), 2.08 – 1.93 (2 H, m, 11-OH), 1.81 – 1.72 (1 H, m, H6), 1.62 (2 H, t, *J* = 11.6Hz, H8), 1.55 – 1.40 (3 H, m, H1,H7,H13), 1.38 (3 H, s, H15), 1.35 – 1.24 (1 H, m, H8), 1.12 (4 H, s, H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.75 (C3), 169.31 (C21), 163.18, 152.23, 144.76, 139.12 (C19), 138.87, 136.40, 134.91, 130.81, 129.99, 125.17, 124.56, 122.40, 120.97, 119.30, 117.20 (C20), 115.76, 113.91, 97.30, 74.54 (C11), 70.62 (C14), 57.98 (C4), 53.45, 53.16, 52.69 (C22), 48.56, 45.38 (C9), 44.61 (C13), 44.01 (C12), 41.74 (C5), 39.79, 36.53 (C6), 35.98 (C10), 34.40 (C2), 30.30 (C8), 26.83 (C7), 26.46, 26.35 (C18), 24.79 (C1), 16.53 (C16), 14.77 (C15), 11.54 (C17) . HR-MS (ESI): Calcd for $C_{43}H_{56}ClN_6O_5S$ ($M+H^+$): 803.3721; Found: 803.3726.

4.2.22.

22-(2-(2-(4-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (82)

White powder; yield: 39%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.43 (1 H, s), 8.29 (1 H, d, *J* = 8.3Hz), 7.83 (1 H, s), 7.55 (1 H, d, *J* = 7.8Hz), 7.36 (1 H, t, *J* = 7.9Hz), 7.20 (2 H, d, *J* = 8.5Hz), 7.07 (1 H, t, *J* = 7.6Hz), 6.83 (2 H, d, *J* = 8.6Hz), 6.43 (1 H, dd, *J* = 17.4, 10.9Hz, H19), 5.68 (1 H, d, *J* = 8.6Hz, H14), 5.34 (3 H, dd, *J* = 19.9, 5.3Hz, H20), 5.16 (1 H, d, *J* = 17.4Hz, H20), 3.82 (2 H, s), 3.39 (3 H, dt, *J* = 25.0, 12.4Hz, H11,H22), 3.18 (4 H, d, *J* = 5.2Hz), 2.73 (4 H, t, *J* = 4.9Hz,H13), 2.23

(3 H, ddt, *J* = 24.5, 19.4, 8.4Hz, H2,H4,H10), 2.08 – 1.86 (2 H, m, 11-OH), 1.82 – 1.72 (1 H, m, H6), 1.63 (2 H, h, *J* = 9.2, 8.8Hz, H8), 1.53 – 1.41 (3 H, m, H1,H7,H13), 1.38 (4 H, s, H8,H15), 1.12 (4 H, s, H18), 0.90 (3 H, d, *J* = 7.0Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.74 (C3), 169.34 (C21), 163.16, 149.80, 144.72, 139.12 (C19), 138.84, 136.41, 130.82, 128.92, 126.09, 125.18, 124.59, 122.41, 120.97, 117.27 (C20), 117.18, 101.69, 74.54 (C11), 70.63 (C14), 57.98 (C4), 55.21, 53.46, 53.46, 53.14, 52.72 (C22), 49.02, 45.38 (C9), 44.61, 44.61 (C13), 44.01 (C12), 41.74 (C5), 39.81, 36.53 (C6), 35.98 (C10), 34.39 (C2), 30.30 (C8), 26.83 (C7), 26.34 (C18), 24.79 (C1), 16.53 (C16), 14.77 (C15), 11.54 (C17). HR-MS (ESI): Calcd for $C_{43}H_{56}ClN_6O_5S$ ($M+H^+$): 803.3721; Found: 803.3682.

4.2.23.

22-(2-(2-(4-((4-(2-methoxyphenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (83)

White powder; yield: 42%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.41 (1 H, s), 8.29 (1 H, d, *J* = 8.3Hz), 7.83 (1 H, s), 7.54 (1 H, d, *J* = 7.7Hz), 7.35 (1 H, t, *J* = 7.9Hz), 6.96 (5 H, ddt, *J* = 51.8, 24.6, 7.8Hz), 6.43 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.39 – 5.28 (3 H, m, H20), 5.16 (1 H, d, *J* = 17.3Hz, H20), 3.85 (5 H, d, *J* = 7.7Hz), 3.49 – 3.28 (3 H, m, H11,H22), 3.11 (4 H, s), 2.81 – 2.69 (4 H, m, H13), 2.35 – 2.11 (3 H, m, H2,H4,H10), 2.08 – 1.92 (2 H, m, 11-OH), 1.77 (1 H, d, *J* = 14.5Hz, H6), 1.62 (2 H, t, *J* = 10.3Hz, H8), 1.55 – 1.39 (3 H, m, H1,H7,H13), 1.39 – 1.22 (4 H, m, H8,H15), 1.11 (4 H, d, *J* = 5.0Hz,H18), 0.90 (3 H,

d, *J* = 6.9Hz,H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.80 (C3), 169.27 (C21), 163.26, 152.24, 144.86, 141.20, 139.13 (C19), 139.13, 138.86, 136.39, 130.78, 125.13, 125.13, 124.59, 122.93, 122.41, 120.96, 118.22, 117.27 (C20), 111.19, 74.54 (C11), 70.54 (C14), 57.99 (C4), 55.33, 53.45, 53.23, 53.09 (C22), 50.47, 45.38 (C9), 44.61 (C13), 43.99 (C12), 41.73 (C5), 39.73, 36.55 (C6), 35.97 (C10), 34.40 (C2), 30.31 (C8), 26.82 (C7), 26.32 (C18), 24.79 (C1), 16.52 (C16), 14.77 (C15), 11.54 (C17). HR-MS (ESI): Calcd for $C_{44}H_{59}N_6O_6S$ ($M+H^+$): 799.4217; Found: 799.4195.

4.2.24.

22-(2-(2-(4-((4-(4-methoxyphenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (84)

White powder; yield: 52%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.42 (1 H, s), 8.28 (1 H, dd, *J* = 8.2, 1.4Hz), 7.87 (1 H, s), 7.54 (1 H, dd, *J* = 7.8, 1.6Hz), 7.35 (1 H, ddd, *J* = 8.6, 7.5, 1.6Hz), 7.06 (1 H, td, *J* = 7.6, 1.4Hz), 6.91 – 6.80 (4 H, m), 6.43 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.36 (2 H, d, *J* = 2.9Hz, H20), 5.33 – 5.30 (1 H, m), 5.15 (1 H, dd, *J* = 17.4, 1.5Hz, H20), 3.84 (2 H, s), 3.76 (3 H, s), 3.50 – 3.25 (4 H, m, H11,H22), 3.12 (4 H, t, *J* = 4.9Hz), 2.76 (4 H, t, *J* = 4.9Hz, H2,H13), 2.34 – 2.10 (3 H, m, H4,H10), 1.98 (2 H, dd, *J* = 16.0, 8.6Hz, 11-OH,H6), 1.76 (1 H, dq, *J* = 14.5, 3.2Hz, H8), 1.70 – 1.54 (2 H, m, H1,H7), 1.54 – 1.40 (2 H, m, H7,H13), 1.37 (4 H, s, H8,H15), 1.11 (4 H, d, *J* = 4.0Hz, H18), 0.89 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.83 (C3), 169.32 (C21), 163.23, 155.23, 153.87, 145.51, 144.51,

139.10 (C19), 138.82, 136.37, 130.78, 125.16, 124.80, 122.43, 120.99, 118.29, 117.20 (C20), 114.42, 82.32, 74.53 (C11), 70.57 (C14), 57.98 (C4), 55.55, 53.44, 53.44, 53.09, 52.93 (C22), 50.41, 45.38 (C9), 44.59 (C13), 44.00 (C12), 41.73 (C5), 39.76, 38.52, 36.53 (C6), 35.97 (C10), 34.40 (C2), 30.30 (C8), 26.82 (C7), 26.33 (C18), 24.78 (C1), 16.53 (C16), 14.77 (C15), 11.56 (C17). HR-MS (ESI): Calcd for $C_{44}H_{59}N_6O_6S$ ($M+H^+$): 799.4217; Found: 799.4133.

4.2.25.

22-(2-(2-(4-((4-(2-hydroxyphenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (85)

White powder; yield: 71%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.45 (1 H, s), 8.29 (1 H, d, *J* = 8.3Hz), 7.86 (1 H, s), 7.55 (1 H, d, *J* = 7.7Hz), 7.36 (1 H, t, *J* = 7.9Hz), 7.16 (1 H, d, *J* = 7.7Hz), 7.07 (2 H, t, *J* = 7.7Hz), 7.00 – 6.91 (1 H, m), 6.85 (1 H, td, *J* = 7.6, 1.6Hz), 6.43 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.68 (1 H, d, *J* = 8.5Hz, H14), 5.45 – 5.28 (4 H, m, H20), 5.16 (1 H, dd, *J* = 17.4, 1.7Hz, H20), 3.85 (2 H, d, *J* = 2.7Hz), 3.51 – 3.32 (3 H, m, H11,H22), 2.93 (4 H, d, *J* = 5.0Hz), 2.77 (5 H, s, H2), 2.35 – 2.10 (4 H, m, H4,H10,H13), 2.08 – 1.93 (2 H, m, 11-OH, H8), 1.82 – 1.72 (1 H, m, H6), 1.62 (1 H, s, H13), 1.51 – 1.39 (2 H, m ,H1,H7), 1.38 (4 H, s, H8,H15), 1.11 (4 H, d, *J* = 6.0Hz, H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.53 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.82 (C3), 169.35 (C21), 163.21, 151.45, 144.60, 139.12 (C19), 138.88, 138.82, 138.82, 136.43, 130.83, 126.47, 125.18, 124.67, 122.38, 121.44, 120.97, 120.04, 117.26 (C20), 114.09, 74.54 (C11), 70.62 (C14), 57.98 (C4), 53.49, 53.44, 53.12, 53.12, 52.32 (C22), 45.38 (C9), 44.60

(C13), 44.00 (C12), 41.74 (C5), 39.83, 36.54 (C6), 35.97 (C10), 34.40 (C2), 30.30 (C8), 26.82 (C7), 26.32 (C18), 24.79 (C1), 16.54 (C16), 14.77 (C15), 11.55 (C17). HR-MS (ESI): Calcd for $C_{43}H_{57}N_6O_6S$ ($M+H^+$): 785.4060; Found: 785.3993.

4.2.26.

22-(2-(2-(4-((4-(3-hydroxyphenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (86)

White powder; yield: 73%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.45 (1 H, s), 8.29 (1 H, d, *J* = 8.3Hz), 7.81 (1 H, s), 7.55 (1 H, dd, *J* = 7.8, 1.6Hz), 7.41 – 7.32 (1 H, m), 7.13 – 7.02 (2 H, m), 6.51 – 6.37 (3 H, m, H19), 6.33 (1 H, dd, *J* = 7.9, 2.3Hz), 5.67 (1 H, d, *J* = 8.5Hz, H14), 5.38 – 5.32 (3 H, m, H20), 5.21 – 5.11 (1 H, m, H20), 3.80 (2 H, s), 3.49 – 3.28 (3 H, m, H11,H22), 3.18 (4 H, t, *J* = 4.8Hz), 2.70 (4 H, t, *J* = 5.0Hz), 2.34 – 2.13 (3 H, m, H2,H10), 2.09 – 1.93 (2 H, m, 11-OH), 1.81 – 1.71 (6 H, m, H4,H6,H8,H13), 1.69 – 1.56 (2 H, m, H1), 1.48 (2 H, dtd, *J* = 25.5, 13.1, 11.5, 4.6Hz, H7), 1.38 (4 H, s, H8,H15), 1.12 (3 H, s, H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.52 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 217.05 (C3), 169.29 (C21), 163.24, 157.10, 152.60, 144.57, 139.03 (C19), 138.87, 136.30, 130.75, 129.92, 125.21, 124.81, 122.50, 121.03, 117.26 (C20), 108.31, 108.24, 106.96, 103.32, 74.57 (C11), 70.60 (C14), 58.02 (C4), 53.46, 53.40, 53.08, 52.75 (C22), 48.76, 45.40 (C9), 44.62 (C13), 44.00 (C12), 41.75 (C5), 39.70, 36.56 (C6), 35.97 (C10), 34.42 (C2), 30.31 (C8), 26.82 (C7), 26.35 (C18), 24.78 (C1), 16.53 (C16), 14.80 (C15), 11.53 (C17). HR-MS (ESI): Calcd for $C_{43}H_{57}N_6O_6S$ ($M+H^+$): 785.4060; Found: 785.4125.

4.2.27.

22-(2-(2-(4-((4-(4-hydroxyphenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (87)

White powder; yield: 65%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.44 (1 H, s), 8.31 – 8.24 (1 H, m), 7.82 (1 H, d, *J* = 1.3Hz), 7.54 (1 H, dt, *J* = 7.7, 1.6Hz), 7.35 (1 H, td, *J* = 7.8, 1.6Hz), 7.06 (1 H, td, *J* = 7.6, 1.5Hz), 6.83 – 6.70 (4 H, m), 6.42 (1 H, ddd, *J* = 17.5, 11.0, 1.7Hz, H19), 5.66 (1 H, d, *J* = 8.4Hz, H14), 5.42 – 5.26 (4 H, m, H20), 5.15 (1 H, dd, *J* = 17.4, 1.7Hz, H20), 3.81 (2 H, s), 3.51 – 3.28 (3 H, m, H11,H22), 3.05 (4 H, t, *J* = 4.8Hz), 2.72 (4 H, t, *J* = 4.8Hz, H13), 2.34 – 2.09 (3 H, m, H2,H4,H10), 2.08 – 1.92 (2 H, m, 11-OH), 1.76 (1 H, dd, *J* = 14.1, 3.1Hz, H6), 1.69 – 1.55 (3 H, m, H8,H7,H13), 1.53 – 1.39 (2 H, m, H1), 1.38 – 1.25 (5 H, m, H8,H15), 1.11 (3 H, d, *J* = 1.5Hz, H18), 0.89 (3 H, d, *J* = 6.9Hz, H17), 0.51 (3 H, d, *J* = 6.9Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 217.03 (C3), 169.39 (C21), 163.26, 150.35, 145.12, 144.52, 139.06 (C19), 138.84, 136.38, 130.80, 125.22, 124.85, 124.84, 122.46, 121.02, 118.56, 117.31 (C20), 116.00, 115.97, 74.55 (C11), 70.60 (C14), 57.99 (C4), 53.42, 53.02, 52.91 (C22), 50.55, 45.39 (C9), 44.58 (C13), 43.98 (C12), 41.73 (C5), 39.78, 36.54 (C6), 35.95 (C10), 34.42 (C2), 30.29 (C8), 29.69, 26.81 (C7), 26.34 (C18), 24.78 (C1), 24.72, 16.54 (C16), 14.78 (C15), 11.58 (C17). HR-MS (ESI): Calcd for $C_{43}H_{57}N_6O_6S$ ($M+H^+$): 785.4060; Found: 785.4081.

4.2.28.

22-(2-(2-(4-((4-(2-nitrophenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (88)

Yellow powder; yield: 43%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.44 (1 H, s), 8.30 (1 H, d, *J* = 8.3Hz), 7.80 (1 H, s), 7.75 (1 H, dd, *J* = 8.1, 1.7Hz), 7.55 (1 H, dd, *J* = 7.8, 1.6Hz), 7.50 – 7.43 (1 H, m), 7.40 – 7.33 (1 H, m), 7.14 (1 H, d, *J* = 8.2Hz), 7.05 (2 H, dd, *J* = 13.2, 5.7Hz), 6.44 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.68 (1 H, d, *J* = 8.6Hz, H14), 5.37 (2 H, d, *J* = 4.1Hz, H20), 5.33 – 5.29 (1 H, m), 5.16 (1 H, dd, *J* = 17.5, 1.6Hz, H20), 3.81 (2 H, s), 3.41 (2 H, p, *J* = 15.1, 14.0Hz, H11,H22), 3.34 – 3.30 (1 H, m), 3.11 (4 H, t, *J* = 4.8Hz), 2.72 (4 H, t, *J* = 4.8Hz, H13), 2.25 (3 H, dq, *J* = 27.8, 9.4, 8.0Hz, H2,H4,H10), 2.06 (1 H, s, 11-OH), 1.99 (1 H, dd, *J* = 16.1, 8.6Hz, H6), 1.76 (2 H, s, H8), 1.63 (3 H, dq, *J* = 14.8, 8.1, 4.3Hz, H1,H7,H13), 1.51 – 1.45 (2 H, m), 1.38 (3 H, s ,H15), 1.36 – 1.25 (2 H, m, H8), 1.12 (3 H, s, H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.53 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.82 (C3), 169.36 (C21), 163.21, 145.92, 143.43, 143.42, 140.24, 139.16 (C20), 138.85, 136.46, 133.44, 130.84, 125.84, 125.14, 124.46, 122.36, 121.77, 121.04, 120.94, 117.31 (C11), 74.54 (C14), 70.60, 57.98 (C4), 53.43, 53.13, 52.84 (C22), 51.57, 45.39 (C9), 44.58 (C13), 44.00 (C12), 41.73 (C5), 39.82, 36.54 (C6), 35.96 (C10), 34.41 (C2), 30.30 (C8), 26.82 (C7), 26.30 (C18), 24.79 (C1), 20.46, 16.55 (C16), 14.77 (C15), 11.56 (C17). HR-MS (ESI): Calcd for $C_{44}H_{56}N_7O_7S$ ($M+H^+$): 814.3962; Found: 814.3950.

4.2.29.

22-(2-(2-(4-((4-(3-nitrophenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)aceta mido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (89)

Yellow powder; yield: 46%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.46 (1 H, s),

8.30 (1 H, d, *J* = 8.3Hz), 7.82 (1 H, s), 7.70 (1 H, t, *J* = 2.4Hz), 7.65 (1 H, dd, *J* = 8.0, 2.1Hz), 7.56 (1 H, dd, *J* = 7.8, 1.6Hz), 7.41 – 7.32 (2 H, m), 7.18 (1 H, dd, *J* = 8.3, 2.5Hz), 7.07 (1 H, t, *J* = 7.6Hz), 6.44 (1 H, dd, *J* = 17.4, 11.0Hz, H19), 5.68 (1 H, d, *J* = 8.5Hz, H14), 5.38 (1 H, d, *J* = 3.9Hz, H20), 5.34 – 5.29 (1 H, m), 5.21 – 5.11 (1 H, m, H20), 3.82 (2 H, s), 3.50 – 3.34 (3 H, m, H11,H22), 3.32 – 3.28 (4 H, m), 2.74 (4 H, s, H13), 2.35 – 2.11 (3 H, m, H2,H4,H10), 2.06 (1 H, d, *J* = 2.7Hz, 11-OH), 2.02 – 1.95 (1 H, m, H6), 1.76 (3 H, s, H8), 1.69 – 1.57 (2 H, m, H1), 1.52 – 1.43 (3 H, m, H7,H13), 1.38 (3 H, s, H15), 1.35 – 1.24 (2 H, m, H8), 1.12 (3 H, s, H18), 0.90 (3 H, d, *J* = 6.9Hz, H17), 0.53 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.68 (C3), 169.34 (C21), 163.16, 151.74, 149.28, 144.80, 139.15 (C19), 138.91, 136.44, 130.84, 129.65, 125.17, 124.46, 122.37, 121.08, 120.95, 117.22 (C20), 113.66, 109.62, 74.54 (C11), 70.66 (C14), 57.97 (C4), 53.44, 53.16, 52.52 (C22), 48.31, 45.38 (C9), 44.63 (C13), 44.02 (C12), 41.75 (C5), 39.84, 36.53 (C6), 35.99 (C10), 34.39 (C2), 30.30 (C8), 26.83 (C7), 26.34 (C18), 24.79 (C1), 16.53 (C16), 14.76 (C15), 11.52 (C17). HR-MS (ESI): Calcd for $C_{44}H_{56}N_7O_7S$ ($M+H^+$): 814.3962; Found: 814.3987.

4.2.30.

22-(2-(2-(4-((4-(4-nitrophenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (90)

Yellow powder; yield: 42%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.49 (1 H, s), 8.29 (1 H, d, *J* = 8.3Hz), 8.18 – 8.05 (2 H, m), 7.82 (1 H, s), 7.56 (1 H, dd, *J* = 7.8, 1.6Hz), 7.43 – 7.32 (1 H, m), 7.13 – 7.00 (1 H, m), 6.87 – 6.75 (2 H, m), 6.44 (1 H, dd,

855 *J* = 17.5, 11.0Hz, H19), 5.68 (1 H, d, *J* = 8.5Hz, H14), 5.38 (2 H, d, *J* = 4.2Hz, H20),
856 5.31 (1 H, td, *J* = 6.3, 3.2Hz), 5.16 (1 H, dd, *J* = 17.5, 1.6Hz, H20), 3.81 (2 H, s),
857 3.50 – 3.38 (6 H, m, H11,H22), 3.37 – 3.30 (1 H, m), 2.72 (4 H, d, *J* = 5.2Hz, H13),
858 2.33 – 2.14 (3 H, m, H2,H4,H10), 2.06 (1 H, s, 11-OH), 2.00 (1 H, dd, *J* = 16.0,
859 8.6Hz, H6), 1.80 – 1.68 (4 H, m, H1,H7,H8), 1.66 – 1.57 (1 H, m, H13), 1.47 (2 H, d,
860 *J* = 10.9Hz), 1.38 (3 H, s, H15), 1.35 – 1.25 (1 H, m, H8), 1.12 (4 H, s, H18), 0.90 (3
861 H, d, *J* = 6.9Hz, H17), 0.53 (3 H, d, *J* = 7.0Hz, H16). ^{13}C NMR (101 MHz,
862 Chloroform-*d*) δ 216.74 (C3), 169.47 (C21), 163.12, 154.79, 144.57, 139.15 (C19),
863 138.88, 138.48, 136.50, 130.89, 125.92, 125.23, 124.52, 122.38, 120.96, 117.24 (C20),
864 112.69, 74.53 (C11), 70.70 (C14), 57.96 (C4), 53.49, 53.42, 53.08, 52.34 (C22), 46.97,
865 45.38 (C9), 44.59 (C13), 44.02 (C12), 41.74 (C5), 39.94, 36.57, 36.51 (C6), 35.98
866 (C10), 34.43, 34.40 (C2), 30.29(C8), 29.68, 26.83 (C7), 26.34 (C18), 24.79 (C1),
867 16.55 (C16), 14.76 (C15), 11.55 (C17). HR-MS (ESI): Calcd for $C_{44}H_{56}N_7O_7S$
868 ($M+H^+$): 814.3962; Found: 814.3915.

869 **4.3. *In vitro* efficacy of pleuromutilin derivatives**

870 **4.3.1 Minimal inhibitory concentration (MIC) and minimum bactericidal**
871 **concentration (MBC) testing**

872 The MIC of these novel pleuromutilin derivatives against methicillin-resistant *S.*
873 *aureus* (ATCC 43300), *S. aureus* (ATCC 29213), *S. aureus* (AD3), *S. aureus* (144)
874 and *E. coli* (ATCC 25922) were determined by using pleuromutilin and tiamulin as
875 positive control. MIC values were determined by the broth micro dilution methods
876 according to CLSI (2012). 2-Fold serial dilutions of each pleuromutilin derivative in

Mueller-Hinton broth medium were prepared in 96-well plates with inoculum size of 5×10^5 CFU/mL. Three parallel experiments for each compound concentration. The plates were incubated at 37 ℃ for 24 h. The MIC value was recorded as the minimum drug concentration that completely inhibits the visible growth of test bacteria.

The MBC values were determined by plating 100 μL aliquots from wells without visible growth onto the agar plates according previously reference [17, 25]. The agar plates were incubated overnight at 37 ℃ for colony count. The MBC was determined as the lowest concentration of compound, which reduces the viable counts for 99.9% of the original inoculum.

4.3.2. Constant concentration time-kill curves

The bactericidal activity of compound **32** was determined by the time-kill curve as reported in our previous work [10]. MRSA grown in Muller-Hinton (MH) broth were diluted to approximately 1×10^6 CFU/mL and treated with 1×MIC, 2×MIC, 4×MIC, 8×MIC, 16×MIC, 32×MIC of compound **32** and tiamulin. Samples (100 μL) were taken from the subculturing inoculums at 0, 3, 6, 9, 12 and 24 h and serially diluted 10-flod with sterile saline. Then 25 μL of the dilutions were plated onto MH agar plates. The total bacterial CFU/mL on the plates were counted to calculate the bacterial colonies after up to 20 h of incubation at 37 ℃. The time-kill curve was constructed by plotting the $\log_{10}$ CFU/mL of bacteria counts in the presence or absence of test compound versus time.

4.3.3. Determination of the postantibiotic effect

Compound **32** and tiamulin were tested against MRSA using time-kill methods according previous work to determine the postantibiotic effect (PAE) [10].

For the PAE testing, the tubes were inoculated with MRSA in the logarithmic phase of growth at a final concentration of 1×10^6 CFU/mL in MH broth as the inoculum. Each test compound at final concentrations of 2×MIC and 4×MIC was added to tubes containing the inoculum. The inoculums containing the test compound were incubated at a 37 □ constant temperature vibration incubator for 2 hours. After the initial incubation, the test compound was removed by centrifuging the tubes three times at 3000 rpm for 10 min, decanting and suspending in pre-warmed MH broth. The bacterial pellet was washed using 0.9% saline and then suspended in 5 mL MH broth in new tubes. The tubes were placed in 37 ℃ and sample solutions were taken from each culture at 0, 1, 2, 4, and 6 h. The sample solutions (100 μL) were diluted 10-fold in saline and plated on MH agar plates. After incubating these plates at 37 ℃ for 20 h, the resulting colonies on the plates were counted. The PAE was calculated by the equation $PAE=T_A-T_C$ and expressed in hour. T_A and T_C are the time required for the bacteria in the test and control groups to increase by 1 $\log_{10}$ CFU/mL.

4.4. *In vivo* efficacy of pleuromutilin derivatives

4.4.1. Neutropenic murine thigh infection model

In this experiment, 6-week-old, specific pathogen-free, female ICR/Swiss mice that weighing 22-28 g were fed and housed. The neutrophils of mice were reduced ($<0.1\times10^9$/L) to establish an experimental model. By injection of cyclophosphamide (Mead Johnson Pharmaceuticals, Evansville, IN) at a dose of 150 mg/kg on 4 days

and injection of cyclophosphamide at a dose of 100 mg/kg on 1 day before the experiment, as described previously [10, 26]. Mice were anesthetized with ether and then injected with 0.1 mL of warm MH broth (about 10^7 CFU/mL) through each thigh to generate a model of thigh infection caused by MRSA.

The mice were divided into 3 groups (3 per group) and 0.9% saline, each test compound (20 mg/kg) and tiamulin (20 mg/kg) were injected intravenously (i.v.) after 2 h of MRSA growth in the thigh. The mice from each group were euthanized 24 h after drug injection. The thigh tissue was collected from animal, weighed, homogenized (Polytron tissue homogenizer, Kinematica, Lucerne, Switzerland) in 3 mL of iced saline and serially followed by plating on MH agar plates. After incubation for 24 h, the resulting colonies were counted. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the South China Agricultural University.

4.4.2. MRSA infection model

The *in vivo* potential of test compound to treat MRSA infection was also determined using the MRSA infection model as described in our previous work [9, 10]. Briefly, 6-week-old, specific pathogen-free, ICR/Swiss mice (the Medical Experimental Animal Center of Guangdong Province, Guangzhou, China), weighing between 23 and 25 g, were used for this model. These mice were randomly divided into five groups with 10 mice in each group. Mice were divided into positive control group (MRSA infection), drug control group (treated with tiamulin (30 mg/kg) after MRSA infection), experimental group (treated with compound **32** (30 mg/kg) after MRSA

infection) and two negative control groups (treated with tiamulin (30 mg/kg) and compound **32** (30 mg/kg) without MRSA infection, respectively). Tiamulin and compound **32** were dissolved in the working solution (5% DMSO, 5% Tween-80, and 90% normal saline). Mice in positive control group, drug control group and experimental group were injected intraperitoneally with 0.5 mL of 10^6 CFU/mL of bacterial solution. Mice in drug control group, the experimental group and the positive control group were administered intravenously with tiamulin, compound **32** and the working solution, respectively. Meanwhile, mice in the negative groups were administered intravenously with tiamulin and compound **32**, respectively. The mice were fed and drank freely, and observed continuously for one week to obtain the mice survival curve. The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee of the South China Agricultural University.

4.5. Cytotoxicity assay

Cell viability was assessed using the MTT assay as described in the references [27, 28]. The cell line used in this experiment was RAW 264.7 cells. The cells were seeded into 96-well plates at a density of 1.0×10^5 cells per well and incubated at 37 ℃ for 24 h. The cells were then treated with these pleuromutilin derivatives (8 μg/mL) and cultured for 16 h incubation at 37 ℃. After that, the cells were incubated with 100 μL/well of MTT (0.5 mg/mL in PBS) for another 4 h under 5% CO_2 37 ℃. The medium was removed and cells were dissolved in 150 μL DMSO. Absorbance at 540

963 nm was recorded using a microplate spectrophotometer after 30 min incubation
964 (BIO-TEK Instrument Inc., USA).

965 **4.6. Effect of derivatives on human liver microsomal CYP3A4 enzyme activity**

966 Inhibition effect of compound **32** on CYP3A4 were evaluated according previous
967 work with minor modifications [8, 29]. Each tube contained 40 μL of human liver
968 microsomes (final concentration of human liver microsomes is 0.2 mg/mL), 20 μL of
969 compound **32** and 20 μL of probe substrates (final concentration of testosterone is 20
970 μmol/L) in 0.1 M Tris (pH 7.4). The final concentrations of compound **32** were 0.5、1、
971 5、10、30 and 50 μM, respectively. After prewarm at 37 ℃ for 5 min, the reaction
972 started with the addition of 20 μL NADPH (final concentration is 1 mmol/L). The
973 tubes were incubated at 37 ℃ for 5 min and then added 100 μL of acetonitrile with 50
974 nM loratadine as the internal standard to quench the reaction. The tubes were
975 centrifuged and supernatants were analyzed by LC/MS/MS.

976 **Acknowledgments**

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Figure Captions

Table 1 In vitro antibacterial activity of the synthesized pleuromutilin derivatives **26-33.**

Table 2 In vitro antibacterial activity of the synthesized pleuromutilin derivatives **72-90.**

Table 3 The PAEs values of compounds **26**, **32** and tiamulin against MRSA ATCC 43300.

Table 1

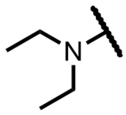
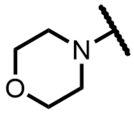
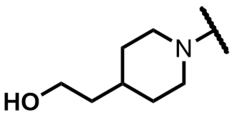
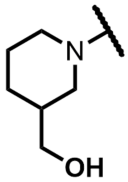
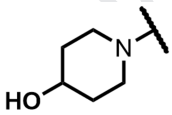
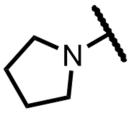
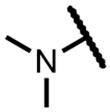
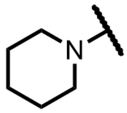
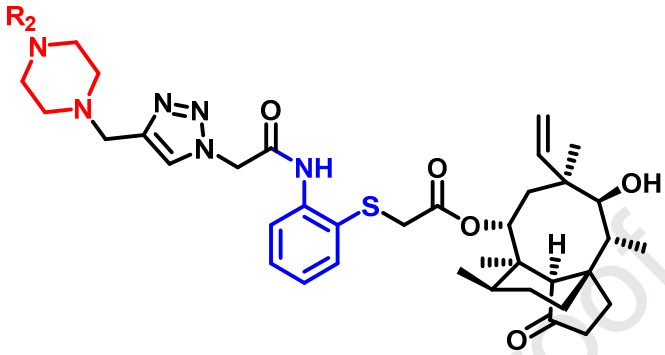
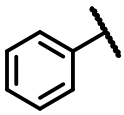
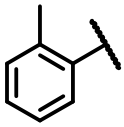
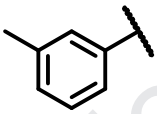
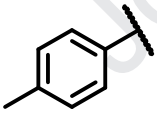
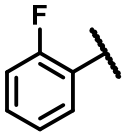
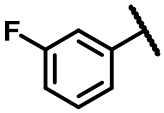
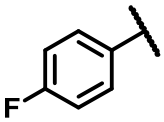
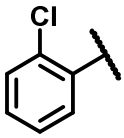
Compound No	R_1	MIC (μg/mL) /MBC (μg/mL)				
		MRSA ATCC 43300	*S.aureus* ATCC 29213	*S.aureus* AD3	*S.aureus* 144	*E. coli* ATCC 25922
26		0.25/0.5	0.25/0.25	0.5/1	0.5/0.5	>32/>32
27		0.5/1	0.5/0.5	1/2	1/1	>32/>32
28		0.5/1	0.5/0.5	1/1	0.5/0.5	>32/>32
29		0.5/0.5	0.5/0.5	0.5/1	0.5/0.5	>32/>32
30		1/2	1/1	1/1	1/2	>32/>32
31		0.25/0.5	0.25/4	0.25/0.5	0.25/0.5	>32/>32
32		0.25/0.5	0.25/0.25	0.25/0.5	0.5/0.5	>32/>32
33		0.5/2	0.5/0.5	0.5/0.5	1/1	>32/>32
Pleuromulin		2/4	1/2	2/4	2/4	-
Tiamulin		0.5/1	0.5/1	1/2	1/2	-

Table 2

Compound No	R_2	MIC (μg/mL) /MBC (μg/mL)				
		MRSA ATCC 43300	*S.aureus* ATCC 29213	*S.aureus* AD3	*S.aureus* 144	*E. coli* ATCC 25922
						
72		0.25/0.5	0.25/0.5	0.5/1	0.5/2	>32/>32
73		0.5/0.5	0.5/0.5	0.5/1	1/2	>32/>32
74		1/1	1/2	2/4	1/4	>32/>32
75		1/1	1/2	2/4	1/8	>32/>32
76		1/4	0.5/0.5	1/2	1/2	>32/>32
77		1/2	0.5/1	0.5/1	1/1	>32/>32
78		1/2	0.5/0.5	0.5/1	1/2	>32/>32
79		1/1	0.5/0.5	0.5/1	1/2	>32/>32
80		1/2	1/2	2/2	2/4	>32/>32

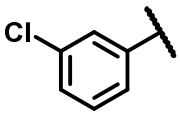
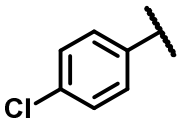
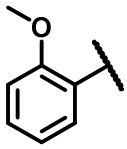
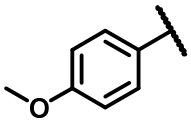
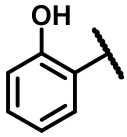
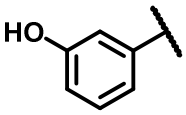
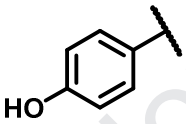
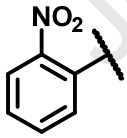
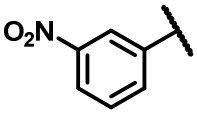
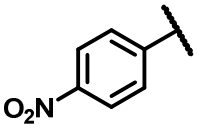
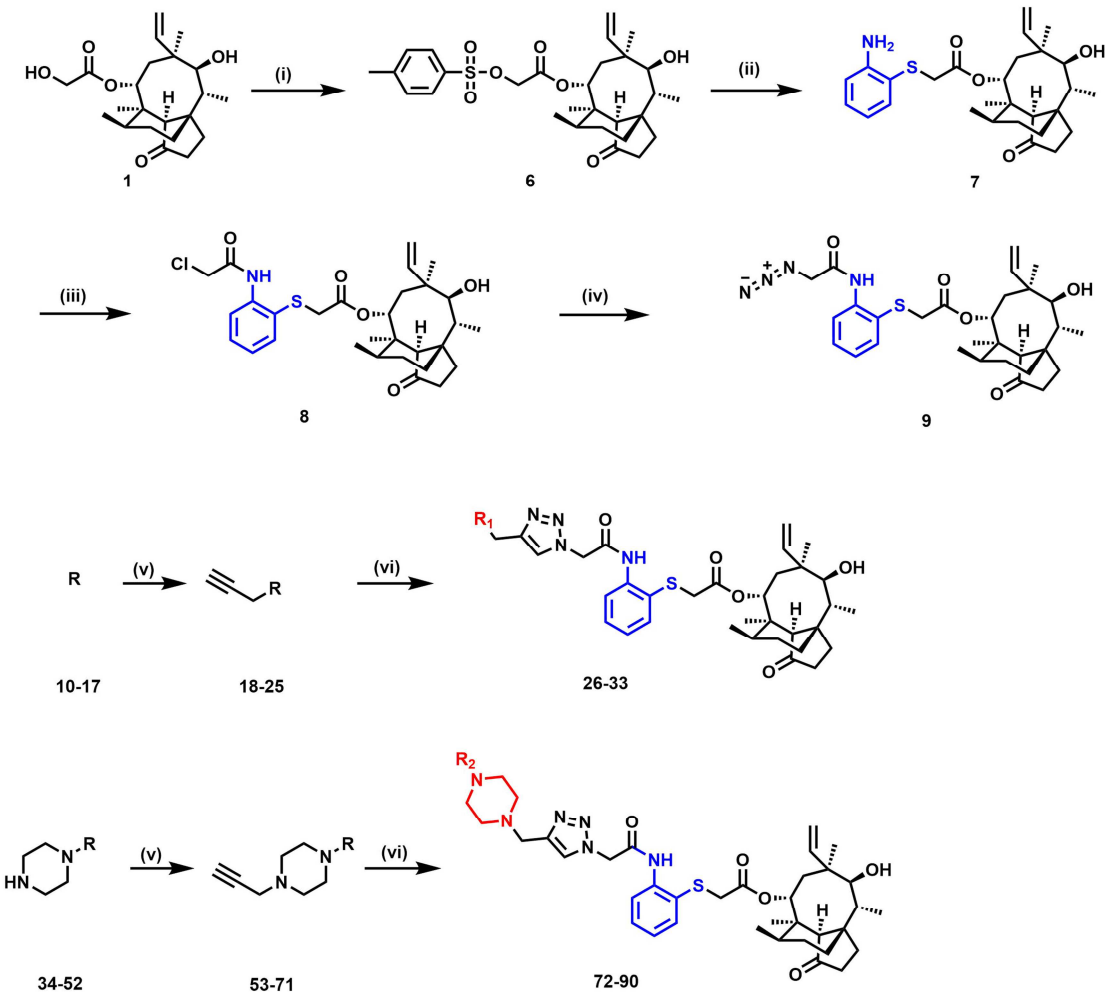
Compound No	R_2	MIC (μg/mL) /MBC (μg/mL)				
		MRSA ATCC 43300	*S.aureus* ATCC 29213	*S.aureus* AD3	*S.aureus* 144	*E. coli* ATCC 25922
81		2/4	1/1	2/2	2/4	>32/>32
82		0.5/1	1/4	2/4	2/2	>32/>32
83		0.5/1	1/2	1/2	1/4	>32/>32
84		1/1	0.5/0.5	1/1	1/2	>32/>32
85		0.5/1	1/1	1/2	1/2	>32/>32
86		0.25/0.5	0.25/0.5	0.25/0.5	0.5/1	>32/>32
87		2/4	2/4	2/4	2/8	>32/>32
88		0.5/1	0.25/0.25	0.5/1	0.5/2	>32/>32
89		0.125/0.25	0.25/0.25	0.25/0.5	0.25/0.5	>32/>32
90		0.125/0.25	0.125/0.125	0.25/0.5	0.25/0.25	>32/>32
Pleuromulin		2/4	1/2	2/4	2/4	-
Tiamulin		0.5/1	0.5/1	1/2	1/2	-

Table 3

Compounds	Concentrations (μg/mL)		PAE (h) Exposure for 2 h
Compound **26**	2 × MIC	0.5	1.81
	4 × MIC	1	1.84
Compound **32**	2 × MIC	0.5	2.03
	4 × MIC	1	2.74
Tiamulin	2 × MIC	1	1.65
	4 × MIC	2	2.04



Scheme 1 Reagent and conditions: (i) tosyl chloride, ethyl acetate, NaOH, 0 □ for 0.5 h, rt for 3 h; (ii) 2-Aminobenzenethiol, ethyl acetate, TEBAC, under N_2, reflux, 6h; (iii) chloride, toluene, DIPEA, reflux, 2 h; (iv) sodium azide, acetone, H_2O, reflux, 4h; (v) 3-Bromopropyne, ethyl acetate, rt, overnight; (vi) compound **9** , $CuSO_4$ ▪$5H_2O$, sodium ascorbate, tert-Butanol: H_2O=1:1, rt, 3 h.

Figure Captions

Figure 1 Structure of pleuromutilin (1), tiamulin (2), valnemulin (3), retapamulin (4), lefamulin (5) and compound **7**.

Figure 2 Time-kill curves for MRSA ATCC 43300 with different concentrations of compound **26** (a), compound **32** (b) and tiamulin (c).

Figure 3 The bacterial growth kinetic curves for MRSA ATCC 43300 exposed to compound **26** (a), compound **32** (b) and tiamulin (c) for 2 h.

Figure 4 Efficacy of tiamulin and compounds **32** against MRSA ATCC 43300 in murine neutropenic thigh models: black circular: growth control; blue square: tiamulin (20 mg/kg); green triangle: compounds **32** (20 mg/kg).

Figure 5 Efficacy of compound **32** (30 mg/kg) and tiamulin (30 mg/kg) in mouse systemic infection model. (Note: tiamulin control group is only injected with tiamulin solution and no pathogenic bacteria, compound **32** control group is only injected with compound **32** solution and no pathogenic bacteria)

Figure 6 Effect on liver microsomal CYP450 enzyme activity, (a) structure of azamulin, (b) Inhibition curve of compound **32** on CYP3A4 (Note: Each point in the figure is three parallel averages)

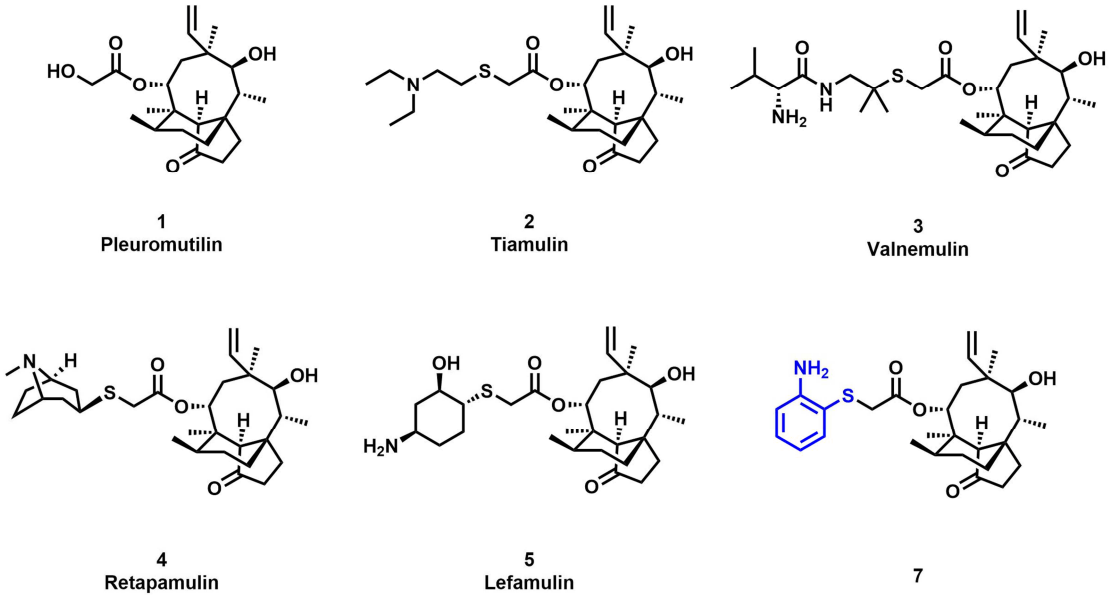
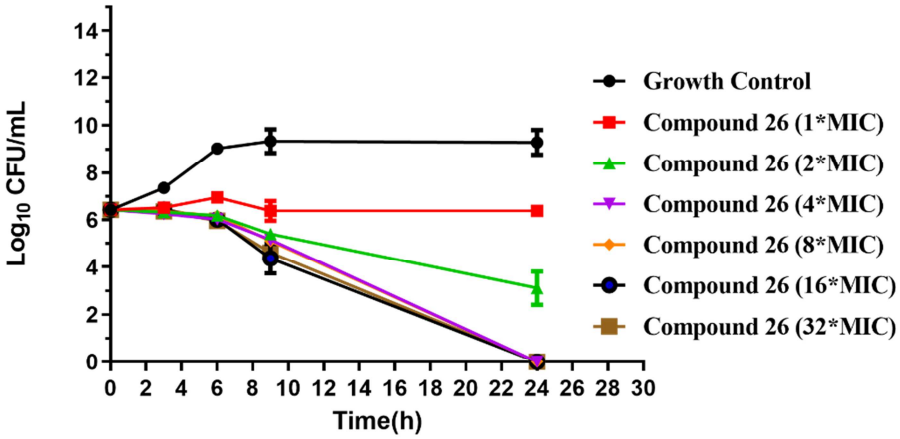
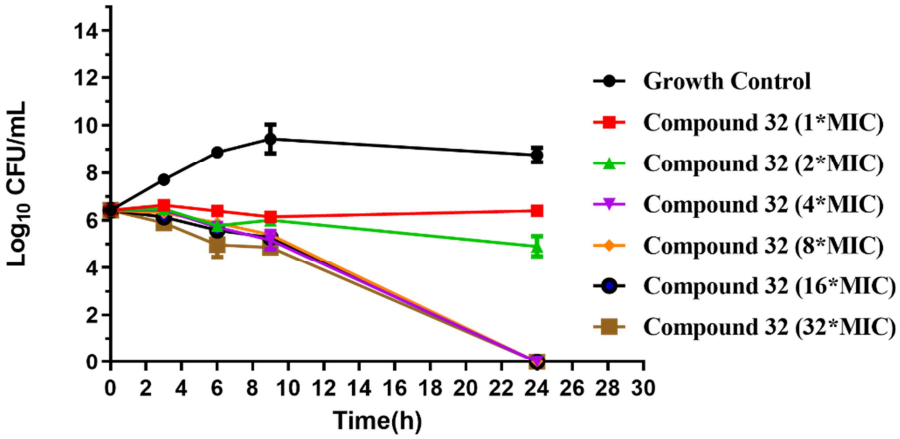


Figure 1

a


Log10 CFU/mL
Time(h)
Growth Control
Compound 26 (1*MIC)
Compound 26 (2*MIC)
Compound 26 (4*MIC)
Compound 26 (8*MIC)
Compound 26 (16*MIC)
Compound 26 (32*MIC)

b


Log10 CFU/mL
Time(h)
Growth Control
Compound 32 (1*MIC)
Compound 32 (2*MIC)
Compound 32 (4*MIC)
Compound 32 (8*MIC)
Compound 32 (16*MIC)
Compound 32 (32*MIC)

c

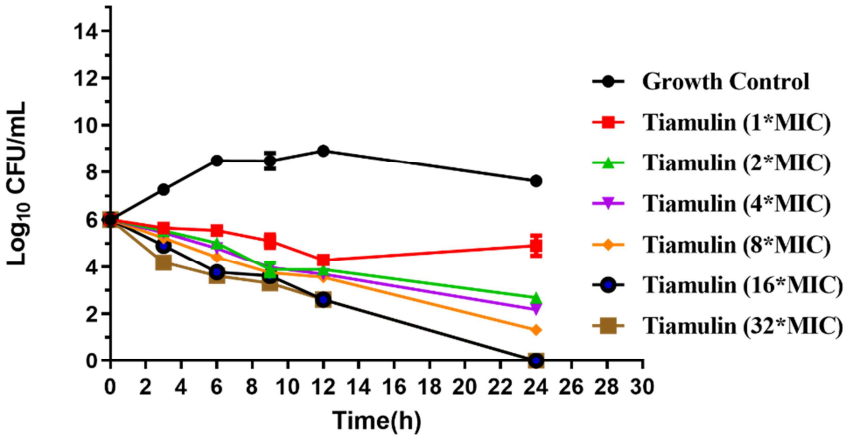
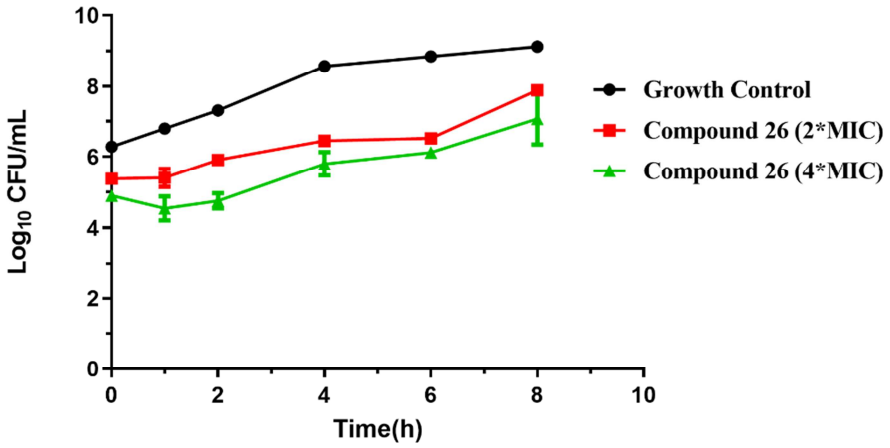
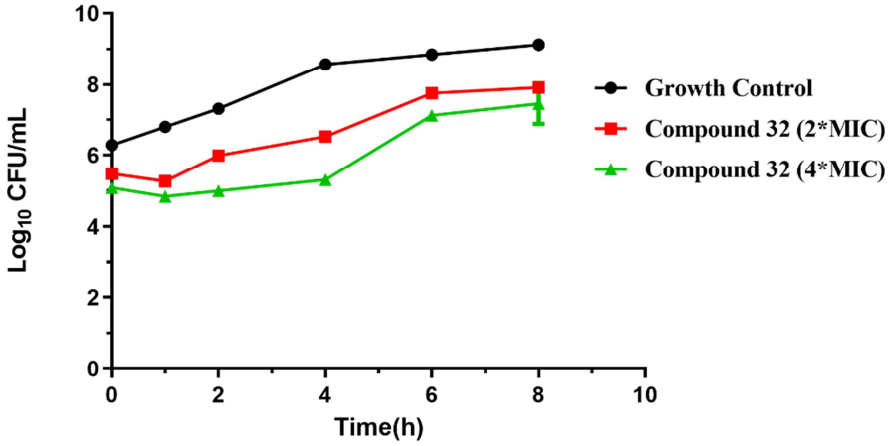

Log10 CFU/mL
Time(h)
Growth Control
Tiamulin (1*MIC)
Tiamulin (2*MIC)
Tiamulin (4*MIC)
Tiamulin (8*MIC)
Tiamulin (16*MIC)
Tiamulin (32*MIC)

Figure 2

a


Log10 CFU/mL
Time(h)
Growth Control
Compound 26 (2*MIC)
Compound 26 (4*MIC)

b


Log10 CFU/mL
Time(h)
Growth Control
Compound 32 (2*MIC)
Compound 32 (4*MIC)

c

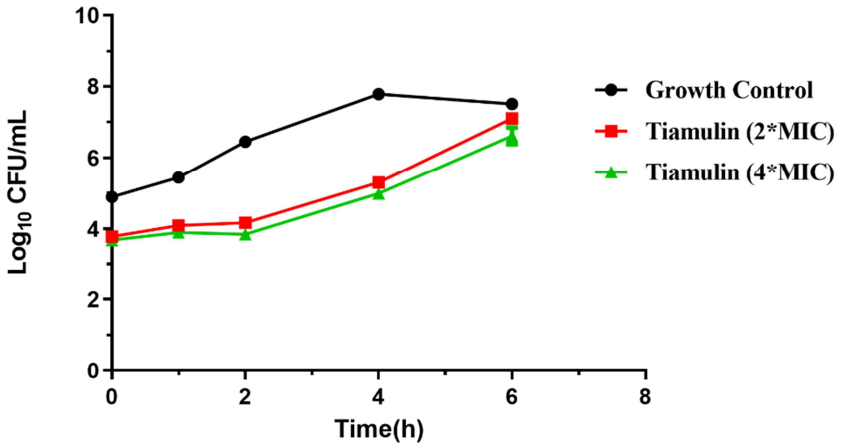

Log10 CFU/mL
Time(h)
Growth Control
Tiamulin (2*MIC)
Tiamulin (4*MIC)

Figure 3

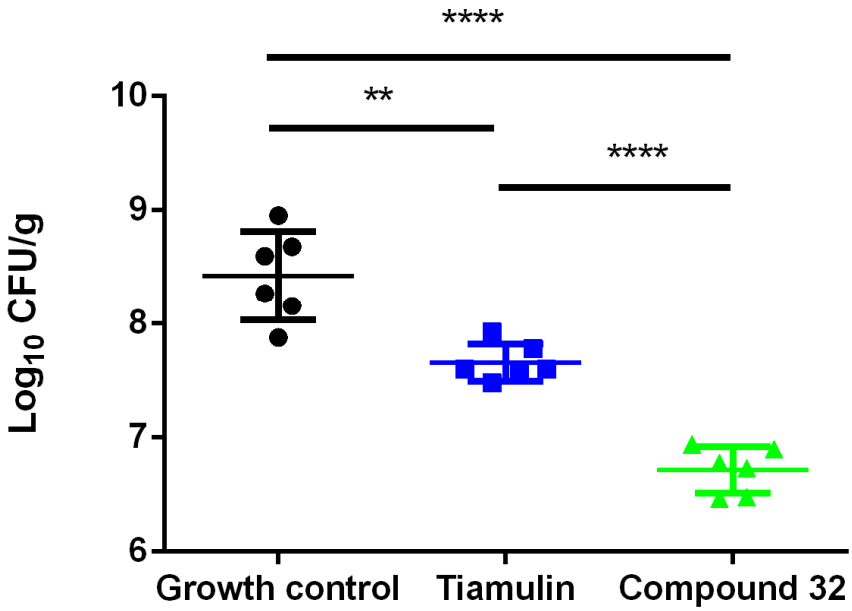


Figure 4

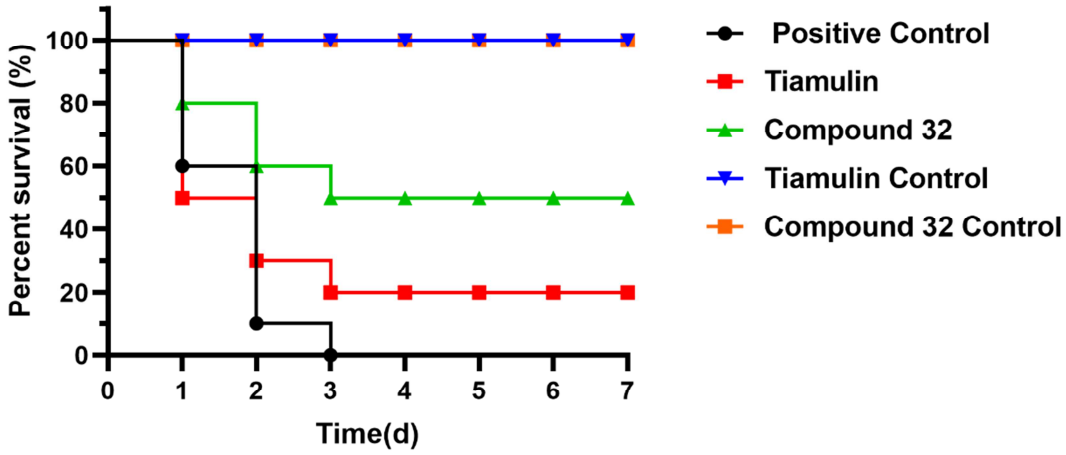
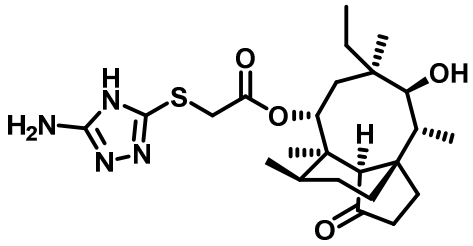


Figure 5

a



Azamulin

b

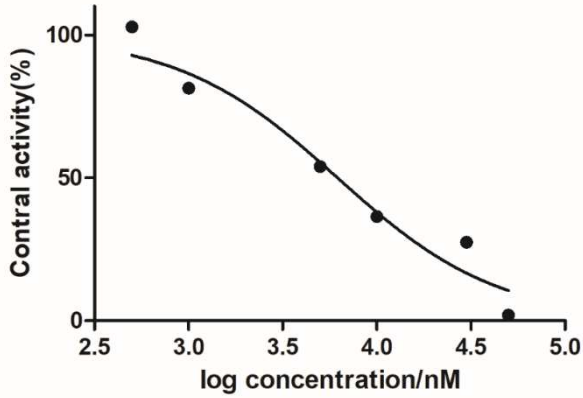


Figure 6

Design, synthesis and biological activities of novel pleuromutilin derivatives with a substituted triazole moiety as potent antibacterial agents

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Highlights

► Synthesis of novel pleuromutilin derivatives bearing both aminophenylthiol and 1,2,3-triazole moiety. ► Compound **32** exhibited superior *in vivo* efficacy to that of tiamulin against MRSA. ► Compound **32** possessed moderate *in vitro* inhibition of CYP3A4.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: