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Research paper

Design, synthesis and biological evaluation of pleuromutilin-Schiff base hybrids as potent anti-MRSA agents *in vitro* and *in vivo*

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ABSTRACT

A series of pleuromutilin derivatives with 1,2,4-triazole-3-substituted Schiff base structure were designed and synthesized under mild conditions. The *in vitro* antibacterial activities of the synthesized derivatives against 4 strains of *Staphylococcus aureus* (MRSA ATCC 43300, *S.aureus* ATCC 29213, *S.aureus* 144 and *S.aureus* AD3) and 1 strain of *E. coli* (ATCC 25922) were evaluated by the broth dilution method. Among these derivatives, compound **60** exhibited superior *in vitro* antibacterial effect against MRSA (MIC = 0.25 μg/mL) than tiamulin (MIC = 0.5 μg/mL), and compound **60** (−2.28 $\log_{10}$ CFU/mL) also displayed superior *in vivo* antibacterial efficacy than tiamulin (−1.40 $\log_{10}$ CFU/mL) in reducing MRSA load in the mouse thigh infection model. The time-kill study and the post-antibiotic effect study indicated that compound **60** showed a faster bactericidal kinetic and longer PAE time (exposure to 2 × MIC and 4 × MIC for 2 h, the PAE was 4.06 and 4.27 h) against MRSA compared with tiamulin (exposure to 2 × MIC and 4 × MIC for 2 h, the PAE was 1.72 and 2.14 h). Meanwhile, most of these compounds had no significant inhibitory effect on RAW 264.7 cells and HepG2 cells at the concentration of 4 μg/mL.

Additionally, the development of resistance study showed that MRSA did not easily develop resistance against compound **60** compared with tiamulin after induction for 8 passages.

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1. Introduction

Staphylococcus aureus is an important bacterium because it can cause a wide range of diseases [1]. Penicillin and its derivatives, including methicillin have been used to treat infections caused by *S. aureus*. However, certain strains of *S. aureus* generated resistance were called methicillin-resistant *S. aureus* (MRSA) [2]. MRSA infections may develop more serious diseases, such as pneumonia, endocarditis and osteomyelitis, etc. [3,4]. MRSA strains have been reported resistant to many clinical antibiotics, such as linezolid, vancomycin and daptomycin [5]. MRSA infections cause a serious threat to public health security around the world [6]. Thus, it is urgent to develop new antibiotics agents with novel modes of action to treat infections caused by MRSA.

Pleuromutilin (1, Fig. 1), a tricyclic diterpenoid natural product, was first isolated from two basidiomycetes species (*Pleurotus mutilus* and *Pleurotus passeckerianus*) by Kavanagh et al., in 1951. It exhibited potent antibacterial activity against Gram-positive bacteria [7,8]. Pleuromutilin selectively interacts with the peptidyl transferase center of the 50S bacterial ribosome subunit and interferes with the translation process of bacterial protein to achieve antibacterial activity [9]. This unique antibacterial mechanism has caused pleuromutilin to receive extensive attention from researchers.

Tiamulin (**2**, Fig. 1) and valnemulin (**3**, Fig. 1) were approved for use as veterinary drugs in 1979 and 1999, respectively [10]. In 2007, retapamulin (4, Fig. 1) was approved by the Food and Drug Administration (FDA) as the first pleuromutilin derivative antibiotic for the treatment of topical skin infection in humans [11]. Subsequently, in 2019, lefamulin (Fig. 5) was approved by the FDA for intravenous and oral treatment of community-acquired bacterial pneumonia (CABP), it was the first systemic pleuromutilin antibiotic in humans [12]. Unfortunately, azamulin (6, Fig. 1) has excellent antibacterial potency, but development was terminated

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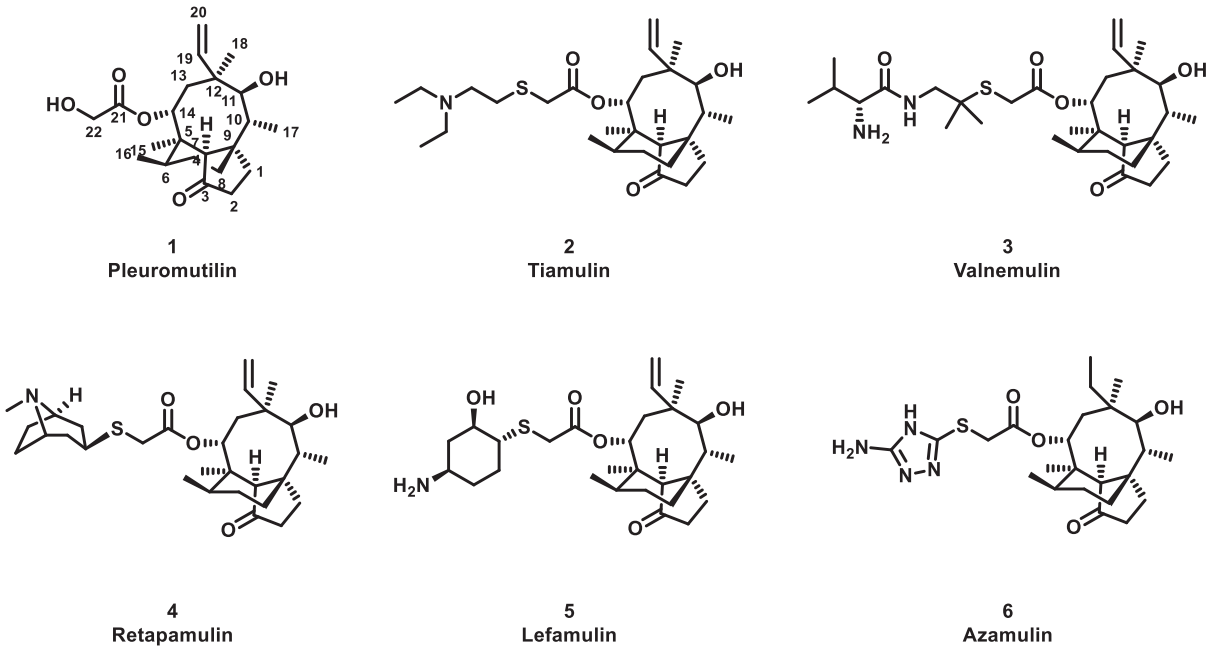


Fig. 1. Structure of pleuromutilin (***1***), tiamulin (***2***), valnemulin (***3***), retapamulin (***4***), lefamulin (***5***) and azamulin (***6***).

after Phase I trials for the low oral bioavailability [13]. In our previous work, a variety of pleuromutilin derivatives were designed and synthesized. These derivatives contain 2-aminophenylthiol, piperazine ring and 1,2,3-triazole structures as linkers, respectively. Some of these pleuromutilin derivatives showed potent antimicrobial activity [14–16].

Compounds containing 1,2,4-triazoles structures have broad-spectrum activities such as antifungal, antibacterial, antitumor and antiparasitic activities, and have potential applications in the field of pharmaceutical chemistry [17]. In recent years, 1,2,4-triazole Schiff bases were found with good pharmacological activity [18,19].

This background inspired us to develop novel pleuromutilin derivatives containing 1,2,4-triazole Schiff bases. In this study, 25 pleuromutilin derivatives containing 1,2,4-triazole-3-substituted Schiff base structure were designed and synthesized. We preliminary evaluated their antibacterial activities *in vitro* and *in vivo*.

2. Results and discussion

2.1. Chemistry

The general synthetic methods for all target pleuromutilin derivatives were illustrated in Scheme 1. Compound **8** was synthesized by compound **7** and CS_2 under heating conditions according to reference [20]. Compound **8** was converted into compound **9** through a cyclization reaction under acidic conditions. Compounds **10**–**34** were obtained by nucleophilic substitution of the amino group in compound **9** and the aldehyde group in a variety of benzaldehyde derivatives. Compound **35** was synthesized from compound **1** and *p*-toluenesulfonyl chloride under alkaline conditions. The iodinated compound **35** and compound **10**–**34** were transformed by nucleophilic substitution reaction to gain the target pleuromutilin derivatives (compound **36**–**60**).

To get the target pleuromutilin derivatives, all those crude products were purified by silica column chromatography. The structures of 25 compounds were characterized by ^{1}H NMR, ^{13}C NMR, HR-MS (ESI) analysis, and the results confirmed that the synthesis of compounds were consistent with the expected structure.

2.2. In vitro antibacterial activity

The MIC and MBC values of those pleuromutilin derivatives were tested to evaluate theirs *in vitro* antibacterial activity. The results were shown in Table 1.

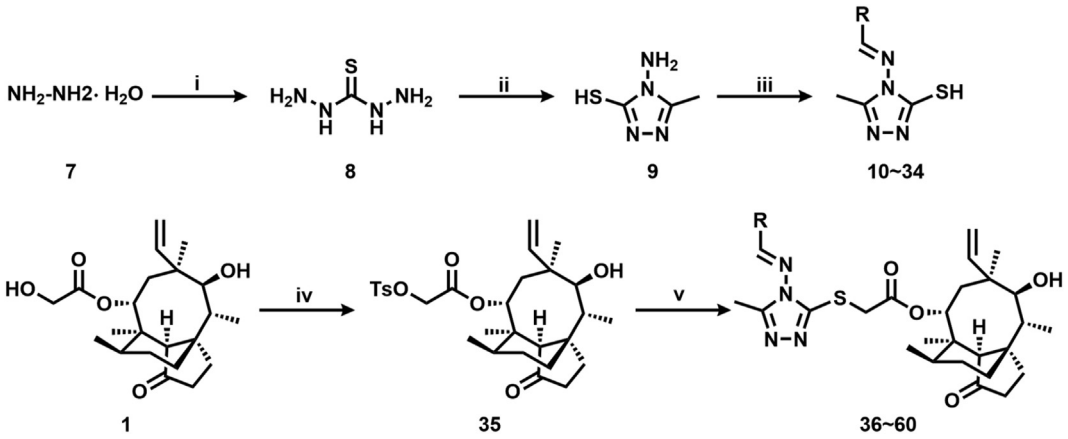
The MIC values of those new pleuromutilin derivatives *in vitro* against the above Gram-positive bacteria ranged from 0.25 to 4 μg/mL. The MICs of the 25 derivatives against *E. coli* (ATCC 25922) were higher than 64 μg/mL. Most of the derivatives exhibited potent antibacterial activities against MRSA. Compounds **48** and **60** (MIC = 0.25 μg/mL) showed the highest antibacterial activity against MRSA among these compounds.

25 pleuromutilin derivatives with 1,2,4-triazole-3-substituted Schiff base structure, the substituent groups include electron-donating groups (methyl, hydroxyl, etc.) and electron-withdrawing groups (fluorine, chlorine, trifluoromethyl, nitro, etc.). Compound **48** containing the hydroxyl group and compound **60** containing the pyridyl group were the most antibacterial activity against MRSA compounds in this series. The results showed that the activity of the compounds containing the electron-donating group may be slightly higher than that containing the electron-withdrawing group, which was consistent with previous research [21].

The ratios of MBC to MIC of the target pleuromutilin derivatives ranged from 1 to 4 indicated that these compounds have excellent bactericidal ability [22]. The results indicated that 25 pleuromutilin derivatives have good bactericidal ability against *S. aureus*. Among these derivatives, compounds **48** and **60** were exhibited a greater bactericidal effect than tiamulin. Therefore, we conducted an in-depth study on the antibacterial activity of these two compounds.

The *in vitro* antibacterial activity of compounds **48** and **60** were tested by the time-kill kinetic approach. The results were shown in Fig. 2.

In a concentration of 1 × MIC, compounds **48** and **60** had a significant inhibitory effect on MRSA. After exposure for 24 h,



Scheme 1. Reagent and conditions: (i) H_2O, CS_2, 90 °C, 1 h (ii) CH_3COOH, 118 °C, 4 h; (iii) absolute ethyl alcohol, Benzaldehyde derivatives, H_2SO_4, reflux, 3 h; (iv) acetonitrile, *p*-toluenesulfonyl chloride, NaOH, rt, 3 h; (v) a). acetonitrile, NaI, 70 °C, 1.5 h; b). acetonitrile, K_2CO_3, compound ***10*–*34***, 70 °C, 2 h.

compounds **48** and **60** showed the bactericidal effect on MRSA and killed 99.9% of MRSA at 4 × MIC and 2 × MIC concentrations, respectively. Especially, compound **60** showed a faster bactericidal kinetic against MRSA compared with tiamulin. However, after the two compounds and tiamulin reach a certain concentration, the bactericidal effect did not have significantly increased. The results indicated that compounds **48**, **60** and tiamulin were time-dependent rather than the concentration-dependent antibacterial agent. In clinical practice, multiple or continuous intravenous administration of time-dependent antibacterial drugs may achieve better therapeutic effects [23].

To evaluate the *in vitro* antibacterial pharmacodynamic activity of compounds **48** and **60**, we conducted the PAE of the above two compounds. PAE refers to bacteria exposed to antibiotics some time, after elimination of the drug, effects sustained by bacterial growth inhibition [24]. The PAE results were shown in Table 2 and the bacterial growth kinetics curve was exhibited in Fig. 3.

After compound **48** was treated with 2 × MIC and 4 × MIC concentration for 2 h, the PAE was 2.55 and 3.22 h, respectively. After exposure at the same concentrations for 2 h, the PAE of compound **60** was 4.06 and 4.27 h, respectively. These results indicated that compounds **48** and **60** exhibited a longer PAE than tiamulin.

As the drug concentration drops below the MIC, it still has a certain antibacterial effect. Therefore, in clinical medication, combined PAE with MIC, MBC and other pharmacodynamic parameters could comprehensively evaluate the efficacy of antibacterial drugs. Besides, the PAE value can be used as a reference for the design of clinical dosing regimen, and provide a theoretical basis for the adjustment of medication interval, which is of great significance for reducing the occurrence of adverse drug reactions [25].

The results of the above experiment showed that the administration interval of compounds **48** and **60** may be longer than that of tiamulin.

2.3. Neutropenic murine thigh infection model

Since compound **60** exhibited superior antibacterial activity *in vitro*, the *in vivo* antibacterial activity of compound **60** was tested by using the neutropenic murine thigh infection model experiment. The results were shown in Fig. 4.

From Fig. 4, tiamulin at 20 mg/kg could reduce the MRSA load (-1.40 $\log_{10}$ CFU/mL) obviously in mice thighs compared to the no-drug control group ($P < 0.0001$, $n = 6$/group). Whereas, at the same dose, compound **60** could reduce the bacterial load (-2.28 $\log_{10}$ CFU/mL) in thighs, compared with the no-drug control group ($P < 0.0001$, $n = 6$/group). Compound **60** exhibited a significant treatment effect against MRSA in thighs compared to the tiamulin group ($P < 0.0001$, $n = 6$/group). The result showed that the antibacterial activity of compound **60** against MRSA *in vivo* was higher than that of tiamulin.

2.4. Cytotoxicity assay

The cytotoxicity of the 25 derivatives were evaluated using one non-cancer cell line RAW 264.7 cells and one cancer cell line HepG2 cells by MTT assay. The results were shown in Figs. 5 and 6.

The results indicated that most of these compounds had no significant inhibitory effect on RAW 264.7 cells and HepG2 cells at the concentration of 4 μg/mL, which was an acceptable concentration. The only exception was compound **39** that reduced RAW 264.7 cells viability by more than 30%.

2.5. Development of resistance

To evaluate if any resistance can be easily induced by those compounds, the bacterial resistance against compound **60** and tiamulin was studied by investigating the resistance development rates of MRSA. The results were shown in Fig. 7.

After induction by sub-MIC concentrations for 8 passages, compound **60** also showed good anti-MRSA activity, with the MIC value reaches 2 μg/mL, which was 8 times the initial MIC value (0.25 μg/mL). However, the MRSA gradually developed resistance to tiamulin (MIC = 32 μg/mL) which lose 64-fold potency compared to the initial MIC value (0.5 μg/mL) after 8 passages. This assay showed that the MRSA did not develop significant resistance against compound **60** as easily as it did against the tiamulin. Therefore, compound **60** has the potential to overcome the resistance to tiamulin.

3. Conclusions

The antibacterial activity of a series of novel pleuromutilin derivatives against MRSA were studied *in vitro* and *in vivo*. The results of the MIC and MBC revealed that compounds **48** and **60** exhibited superior *in vitro* antibacterial activity against *S. aureus* and MRSA than tiamulin. The time-kill study and PAE study indicated that compound **60** showed a faster bactericidal kinetic and longer PAE time for MRSA compared with tiamulin. Meanwhile, compound **60**

Table 1
MIC and MBC (μg/mL) values of compounds **36**–**60**, pleuromutilin and tiamulin against MRSA (ATCC 43300), *S. aureus* (ATCC 29213), *S. aureus* (AD3), *S. aureus* (144) and *E. coli* (ATCC 25922).

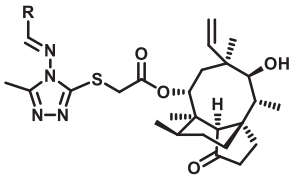
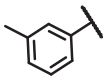
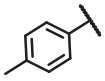
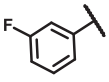
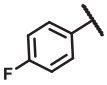
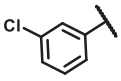
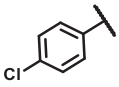
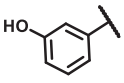
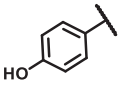
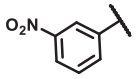
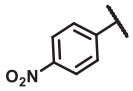
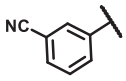
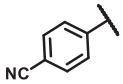
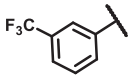
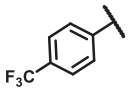
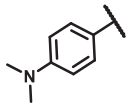
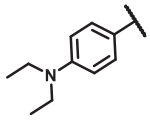
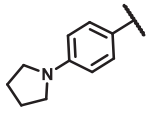
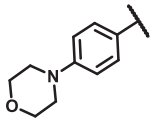
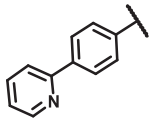
Compound No	R	MIC/MBC(μg/mL)				
		MRSA ATCC 43300	*S.aureus* ATCC 29213	*S.aureus* AD3	*S.aureus* 144	*E. coli* ATCC 25922
						
36		0.5/1	1/2	1/4	2/4	>64/>64
37		1/2	1/2	1/4	2/4	>64/>64
38		1/2	1/2	1/4	2/4	>64/>64
39		0.5/1	1/2	1/4	2/4	>64/>64
40		0.5/1	1/2	1/4	2/4	>64/>64
41		0.5/1	1/4	1/4	2/4	>64/>64
42		1/2	1/4	1/4	2/4	>64/>64
43		1/2	1/2	1/4	2/4	>64/>64
44		1/2	1/4	2/4	2/4	>64/>64
45		0.5/2	1/2	1/4	2/4	>64/>64
46		0.5/1	1/2	1/4	2/4	>64/>64
47		0.5/1	0.5/2	1/4	2/4	>64/>64
48		0.25/1	0.5/2	0.5/2	1/2	>64/>64
49		0.5/1	1/4	2/8	2/8	>64/>64

Table 1 (*continued*)

Compound No	R	MIC/MBC(μg/mL)				
		MRSA ATCC 43300	*S.aureus* ATCC 29213	*S.aureus* AD3	*S.aureus* 144	*E. coli* ATCC 25922
50		1/2	2/8	2/8	2/4	>64/>64
51		1/2	2/4	2/8	2/8	>64/>64
52		1/2	1/4	2/8	2/4	>64/>64
53		1/4	1/4	2/4	2/4	>64/>64
54		2/4	2/4	2/4	4/4	>64/>64
55		2/4	2/4	2/8	4/8	>64/>64
56		0.5/1	0.5/2	1/4	2/4	>64/>64
57		4/4	4/8	4/16	4/8	>64/>64
58		1/2	2/4	2/4	2/4	>64/>64
59		0.5/1	1/2	1/4	2/4	>64/>64
60		0.25/1	0.5/1	0.5/2	1/4	>64/>64
Pleuromulin		2/4	2/4	2/4	2/4	>64/>64
Tiamulin		0.5/1	1/1	1/2	1/2	>64/>64

was found to display a potent *in vivo* antibacterial effect in the neutropenic murine thigh infection model study. In the cytotoxicity assay study, most of these compounds had no significant inhibitory effect on RAW 264.7 cells and HepG2 cells at the concentration of 4 μg/mL. The development of resistance study showed that MRSA did not easily develop resistance against compound **60** compared with tiamulin. According to the current experimental results, compound **60** was worthy of further development as a potential drug against MRSA infection.

4. Experimental

4.1. Materials

The raw material pleuromutilin (>90% pure) was purchased from Great Enjoyhood Biochemical Co. Ltd., (Daying, China). The benzaldehyde derivatives were purchased from Bide Technology Co. Ltd., (Shanghai, China). The other general reagents were all of the analytical grades and obtained from Guangzhou Chemical Reagent Factory (Guangzhou, China). Purification of the target

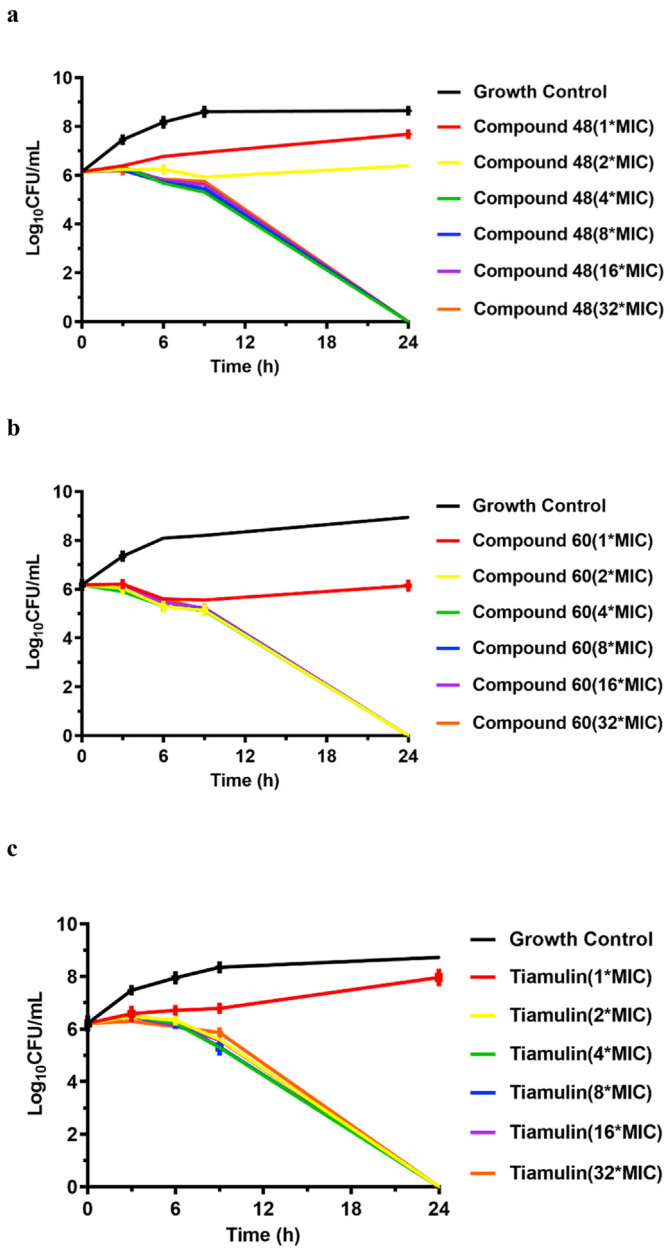


Fig. 2. Time-kill curves for MRSA ATCC 43300 with different concentrations of compounds ***48*** (a), ***60*** (b) and tiamulin (c).

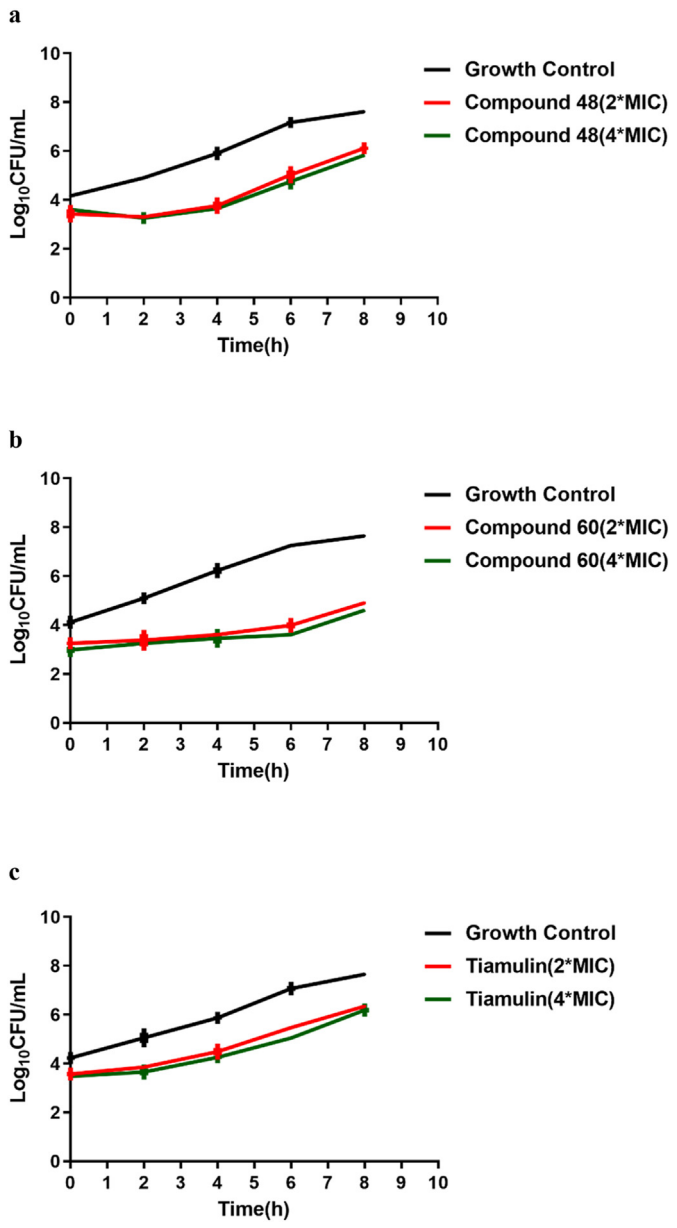


Fig. 3. The bacterial growth kinetic curves for MRSA ATCC 43300 exposed to compound ***48*** (a), compound ***60*** (b) and tiamulin (c) for 2 h.

Table 2
The PAEs values of compounds **48**, **60** and tiamulin against MRSA ATCC 43300.

Compounds	Concentrations	PAE (h)	
		Exposure for 0 h[a]	Exposure for 2 h
Compound **48**	2 × MIC	2.51	2.55
	4 × MIC		3.22
Compound **60**	2 × MIC	2.04	4.06
	4 × MIC		4.27
Tiamulin	2 × MIC	2.44	1.72
	4 × MIC		2.14

[a] Exposure for 0 h is the time required for the bacteria in the control groups to increase by 1 $\log_{10}$ CFU/mL.

compounds by column chromatography was carried out using silica gel (200–300 mesh, Branch of Qingdao Haiyang Chemical Co. Ltd.,

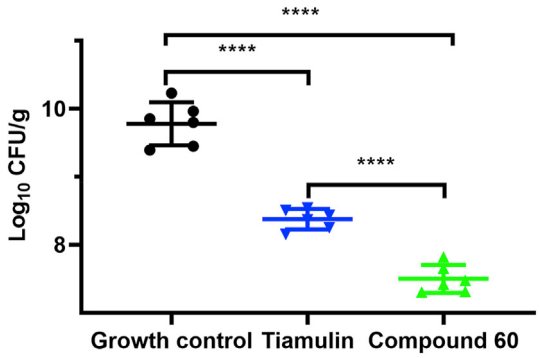


Fig. 4. Efficacy of tiamulin and compound ***60*** against MRSA ATCC 43300 in murine neutropenic thigh models: circular: growth control; inverted triangle: tiamulin (20 mg/kg); regular triangle: compound ***60*** (20 mg/kg).

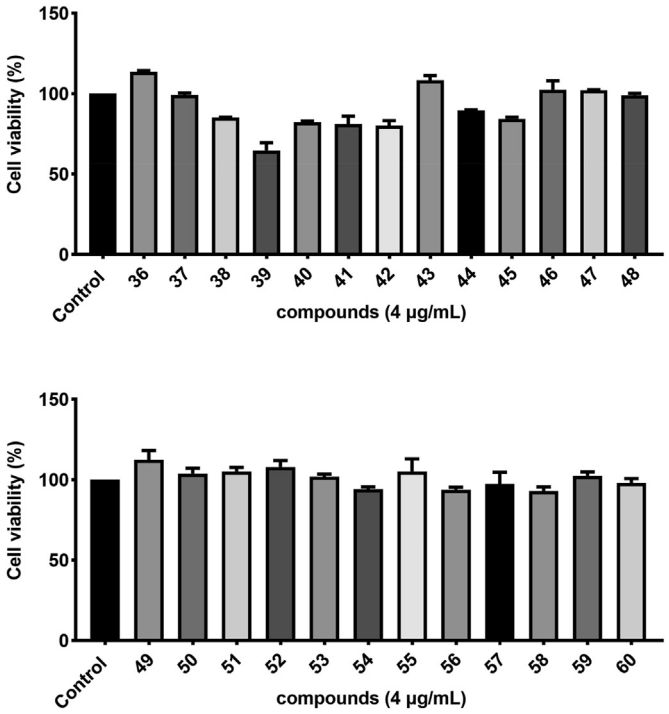


Fig. 5. The cytotoxicity assay of 25 pleuromutilin derivatives to RAW 264.7 cells at the concentration of 4 μg/mL.

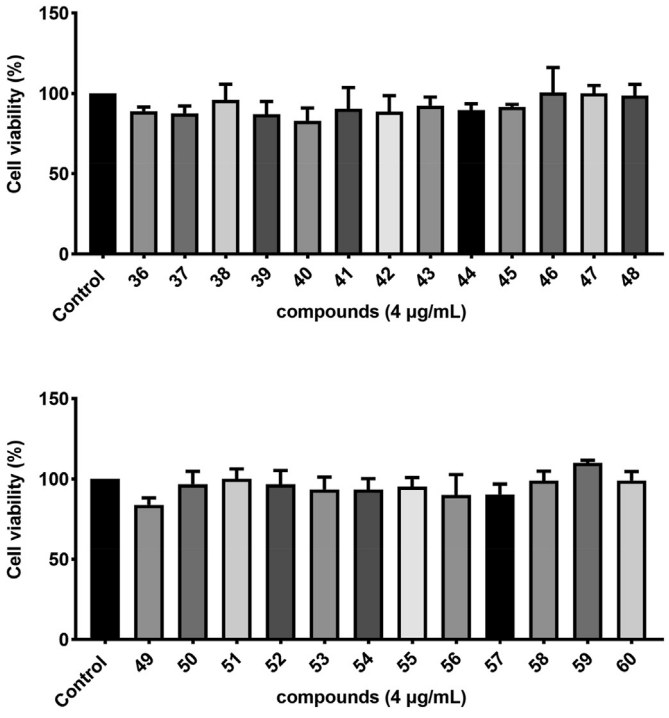


Fig. 6. The cytotoxicity assay of 25 pleuromutilin derivatives to HepG2 cells at the concentration of 4 μg/mL.

Shandong, China). 1H NMR and 13C NMR spectra were measured on Bruker AV-400 or Bruker AV-600 spectrometer in CDCl3. The chemical shift values (δ) are reported in ppm relative to tetramethylsilane as an internal standard. High-resolution mass spectra were recorded by Waters Acquity UPLC-LCT Premier XE with an

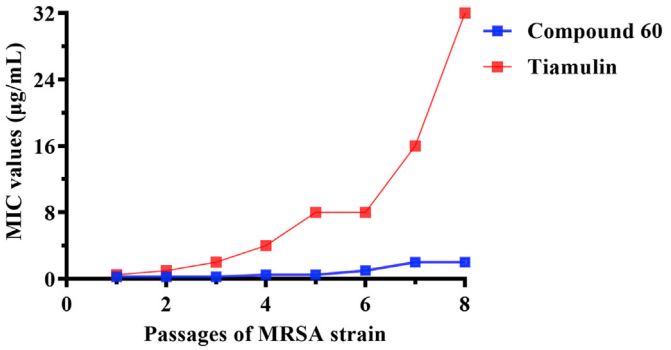


Fig. 7. Development of MRSA ATCC 43300 resistance to compound ***60*** and tiamulin, each MIC value of every passage was an average of three parallels.

electrospray ionization (ESI) source.

4.2. Synthesis

A general synthetic route based on the compound 22-O-tosylpleuromutilin (compound **35**) and a variety of 4-amino-5-methyl-1,2,4-triazole-3-substituted Schiff base derivatives (compound **10**—**34**) were used to prepare a series of pleuromutilin derivatives.

4.2.1. Synthesis of 1,3-Diamino-2-Thiourea (8)

Compound **7** (20 mL, 400 mmol) was dissolved in water (60 mL). Then CS_2 (6 mL, 100 mmol) was added to the solution and stirred at 90 °C for 1 h until the reaction was completed. The reaction mixture was cooled to room temperature. The solid products were precipitated from the reaction solution. Then the above products were collected by vacuum filtration. The crude products were recrystallized with water to afford compound **8** (white solid, yield 83.7%).

4.2.2. Synthesis of 4-amino-5-methyl-1,2,4-triazole-3-thiol (9)

Compound **8** (3 g, 28.2 mmol) was dissolved in acetic acid (10 mL, 17.5 mmol). The reaction was refluxed for 4 h. After compound **8** was completely consumed, the reaction mixture was cooled to room temperature. Then the unreacted acetic acid was removed by vacuum distillation. The solids were precipitated from the reaction solution and collected by vacuum filtration. The crude products were purified by recrystallization with water to obtain compound **9** (white solid, yield 72.5%).

4.2.3. General procedure for the synthesis of 4-amino-5-methyl-1,2,4-triazole-3-substituted Schiff base derivatives (compounds 10—34)

Compound **9** (1 g, 7.7 mmol) was added to anhydrous ethanol (30 mL). The reaction mixture was heated and stirred until completely dissolved. Then Benzaldehyde derivatives (0.78 mL, 7.7 mmol) and concentrated sulfuric acid (1 mL) were slowly added to the reaction solution. The reaction mixture was stirred for 4 h at 78 °C until compound **9** completely disappeared. The reaction mixture was cooled to room temperature. Then a large amount of solid was precipitated from the reaction solution, the solid was collected by vacuum filtration. The crude products were purified by recrystallization with ethyl alcohol to afford compound **10**—**34** in 35%—78% yield.

4.2.4. 22-O-tosylpleuromutilin (35)

Compound **35** (22-O-tosylpleuromutilin) was synthesized according to a reported method [15]. Pleuromutilin **1** (5.0 g, 13.21 mmol) and *p*-toluenesulfonyl chloride (2.7 g, 14.53 mmol) were dissolved in dichloromethane (15.0 mL) under an ice bath.

Then sodium hydroxide granules (1.5 g, 26.42 mmol) were dissolved in water (10 mL) and dropped slowly into the above mixture solution. The mixture was stirred at room temperature until the pleuromutilin was completely consumed. The solution was extracted twice with $CHCl_3$ (30 mL). Then the organic phases were combined and washed three times with saturated brine. The organic phases were collected and dried with dried over anhydrous sodium sulfate and evaporation under reduced pressure. The crude product was purified by recrystallization with isopropanol to obtain a white solid powder in 85.7% yield.

4.2.5. General procedure for the synthesis of target pleuromutilin derivatives (36–60)

Compound **6** (1 g, 1.87 mmol) was dissolved in acetonitrile (10 mL). The sodium iodide (0.31 g, 2.07 mmol) was added into the mixture solution and stirred at 70 °C for 1 h. After the compound **6** reaction was completed. Then compounds **10**–**34** (0.38 g, 2.07 mmol) and potassium carbonate (0.52 g, 3.76 mmol) were added to the above reaction. Stirring was continued for 2 h at 70 °C. After completion of the reaction, the ice water (50 mL) and $CHCl_3$ (50 mL) were added to the solution. The organic phase was washed with brine three times. Then the organic solvent was dried (Na_2SO_4) and concentrated under reduced pressure to give the crude product. The above crude product was purified by silica gel column chromatography (dichloromethane: methanol = 40: 1 v/v) to obtain the pure compounds **36**–**60** in 61%–83% yield.

4.2.6. 22-[(4-(benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl) thio]deoxy pleuromutilin (36)

White powder; yield: 68%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.60 (1H, s), 7.90–7.86 (2 H, m), 7.62–7.58 (1 H, m), 7.55–7.50 (2 H, m), 6.42 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.30 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.11–3.98 (2H, m, H22), 3.35 (1H, dd, J = 10.0, 6.4Hz, H11), 2.51 (3H, s), 2.33–2.22 (2 H, m, H2), 2.21–2.16 (1H, m, H10), 2.09 (1 H, d, J = 2.8Hz, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.77 (2 H, s, H6, H13), 1.65 (2 H, dtd, J = 11.1, 9.3, 5.3Hz, H1), 1.51 (2 H, dd, J = 12.9, 3.3Hz, H7), 1.48–1.43 (1 H, m, H4), 1.42 (3 H, s, H15), 1.37–1.32 (2 H, m, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.81 (C3), 166.88 (C21), 162.50, 150.97, 144.71, 138.85 (C19), 133.02, 131.76, 129.15, 128.94, 117.20 (C20), 99.99, 74.61 (C11), 70.52 (C14), 58.08 (C4), 52.53, 45.42 (C9), 44.52 (C13), 44.02 (C12), 41.85 (C5), 41.76, 36.67 (C6), 36.02 (C10), 34.43 (C2), 30.40 (C8), 26.83 (C7), 26.42 (C18), 24.82 (C1), 16.73 (C16), 14.76 (C15), 11.43 (C17), 11.20. HR-MS(ESI$^+$): Calcd for $C_{32}H_{42}N_4O_4S$ (M+H$^+$): 579.3000; Found: 579.2989.

4.2.7. 22-[(4-(2-methyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (37)

White powder; yield: 71%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.90 (1H, d, J = 0.5Hz), 8.00–7.98 (1 H, m), 7.46 (1 H, td, J = 7.5, 1.4Hz), 7.36–7.29 (2 H, m), 6.41 (1 H, dd, J = 17.5, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.31–5.27 (1 H, m, H20), 5.18 (1H, dd, J = 17.4, 1.5Hz, H20), 4.12–3.97 (2H, m, H22), 3.35 (1H, dd, J = 10.1, 6.5Hz, H11), 2.58 (3H, s), 2.52 (3 H, s), 2.32–2.28 (1 H, m, H2), 2.27–2.22 (1H, m, H2), 2.18 (1H, dd, J = 19.4, 9.4Hz, H10), 2.09 (1 H, d, J = 2.8Hz, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.80 (2 H, s, H7), 1.75 (1 H, dq, J = 14.6, 3.2Hz, H6), 1.65 (2 H, dtd, J = 11.1, 9.0, 5.3Hz, H1), 1.54–1.49 (1 H, m, H13), 1.48–1.42 (1 H, m, H4), 1.41 (3 H, s, H15), 1.37–1.31 (2 H, m, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.81 (C3), 166.80 (C21), 161.57, 151.07, 144.32, 139.41 (C19), 138.85, 132.64, 131.42, 129.97, 128.23, 126.62, 117.18 (C20), 74.61 (C11), 70.51 (C14), 58.09 (C4), 45.42 (C9), 44.53 (C13), 44.01 (C12), 41.85 (C5), 36.67 (C6), 36.07, 36.02 (C10), 34.43 (C2), 30.39 (C8), 26.82 (C7), 26.43 (C18), 24.82 (C1), 19.85, 16.72 (C16), 14.76 (C15), 11.42 (C17), 11.18. HR-MS(ESI$^+$): Calcd for $C_{33}H_{44}N_4O_4S$ (M+H$^+$): 593.3156; Found: 593.3146.

4.2.8. 22-[(4-(3-methyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (38)

White powder; yield: 62%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.55 (1H, s), 7.73–7.62 (2 H, m), 7.48–7.35 (2 H, m), 6.42 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5, H14), 5.30 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.11–3.96 (2H, m, H22), 3.35 (1H, dd, J = 10.3, 6.5Hz, H11), 2.51 (3 H, s), 2.45 (3 H, d, J = 0.9Hz), 2.33–2.15 (3 H, m, H2, H10), 2.09 (1H, d, J = 2.7Hz, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6, H13), 1.79 (2 H, s, H7), 1.65 (2 H, dtd, J = 11.1, 9.1, 5.3Hz, H1), 1.55–1.42 (3 H, m, H4, H6, H13), 1.42 (3 H, s, H15), 1.38–1.32 (2 H, m, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.81 (C3), 166.89 (C21), 163.03, 150.89, 144.72, 139.07 (C19), 138.86, 133.91, 131.66, 129.27, 129.03, 126.37, 117.19 (C20), 74.61 (C11), 70.50 (C14), 58.09 (C4), 45.42 (C9), 44.52 (C13), 44.02 (C12), 41.85 (C5), 36.68 (C6), 36.00 (C10), 34.43 (C2), 30.40 (C8), 26.83 (C7), 26.43 (C18), 24.82 (C1), 21.28, 16.73 (C16), 14.76 (C15), 11.43 (C17), 11.18. HR-MS(ESI$^+$): Calcd for $C_{33}H_{44}N_4O_4S$ (M+H$^+$): 593.3156; Found: 593.3145.

4.2.9. 22-[(4-(4-methyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (39)

White powder; yield: 59%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.54 (1H, s), 7.81–7.66 (2 H, m), 7.34–7.31 (2 H, m), 6.41 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.30 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.4, 1.5Hz, H20), 4.12–3.93 (2H, m, H22), 3.35 (1H, dd, J = 10.4, 6.5Hz, H11), 2.50 (3H, s), 2.46 (3 H, s), 2.33–2.15 (3 H, m, H2, H10), 2.11–2.08 (1H, m, 11-OH), 2.03 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.78 (2 H, s, H7), 1.65 (2 H, dtd, J = 11.0, 9.0, 5.3Hz, H1), 1.54–1.49 (2 H, m, H6, H13), 1.45 (1 H, ddd, J = 13.4, 9.6, 2.7Hz, H4), 1.41 (3 H, s, H15), 1.38–1.31 (2 H, m, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.82 (C3), 166.90 (C21), 162.95, 150.83, 144.73, 144.00, 138.86 (C19), 129.89, 129.05, 128.97, 117.19 (C20), 74.61 (C11), 70.48 (C14), 58.09 (C4), 45.42 (C9), 45.01, 44.52 (C13), 44.02 (C12), 41.85 (C5), 36.68, 36.54 (C6), 36.01 (C10), 35.95, 34.43 (C2), 30.40 (C8), 26.83 (C7), 26.42 (C18), 24.82 (C1), 21.77, 16.73 (C16), 14.76 (C15), 11.43 (C17), 11.16. HR-MS(ESI$^+$): Calcd for $C_{33}H_{44}N_4O_4S$ (M+H$^+$): 593.3156; Found: 593.3146.

4.2.10. 22-[(4-(2-fluoro-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (40)

White powder; yield: 67%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.89 (1H, d, J 0.6), 8.21–7.98 (1 H, m), 7.58 (1 H, dddd, J = 8.4, 7.3, 5.4, 1.8Hz), 7.30 (1 H, dddd, J = 7.9, 7.4, 1.1, 0.7Hz), 7.21 (1 H, ddd, J = 10.3, 8.4, 1.0Hz), 6.41 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.30 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.4, 1.5Hz, H20), 4.15–3.91 (2H, m, H22), 3.35 (1H, dd, J = 10.2, 6.5Hz, H11), 2.53 (3H, s), 2.33–2.14 (3 H, m, H2, H10), 2.11–2.08 (1H, m, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.78 (2 H, s, H7), 1.65 (2 H, tdd, J = 11.1, 8.6, 5.3Hz, H1), 1.52 (2 H, dd, J = 14.8, 10.5Hz, H6, H13), 1.45 (1 H, ddd, J = 13.3, 9.5, 2.6Hz, H4), 1.42 (3 H, s, H15), 1.37–1.33 (2 H, m, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.82 (C3), 166.76 (C21), 155.00, 154.96, 150.87, 145.08, 138.84 (C19), 134.79, 127.49, 124.90, 117.19 (C20), 116.37, 116.24, 74.61 (C11), 70.52 (C14), 58.09 (C4), 45.42 (C9), 44.52 (C13), 44.01 (C12), 41.85 (C5), 36.69 (C6), 36.01 (C10), 35.91, 34.43 (C2), 30.40 (C8), 26.82 (C7), 26.42 (C18), 24.82 (C1), 16.71 (C16), 14.74 (C15), 11.42 (C17), 11.33. HR-MS (ESI$^+$): Calcd for $C_{32}H_{41}FN_4O_4S$ (M+H$^+$): 597.2906; Found:

597.2896.

4.2.11. 22-[(4-(3-fluoro-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (41)

White powder; yield: 73%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.64 (1H, d, J = 1.0Hz), 7.64 (1 H, ddd, J = 9.1, 2.6, 1.5Hz), 7.61 (1 H, dt, J = 7.6, 1.2Hz), 7.51 (1 H, td, J = 8.0, 5.4Hz), 7.31−7.28 (1 H, m), 6.41 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.29 (1 H, dd, J = 11.0, 1.4Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.14−3.97 (2H, m, H22), 3.35 (1H, dd, J = 10.4, 6.5Hz, H11), 2.52 (3 H, d, J = 0.7Hz), 2.31−2.16 (3 H, m, H2, H10), 2.12−2.07 (1H, m, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.7Hz, H13), 1.76 (2 H, s, H7), 1.65 (2 H, tdd, J = 12.6, 7.1, 3.4Hz, H1), 1.54−1.43 (3 H, m, H4, H6, H13), 1.42 (3 H, s, H15), 1.37−1.32 (2 H, m, H8), 1.16 (3 H, s, H18), 0.88 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.76 (C3), 166.85 (C21), 160.29, 160.27, 151.19, 144.65, 138.86 (C19), 130.86, 125.31, 120.06, 119.92, 117.18 (C20), 114.76, 74.60 (C11), 70.62 (C14), 58.07 (C4), 45.41 (C9), 44.53 (C13), 44.02 (C12), 41.84 (C5), 36.66 (C6), 36.19, 36.02 (C10), 34.42 (C2), 30.39 (C8), 26.83 (C7), 26.42 (C18), 24.81 (C1), 16.73 (C16), 14.75 (C15), 11.42 (C17), 11.23. HR-MS (ESI$^+$): Calcd for $C_{32}H_{41}FN_4O_4S$ (M+H$^+$): 597.2906; Found: 597.2893.

4.2.12. 22-[(4-(4-fluoro-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (42)

White powder; yield: 66%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.59 (1H, s), 7.92−7.84 (2 H, m), 7.25−7.17 (2 H, m), 6.41 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.72 (1 H, d, J = 8.5Hz, H14), 5.29 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.11−3.96 (2H, m, H22), 3.35 (1H, dd, J = 10.0, 6.4Hz, H11), 2.50 (3H, s), 2.34−1.96 (5 H, m, H2, H4, H10, 11-OH), 1.80 (1H, s, H7), 1.75 (1 H, dq, J = 14.5, 3.1Hz, H6), 1.65 (2 H, tdd, J = 10.8, 8.2, 5.2Hz, H1), 1.54−1.48 (2 H, m, H7, H13), 1.45 (1 H, ddd, J = 13.4, 9.7, 2.8Hz, H13), 1.41 (3 H, s, H15), 1.38−1.31 (2 H, m, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.70 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.78 (C3), 166.87 (C21), 164.79, 161.06, 150.96, 144.65, 138.88 (C19), 131.17, 131.11, 117.16 (C20), 116.62, 116.47, 74.60 (C11), 70.58 (C14), 58.07 (C4), 45.41 (C9), 44.53 (C13), 44.02 (C12), 41.85 (C5), 36.66 (C6), 36.07, 36.02 (C10), 34.43 (C2), 30.39 (C8), 26.83 (C7), 26.44 (C18), 24.81 (C1), 16.73 (C16), 14.75 (C15), 14.71, 11.42 (C17), 11.18. HR-MS (ESI$^+$): Calcd for $C_{32}H_{41}FN_4O_4S$ (M+H$^+$): 597.2906; Found: 597.2894.

4.2.13. 22-[(4-(2-chloro-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (43)

White powder; yield: 68%; ^{1}H NMR (600 MHz, Chloroform-d) δ 9.08 (1H, d, J = 0.5Hz), 8.17 (1 H, dt, J = 7.7, 1.3Hz), 7.55−7.46 (2 H, m), 7.42 (1 H, dddd, J = 7.8, 5.5, 2.9, 0.7Hz), 6.42 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.74 (1 H, d, J = 8.5Hz, H14), 5.30 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.4, 1.5Hz, H20), 4.14−3.99 (2H, m, H22), 3.35 (1H, dd, J = 10.4, 6.5Hz, H11), 2.55 (3H, s), 2.33−2.14 (3 H, m, H2, H10), 2.10−2.08 (1H, m, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.76 (2 H, s, H7), 1.65 (2 H, tdd, J = 10.8, 8.2, 5.3Hz, H1), 1.56−1.43 (3 H, m, H4, H6, H13), 1.42 (3 H, s, H15), 1.36 (2 H, dd, J = 16.5, 3.1Hz, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.72 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.82 (C3), 166.68 (C21), 157.48, 150.96, 145.15, 138.83 (C19), 136.33, 133.67, 130.29, 129.59, 128.05, 127.43, 117.21 (C20), 74.62 (C11), 70.51 (C14), 58.10 (C4), 45.42 (C9), 44.53 (C13), 44.01 (C12), 41.87 (C5), 36.69 (C6), 36.02 (C10), 35.88, 34.44 (C2), 30.40 (C8), 29.67, 26.83 (C7), 26.43 (C18), 24.82 (C1), 16.73 (C16), 14.77 (C15), 11.42 (C17). HR-MS (ESI$^+$): Calcd for $C_{32}H_{41}ClN_4O_4S$ (M+H$^+$): 613.2610; Found: 613.2595.

4.2.14. 22-[(4-(3-chloro-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (44)

White powder; yield: 77%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.62 (1H, s), 7.90 (1 H, t, J = 1.9Hz), 7.73 (1 H, dt, J = 7.7, 1.4Hz), 7.56 (1 H, ddd, J = 8.0, 2.1, 1.1Hz), 7.47 (1 H, t, J = 7.9Hz), 6.41 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.29 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.4, 1.5Hz, H20), 4.13−3.97 (2H, m, H22), 3.35 (1H, dd, J = 10.4, 6.5Hz, H11), 2.52 (3H, s), 2.34−2.14 (3 H, m, H2, H10), 2.11−2.08 (1H, m, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.77 (2 H, s, H7), 1.65 (2 H, dddd, J = 13.1, 11.1, 7.9, 4.8Hz, H1), 1.54−1.43 (3 H, m, H4, H6, H13), 1.42 (3 H, s, H15), 1.38−1.31 (2 H, m, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.1Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.77 (C3), 166.86 (C21), 160.16, 151.21, 144.62, 138.85 (C19), 135.42, 132.83, 130.41, 128.29, 127.24, 117.19 (C20), 74.60 (C11), 70.62 (C14), 58.07 (C4), 45.41 (C9), 44.53 (C13), 44.02 (C12), 41.84 (C5), 36.66 (C6), 36.24, 36.02 (C10), 34.42 (C2), 30.39 (C8), 26.83 (C7), 26.43 (C18), 24.81 (C1), 16.73 (C16), 14.75 (C15), 11.43 (C17), 11.25. HR-MS (ESI$^+$): Calcd for $C_{32}H_{41}ClN_4O_4S$ (M+H$^+$): 613.2610; Found: 613.2595.

4.2.15. 22-[(4-(4-chloro-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (45)

White powder; yield: 64%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.60 (1H, s), 7.88−7.74 (2 H, m), 7.62−7.40 (2 H, m), 6.41 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.72 (1 H, d, J = 8.5Hz, H14), 5.29 (1 H, dd, J = 11.0, 1.4Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.11−3.98 (2H, m, H22), 3.35 (1H, dd, J = 10.0, 6.5Hz, H11), 2.51 (3 H, d, J = 0.6Hz), 2.31−2.15 (3 H, m, H2, H10), 2.09 (1H, d, J = 2.8Hz, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.77 (2 H, s, H7), 1.69−1.60 (2 H, m, H1), 1.55−1.42 (3 H, m, H4, H6, H13), 1.42 (3 H, s, H15), 1.38−1.31 (2 H, m, H8), 1.16 (3 H, s, H18), 0.88 (3 H, d, J = 7.0Hz, H17), 0.70 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.77 (C3), 166.85 (C21), 160.61, 151.06, 144.66, 139.29 (C19), 138.87, 130.27, 130.01, 129.55, 117.17 (C20), 74.60 (C11), 70.61 (C14), 58.07 (C4), 45.41 (C9), 44.53 (C13), 44.02 (C12), 41.85 (C5), 36.66 (C6), 36.11, 36.02 (C10), 34.42 (C2), 30.39 (C8), 29.67, 26.83 (C7), 26.44 (C18), 24.81 (C1), 16.73 (C16), 14.76 (C15), 11.42 (C17), 11.22. HR-MS (ESI$^+$): Calcd for $C_{32}H_{41}ClN_4O_4S$ (M+H$^+$): 613.2610; Found: 613.2600.

4.2.16. 22-[(4-(2-hydroxyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (46)

White powder; yield: 58%; ^{1}H NMR (600 MHz, Chloroform-d) δ 10.26 (1H, s), 8.76 (1 H, s), 7.52 (1 H, ddd, J = 8.6, 7.3, 1.7Hz), 7.43 (1 H, dd, J = 7.8, 1.7Hz), 7.12−7.08 (1 H, m), 7.05 (1 H, td, J = 7.5, 1.1Hz), 6.41 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.72 (1 H, d, J = 8.5Hz, H14), 5.29 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.11−3.92 (2H, m, H22), 3.35 (1H, dd, J = 10.4, 6.5Hz, H11), 2.51 (3H, s), 2.32−2.15 (3 H, m, H2, H10), 2.11−2.02 (2H, m, H13, 11-OH), 1.78−1.73 (3 H, m, H7, H13), 1.65 (2 H, tdd, J = 11.1, 7.5, 5.2Hz, H1), 1.51−1.48 (1 H, m, H6), 1.45 (1 H, ddd, J = 13.4, 9.6, 2.7Hz, H4), 1.42 (3 H, s, H15), 1.37−1.30 (2 H, m, H8), 1.16 (3 H, s, H18), 0.88 (3 H, d, J = 7.0Hz, H17), 0.70 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.74 (C3), 167.48 (C21), 166.85, 159.91, 150.02, 145.00, 138.88 (C19), 135.33, 133.26, 120.26, 117.87, 117.17 (C20), 115.83, 74.59 (C11), 70.70 (C14), 58.05 (C4), 45.41 (C9), 44.54 (C13), 44.03 (C12), 41.83 (C5), 36.64 (C6), 36.18, 36.00 (C10), 34.42 (C2), 30.38 (C8), 26.82 (C7), 26.39 (C18), 24.81 (C1), 16.74 (C16), 14.75 (C15), 11.43 (C17), 11.07. HR-MS (ESI$^+$): Calcd for $C_{32}H_{42}N_4O_5S$ (M+H$^+$): 595.2949; Found: 595.2935.

4.2.17. 22-[(4-(3-hydroxyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (47)

White powder; yield: 56%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.88 (1H, s), 8.54 (1 H, s), 7.48 (1 H, dd, J = 2.5, 1.5Hz),

7.38–7.31 (2 H, m), 7.18 (1 H, ddd, J = 7.9, 2.5, 1.3Hz), 6.39 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.71 (1 H, d, J = 8.5Hz, H14), 5.27 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.16 (1H, dd, J = 17.4, 1.5Hz, H20), 4.10–3.93 (2H, m, H22), 3.35 (1H, dd, J = 9.9, 6.2Hz, H11), 2.52 (3H, s), 2.31–2.14 (3 H, m, H2, H10), 2.09–2.06 (1H, m, 11-OH), 2.01 (1 H, dd, J = 16.0, 8.6Hz, H13), 1.92 (2 H, s, H7), 1.74 (1 H, dt, J = 14.5, 3.2Hz, H13), 1.65–1.58 (3 H, m, H1, H6), 1.44 (1 H, ddt, J = 12.1, 9.5, 5.0Hz, H4), 1.39 (3 H, s, H15), 1.35–1.28 (2 H, m, H8), 1.14 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.68 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 217.10 (C3), 166.79 (C21), 163.84, 157.64, 150.93, 145.18, 138.78 (C19), 132.69, 130.29, 121.34, 121.14, 117.26 (C20), 115.02, 74.63 (C11), 70.62 (C14), 58.09 (C4), 45.43 (C9), 44.49 (C13), 44.00 (C12), 41.84 (C5), 36.67 (C6), 36.00 (C10), 35.86, 34.45 (C2), 30.38 (C8), 26.80 (C7), 26.41 (C18), 24.81 (C1), 16.71 (C16), 14.76 (C15), 11.46 (C17), 11.08. HR-MS (ESI$^+$): Calcd for $C_{32}H_{42}N_4O_5S$ (M+H$^+$): 595.2949; Found: 595.2936.

4.2.18. 22-[(4-(4-hydroxyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (48)

White powder; yield: 66%; ^{1}H NMR (600 MHz, Chloroform-d) δ 9.97 (1H, s), 8.46 (1 H, s), 7.79–7.73 (2 H, m), 7.11–7.05 (2 H, m), 6.40 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.72 (1 H, d, J = 8.6Hz, H14), 5.29 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.17 (1H, dd, J = 17.4, 1.5Hz, H20), 4.11–3.91 (2H, m, H22), 3.35 (1H, dd, J = 10.3, 6.4Hz, H11), 2.51 (3H, s), 2.32–2.14 (3 H, m, H2, H10), 2.11–2.07 (1H, m, 11-OH), 2.02 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.85 (2 H, s, H7), 1.78–1.72 (1 H, m, H13), 1.68–1.60 (2 H, m, H1), 1.57 (1 H, d, J = 10.4Hz, H8), 1.54–1.42 (2 H, m, H4, H6), 1.41 (3 H, s, H15), 1.35–1.31 (1 H, m, H8), 1.14 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.70 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 217.01 (C3), 166.87 (C21), 164.88, 162.44, 150.74, 145.25, 138.82 (C19), 131.41, 122.84, 117.24 (C20), 116.69, 74.63 (C11), 70.59 (C14), 58.10 (C4), 45.43 (C9), 45.03, 44.51 (C13), 44.01 (C12), 41.85 (C5), 41.46, 36.68 (C6), 36.01 (C10), 35.70, 34.45 (C2), 30.39 (C8), 26.82 (C7), 26.41 (C18), 24.81 (C1), 16.72 (C16), 14.77 (C15), 11.46 (C17), 10.93. HR-MS (ESI$^+$): Calcd for $C_{32}H_{42}N_4O_5S$ (M+H$^+$): 595.2949; Found: 595.2938.

4.2.19. 22-[(4-(2-nitro-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (49)

White powder; yield: 57%; ^{1}H NMR (600 MHz, Chloroform-d) δ 9.29 (1H, s), 8.21 (2 H, ddd, J = 8.0, 6.9, 1.4Hz), 7.86–7.68 (2 H, m), 6.42 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.74 (1 H, d, J = 8.4Hz, H14), 5.30 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.4, 1.5Hz, H20), 4.14–4.00 (2H, m, H22), 3.35 (1H, dd, J = 10.3, 6.5Hz, H11), 2.59 (3H, s), 2.33–2.14 (3 H, m, H2, H10), 2.10 (1H, d, J = 2.8Hz, 11-OH), 2.05 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.77 (2 H, s, H7), 1.65 (2 H, dddd, J = 11.0, 9.0, 7.0, 3.5, H1), 1.53–1.49 (2 H, m, H6, H13), 1.48–1.43 (1 H, m, H4), 1.42 (3 H, s, H15), 1.39–1.33 (2 H, m, H8), 1.17 (3 H, s, H18), 0.87 (3 H, d, J = 7.1Hz, H17), 0.72 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.83 (C3), 166.74 (C21), 156.36, 150.71, 145.93, 138.85 (C19), 134.12, 132.71, 129.49, 127.28, 125.23, 117.19 (C20), 74.61 (C11), 70.59 (C14), 58.08 (C4), 45.42 (C9), 44.51 (C13), 44.02 (C12), 41.86 (C5), 36.68 (C6), 36.01 (C10), 35.77, 34.43 (C2), 30.40 (C8), 26.83 (C7), 26.42 (C18), 24.81 (C1), 16.73 (C16), 14.77 (C15), 11.66, 11.42 (C17). HR-MS (ESI$^+$): Calcd for $C_{32}H_{41}N_5O_6S$ (M+H$^+$): 624.2851; Found: 624.2838.

4.2.20. 22-[(4-(3-nitro-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (50)

White powder; yield: 69%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.83 (1H, s), 8.72–8.71 (1 H, m), 8.43 (1 H, ddd, J = 8.2, 2.3, 1.1Hz), 8.23 (1 H, dt, J = 7.7, 1.4Hz), 7.75 (1 H, t, J = 8.0Hz), 6.40 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.28 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.15–4.01 (2H, m, H22), 3.36 (1H, dd, J = 10.1, 6.4Hz, H11), 2.56 (3H, s), 2.33–2.14 (3 H, m, H2, H10), 2.11–2.08 (1H, m, 11-OH), 2.05 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.75 (2 H, d, J = 4.5Hz, H7), 1.65 (2 H, dddd, J = 13.8, 10.9, 8.1, 3.6Hz, H1), 1.53–1.43 (3 H, m, H4, H6, H13), 1.42 (3 H, s, H15), 1.39–1.31 (2 H, m, H8), 1.16 (3 H, s, H18), 0.88 (3 H, d, J = 7.1Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.73 (C3), 166.82 (C21), 158.08, 151.49, 148.80, 144.62, 138.87 (C19), 133.91, 133.67, 130.34, 126.95, 123.48, 117.15 (C20), 74.59 (C11), 70.76 (C14), 58.05 (C4), 45.41 (C9), 44.55 (C13), 44.03 (C12), 41.85 (C5), 36.63 (C6), 36.41, 36.02 (C10), 34.41 (C2), 30.37 (C8), 26.83 (C7), 26.44 (C18), 24.81 (C1), 16.73 (C16), 14.76 (C15), 11.42 (C17), 11.33. HR-MS (ESI$^+$): Calcd for $C_{32}H_{41}N_5O_6S$ (M+H$^+$): 624.2851; Found: 624.2836.

4.2.21. 22-[(4-(4-nitro-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (51)

White powder; yield: 58%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.80 (1H, s), 8.40–8.35 (2 H, m), 8.09–8.03 (2 H, m), 6.40 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.28 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.17–4.03 (2H, m, H22), 3.36 (1H, dd, J = 10.3, 6.5Hz, H11), 2.56 (3H, s), 2.31–2.15 (3 H, m, H2, H10), 2.10 (1H, d, J = 2.7, 11-OH), 2.05 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.75 (2 H, s, H7), 1.70–1.61 (2 H, m, H1), 1.54–1.48 (2 H, m, H6, H13), 1.48–1.43 (1 H, m, H4), 1.43 (3 H, s, H15), 1.38–1.32 (2 H, m, H8), 1.17 (3 H, s, H18), 0.88 (3 H, d, J = 7.1Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.74 (C3), 166.76 (C21), 157.40, 151.56, 150.13, 144.73, 138.89 (C19), 137.49, 129.49, 124.29, 117.15 (C20), 74.59 (C11), 70.79 (C14), 58.05 (C4), 53.40 (C22), 45.41 (C9), 44.53 (C13), 44.04 (C12), 41.85 (C5), 36.63 (C6), 36.31, 36.03 (C10), 34.41 (C2), 30.36 (C8), 26.83 (C7), 26.47 (C18), 24.81 (C1), 16.74 (C16), 14.76 (C15), 11.42 (C17), 11.36. HR-MS (ESI$^+$): Calcd for $C_{32}H_{41}N_5O_6S$ (M+H$^+$): 624.2851; Found: 624.2835.

4.2.22. 22-[(4-(3-nitrile-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (52)

White powder; yield: 70%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.73 (1H, s), 8.18 (1 H, t, J = 1.7Hz), 8.10 (1 H, dt, J = 7.9, 1.4Hz), 7.86 (1 H, dt, J = 7.8, 1.3Hz), 7.67 (1 H, t, J = 7.8Hz), 6.40 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.28 (1 H, dd, J = 11.0, 1.4Hz, H20), 5.18 (1H, dd, J = 17.4, 1.5Hz, H20), 4.21–3.90 (2H, m, H22), 3.36 (1H, dd, J = 10.3, 6.5Hz, H11), 2.54 (3H, s), 2.32–2.15 (3 H, m, H2, H10), 2.10 (1H, d, J = 2.7Hz, 11-OH), 2.05 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.78–1.73 (3 H, m, H7, H13), 1.68–1.61 (2 H, m, H1), 1.51 (1 H, d, J = 10.2Hz, H6), 1.45 (1 H, ddd, J = 13.0, 9.5, 2.5Hz, H4), 1.42 (3 H, s, H15), 1.39–1.31 (2 H, m, H8), 1.16 (3 H, s, H18), 0.88 (3 H, d, J = 7.0Hz, H17), 0.70 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.73 (C3), 166.82 (C21), 158.23, 151.43, 144.60, 138.88 (C19), 135.56, 133.23, 132.56, 131.96, 130.11, 117.58, 117.15 (C20), 113.85, 74.59 (C11), 70.75 (C14), 58.05 (C4), 45.41 (C9), 44.54 (C13), 44.03 (C12), 41.84 (C5), 36.63 (C6), 36.37, 36.02 (C10), 34.41 (C2), 30.37 (C8), 26.84 (C7), 26.46 (C18), 24.81 (C1), 16.73 (C16), 14.76 (C15), 11.42 (C17), 11.30. HR-MS (ESI$^+$): Calcd for $C_{33}H_{41}N_5O_4S$ (M+H$^+$): 604.2952; Found: 604.2942.

4.2.23. 22-[(4-(4-nitrile-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio] deoxy pleuromutilin (53)

White powder; yield: 72%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.73 (1H, s), 8.03–7.96 (2 H, m), 7.87–7.76 (2 H, m), 6.40 (1 H, dd, J = 17.5, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.27 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.16–4.00 (2H, m, H22), 3.36 (1H, dd, J = 10.3, 6.4Hz, H11), 2.54 (3H, s), 2.31–2.14 (3 H, m, H2, H10), 2.09 (1H, d, J = 2.8Hz, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.76 (2 H, s, H7), 1.65 (2 H, dddd, J = 13.7, 10.8, 8.1, 3.5Hz, H1), 1.54–1.43 (3 H, m, H4, H6, H13), 1.42 (3 H, s, H15), 1.39–1.29 (2 H, m, H8), 1.16 (3 H, s, H18), 0.88 (3 H, d, J = 7.0Hz,

H17), 0.70 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.73 (C3), 166.76 (C21), 158.10, 151.45, 144.71, 138.89 (C19), 135.85, 132.81, 129.07, 117.81, 117.14 (C20), 115.99, 74.58 (C11), 70.76 (C14), 58.05 (C4), 45.41 (C9), 44.53 (C13), 44.03 (C12), 41.85 (C5), 40.81, 36.63 (C6), 36.27, 36.03 (C10), 34.42 (C2), 30.37 (C8), 26.83 (C7), 26.47 (C18), 25.48, 24.81 (C1), 16.73 (C16), 14.75 (C15), 11.42 (C17), 11.32. HR-MS (ESI^+): Calcd for $C_{33}H_{41}N_5O_4S$ ($M+H^+$): 604.2952; Found: 604.2943.

4.2.24. 22-[(4-(3-trifluoromethyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio]deoxy pleuromutilin (54)

White powder; yield: 55%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.74 (1H, s), 8.13 (1 H, d, J = 1.8Hz), 8.07 (1 H, dt, J = 7.7, 1.5Hz), 7.85 (1 H, ddt, J = 7.9, 1.9, 1.0Hz), 7.68 (1 H, t, J = 7.8Hz), 6.41 (1 H, dd, J = 17.5, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.28 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.14−3.99 (2H, m, H22), 3.35 (1H, dd, J = 10.4, 6.5Hz, H11), 2.54 (3H, s), 2.32−2.16 (3 H, m, H2, H10), 2.11−2.08 (1H, m, 11-OH), 2.05 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.75 (2 H, s, H7), 1.68−1.61 (2 H, m, H1), 1.54−1.43 (3 H, m, H4, H6, H13), 1.42 (3 H, s, H15), 1.39−1.30 (2 H, m, H8), 1.16 (3 H, s, H18), 0.88 (3 H, d, J = 7.1Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.74 (C3), 166.85 (C21), 159.76, 151.30, 144.61, 138.85 (C19), 132.70, 131.80, 129.79, 129.25, 129.22, 125.49, 125.46, 117.16 (C20), 74.60 (C11), 70.67 (C14), 58.06 (C4), 45.41 (C9), 44.54 (C13), 44.02 (C12), 41.84 (C5), 36.65 (C6), 36.31, 36.02 (C10), 34.42 (C2), 30.38 (C8), 26.82 (C7), 26.42 (C18), 24.81 (C1), 16.72 (C16), 14.75 (C15), 11.41 (C17), 11.26. HR-MS (ESI^+): Calcd for $C_{33}H_{41}F_3N_4O_4S$ ($M+H^+$): 647.2874; Found: 647.2861.

4.2.25. 22-[(4-(4-trifluoromethyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio]deoxy pleuromutilin (55)

White powder; yield: 51%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.73 (1H, s), 8.03−7.94 (2 H, m), 7.79 (2 H, d, J = 8.1Hz), 6.41 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.6Hz, H14), 5.29 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.4, 1.5Hz, H20), 4.16−3.98 (2H, m, H22), 3.36 (1H, dd, J = 10.3, 6.4Hz, H11), 2.54 (3 H, d, J = 0.9Hz), 2.32−2.16 (3 H, m, H2, H10), 2.11−2.08 (1H, m, 11-OH), 2.05 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.74 (2 H, s, H7), 1.65 (2 H, dddd, J = 13.8, 10.7, 8.1, 3.6Hz, H1), 1.53−1.43 (3 H, m, H4, H6, H13), 1.42 (3 H, s, H15), 1.39−1.31 (2 H, m, H8), 1.16 (3 H, s, H18), 0.88 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.74 (C3), 166.80 (C21), 159.34, 151.32, 144.68, 138.87 (C19), 135.09, 129.03, 126.15, 126.13, 126.10, 126.08, 124.40, 117.16 (C20), 74.60 (C11), 70.69 (C14), 58.06 (C4), 45.41 (C9), 44.54 (C13), 44.03 (C12), 41.85 (C5), 36.65 (C6), 36.21, 36.02 (C10), 34.42 (C2), 30.38 (C8), 26.83 (C7), 26.44 (C18), 24.81 (C1), 16.73 (C16), 14.75 (C15), 11.41 (C17), 11.28. HR-MS (ESI^+): Calcd for $C_{33}H_{41}F_3N_4O_4S$ ($M+H^+$): 647.2874; Found: 647.2862.

4.2.26. 22-[(4-(4-dimethylamino-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio]deoxy pleuromutilin (56)

White powder; yield: 50%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.32 (1H, s), 7.75−7.69 (2 H, m), 6.77−6.69 (2 H, m), 6.43 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.32 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.19 (1H, dd, J = 17.4, 1.5Hz, H20), 4.07−3.94 (2H, m, H22), 3.35 (1H, s, H11), 3.11 (6H, s), 2.46 (3 H, s), 2.34−2.15 (3 H, m, H2, H10), 2.10 (1H, d, J = 2.8Hz, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.77 (1 H, q, J = 3.2Hz, H7), 1.75 (1 H, q, J = 3.2Hz, H7), 1.69−1.61 (2 H, m, H1), 1.56−1.43 (2 H, m, H6, H13), 1.42 (3 H, s, H15), 1.35 (2 H, dd, J = 15.3, 3.9Hz, H4, H8), 1.17 (3 H, s, H18), 1.12 (1 H, td, J = 14.1, 4.4Hz, H8), 0.88 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.88 (C3), 167.03 (C21), 164.81, 153.58, 150.44, 144.90, 138.87 (C19), 132.87, 130.94, 118.75, 117.20 (C20), 111.55, 74.63 (C11), 70.31 (C14), 58.11 (C4), 45.42 (C9), 44.50 (C13), 44.01 (C12), 41.85 (C5), 40.07, 39.81, 36.71 (C6), 36.01 (C10), 35.63, 34.45 (C2), 30.42 (C8), 29.67, 26.84 (C7), 26.43 (C18), 24.82 (C1), 16.74 (C16), 14.78 (C15), 11.44 (C17), 11.02. HR-MS (ESI^+): Calcd for $C_{34}H_{47}N_5O_4S$ ($M+H^+$): 622.3422; Found: 622.3410.

4.2.27. 22-[(4-(4-diethylamino-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio]deoxy pleuromutilin (57)

White powder; yield: 65%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.28 (1H, s), 7.74−7.64 (2 H, m), 6.76−6.65 (2 H, m), 6.42 (1 H, dd, J = 17.5, 11.0Hz, H19), 5.72 (1 H, d, J = 8.5Hz, H14), 5.31 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.19 (1H, dd, J = 17.4, 1.5Hz, H20), 4.09−3.89 (2H, m, H22), 3.46 (4H, q, J = 7.1Hz), 3.35 (1 H, dd, J = 10.4, 6.5Hz, H11), 2.44 (3 H, s), 2.33−2.16 (3 H, m, H2, H10), 2.09 (1H, d, J = 2.7Hz, 11-OH), 2.03 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.82 (2 H, s, H7), 1.75 (1 H, dq, J = 14.3, 3.2Hz, H13), 1.68−1.61 (2 H, m, H1), 1.54−1.45 (2 H, m, H4, H6), 1.41 (3 H, s, H15), 1.35 (2 H, dd, J = 15.6, 3.5Hz, H8), 1.24 (6 H, t, J = 7.1Hz), 1.17 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.88 (C3), 167.03 (C21), 165.00, 151.38, 150.40, 144.92, 138.88 (C19), 131.27, 117.99, 117.20 (C20), 111.13, 100.00, 74.63 (C11), 70.28 (C14), 58.12 (C4), 45.95, 45.42 (C9), 44.65 (C13), 44.50, 44.02 (C12), 41.85 (C5), 39.72, 36.71 (C6), 36.01 (C10), 35.57, 34.91, 34.45 (C2), 30.42 (C8), 26.84 (C7), 26.43 (C18), 24.82 (C1), 16.74 (C16), 14.79 (C15), 12.50, 11.43 (C17), 10.98. HR-MS (ESI^+): Calcd for $C_{36}H_{51}N_5O_4S$ ($M+H^+$): 650.3735; Found: 650.3723.

4.2.28. 22-[(4-(4-pyrrolidinyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl)thio]deoxy pleuromutilin (58)

White powder; yield: 55%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.29 (1H, s), 7.79−7.61 (2 H, m), 6.66−6.52 (2 H, m), 6.42 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.72 (1 H, d, J = 8.5Hz, H14), 5.31 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.19 (1H, dd, J = 17.5, 1.5Hz, H20), 4.09−3.89 (2H, m, H22), 3.43−3.38 (4H, m), 3.37−3.32 (1H, m, H11), 2.44 (3 H, s), 2.34−2.15 (3 H, m, H2, H10), 2.08 (5H, td, J = 7.0, 3.5Hz, 11-OH), 2.03 (1 H, dd, J = 16.1, 8.5Hz, H13), 1.83 (2 H, s, H7), 1.75 (1 H, dq, J = 14.5, 3.2Hz, H13), 1.68−1.60 (2 H, m, H1), 1.56−1.45 (2 H, m, H4, H6), 1.41 (3 H, s, H15), 1.35 (2 H, dd, J = 15.6, 3.2Hz, H8), 1.17 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.71 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.88 (C3), 167.04 (C21), 165.14, 151.19, 150.41, 144.93, 138.87 (C19), 131.10, 118.20, 117.20 (C20), 111.67, 74.63 (C11), 70.29 (C14), 58.12 (C4), 47.65, 45.53 (C9), 45.42, 44.50 (C13), 44.02 (C12), 42.21, 41.85 (C5), 36.93, 36.71 (C6), 36.01 (C10), 35.59, 34.45 (C2), 30.42 (C8), 27.20 (C7), 26.84, 26.43 (C18), 25.42, 24.82 (C1), 16.74 (C16), 14.79 (C15), 11.43 (C17), 11.01. HR-MS (ESI^+): Calcd for $C_{36}H_{49}N_5O_4S$ ($M+H^+$): 648.3578; Found: 648.3565.

4.2.29. 22-[(4-(4-morpholinyl-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl) thio]deoxy pleuromutilin (59)

White powder; yield: 69%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.39 (1H, s), 7.80−7.71 (2 H, m), 6.97−6.92 (2 H, m), 6.42 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.72 (1 H, d, J = 8.5Hz, H14), 5.30 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.08−3.94 (2H, m, H22), 3.90−3.86 (4H, m), 3.37−3.32 (5H, m, H11), 2.46 (3 H, s), 2.33−2.15 (3 H, m, H2, H10), 2.09 (1H, d, J = 2.7Hz, 11-OH), 2.03 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.81 (2 H, s, H7), 1.75 (1 H, dq, J = 14.5, 3.2Hz, H13), 1.68−1.61 (2 H, m, H1), 1.55−1.50 (1 H, m, H6), 1.50−1.42 (1 H, m, H4), 1.41 (3 H, s, H15), 1.34 (2 H, dd, J = 15.1, 6.5Hz, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.1Hz, H17), 0.70 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.85 (C3), 166.96 (C21), 163.64, 154.37, 150.55, 144.81, 138.87 (C19), 130.76, 121.83, 117.19 (C20), 114.11, 74.62 (C11), 70.40 (C14), 66.50, 58.10 (C4), 47.53, 45.42 (C9), 44.51 (C13), 44.02 (C12), 43.70, 41.85 (C5), 36.69 (C6), 36.01 (C10), 35.76, 34.79, 34.44 (C2), 30.41 (C8), 29.67, 26.84 (C7), 26.44 (C18), 24.82 (C1), 16.74 (C16), 14.77 (C15), 11.43 (C17), 11.08.

HR-MS (ESI$^+$): Calcd for $C_{36}H_{49}N_5O_5S$ (M+H$^+$): 664.3527; Found: 664.3513.

4.2.30. 22-[(4-(4-(2-pyridine)-benzylidene amino)-5-methyl-4H-1,2,4-triazol-3-yl) thio]deoxy pleuromutilin (60)

White powder; yield: 71%; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.75 (1H, dt, J = 4.8, 1.4Hz), 8.66 (1 H, s), 8.19–8.16 (2 H, m), 8.00–7.96 (2 H, m), 7.84–7.81 (2 H, m), 7.34–7.30 (1 H, m), 6.42 (1 H, dd, J = 17.4, 11.0Hz, H19), 5.73 (1 H, d, J = 8.5Hz, H14), 5.30 (1 H, dd, J = 11.0, 1.5Hz, H20), 5.18 (1H, dd, J = 17.5, 1.5Hz, H20), 4.12–4.00 (2H, m, H22), 3.35 (1H, dd, J = 10.4, 6.5Hz, H11), 2.54 (3H, s), 2.33–2.14 (3 H, m, H2, H10), 2.11–2.08 (1H, m, 11-OH), 2.04 (1 H, dd, J = 16.1, 8.6Hz, H13), 1.82 (2 H, s, H7), 1.75 (1 H, dq, J = 14.6, 3.2Hz, H13), 1.65 (2 H, dddd, J = 13.6, 10.7, 8.1, 3.5Hz, H1), 1.52 (1 H, d, J = 10.4Hz, H6), 1.48–1.43 (1 H, m, H4), 1.42 (3 H, s, H15), 1.35 (2 H, dd, J = 15.5, 4.0Hz, H8), 1.16 (3 H, s, H18), 0.87 (3 H, d, J = 7.0Hz, H17), 0.72 (3 H, d, J = 7.1Hz, H16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 216.80 (C3), 166.89 (C21), 161.72, 155.84, 151.03, 149.99, 144.77, 143.62, 138.86 (C19), 136.97, 132.06, 129.32, 127.53, 123.04, 120.91, 117.20 (C20), 74.61 (C11), 70.55 (C14), 58.08 (C4), 45.52, 45.42 (C9), 44.61 (C13), 44.53, 44.02 (C12), 41.85 (C5), 36.68 (C6), 36.07, 36.02 (C10), 34.43 (C2), 30.40 (C8), 26.83 (C7), 26.44 (C18), 24.81 (C1), 16.74 (C16), 14.77 (C15), 11.43 (C17), 11.26. HR-MS (ESI$^+$): Calcd for $C_{37}H_{45}N_5O_4S$ (M+H$^+$): 656.3265; Found: 656.3253.

4.3. In vitro efficacy of pleuromutilin derivatives

4.3.1. Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) testing

The MIC and MBC values were determined according to the Clinical and Laboratory Standards Institute (CLSI). The MIC values of the target pleuromutilin derivatives against methicillin-resistant *S. aureus* (ATCC 43300), *S. aureus* (ATCC 29213), *S. aureus* (AD3), *S. aureus* (144) and *E. coli* (ATCC 25922) were determined using by pleuromutilin and tiamulin as positive controls. The synthetic compounds were dissolved in the aqueous solution of 2.5% DMSO and 2.5% tween-80 to make a stock solution with a concentration of 1280 μg/mL. The stock solution was diluted twice continuously with sterile water and diluted 10 times with MH broth to give a concentration range of 320.03 μg/mL. The amounts of bacteria were added in each well was 5×10^5 CFU/mL. Three parallel experiments were set for each compound concentration. The 96-well-plate was incubated at 37 °C for 18–24 h. The MIC values were recorded as the lowest drug concentration that completely inhibits the visible growth of test bacteria.

After recording the MIC value, the 96-well plate was placed at 37 °C for 24 h, and the MBC values were determined by plating 100 μL aliquots from wells without visible growth onto the MH agar plates. Then, the MH agar plate was further cultured at 37 °C for 24 h, and the minimum concentration of the compound required to reduce the bacterial count by more than 99.9% on the plate was MBC.

4.3.2. Constant concentration time-kill curves

Compounds **48** and **60** were screened according to MIC results. The Time–kill curve experiments of the above two compounds were performed in triplicate according to the previous report [26]. The logarithmic phase of MRSA ATCC 43300 was diluted to 10^6 CFU/mL with MH broth. Compounds **48**, **60** and tiamulin were added into the bacteria solution with the final concentration of 1 × MIC, 2 × MIC, 4 × MIC, 8 × MIC, 16 × MIC, and 32 × MIC, respectively. All samples were cultured at 37 °C with shaking. Samples (100 μL) were taken from the mixtures at 0, 3, 6, 9 and 24 h and were continuously diluted decuple with sterile saline. Then 25 μL of the diluted sample were plated on the sterile MH agar plate and incubated at 37 °C for 18–24 h. The total bacterial CFU/mL on the plates were counted to calculate the bacterial colonies. The time-kill curve was constructed by plotting $\log_{10}$ CFU/mL of bacteria counts versus time.

4.3.3. Determination of the post-antibiotic effect (PAE)

The post-antibiotic effect (PAE) of compounds **48** and **60** against MRSA were determined using time-kill methods according to previous methods [27]. In this experiment, MRSA ATCC 43300 in the logarithmic phase was diluted to 10^6 CFU/mL with MH broth. The above compounds at final concentrations of 2 × MIC and 4 × MIC were added to the tubes containing the inoculum. Then, the samples were shaken at 37 °C for 2 h. After the incubation was completed. The drugs were removed from the sample by diluting 1000 times with the preheated MH broth. The tubes were placed in 37 °C and the culture solution (100 μL) was extracted at times 0, 2, 4, 6 and 8 h. After the culture solution was diluted 10-fold with sterile saline and plated onto MH agar plates. The colonies were counted after 20 h of incubation in 37 °C. The PAE value was presented in the hour and calculated by the equation $PAE = T_A - T_C$. (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. Neutropenic murine thigh infection model

The neutropenic murine thigh infection model experiment was performed as described in the literature [16]. Six-week-old SPF-ICR female that weighing 23–27 g mice were used for this study. The mice were injected with cyclophosphamide (Mead Johnson Pharmaceuticals, Evansville, IN) at a dose of 150 mg/kg on 4 days and at a dose of 100 mg/kg on 1 day before the experiment to reduce neutrophils and achieve immunosuppression ($<0.1 \times 10^9$/L). 0.1 mL MH broth (MRSA ATCC 43300 concentration was approximately 10^7 CFU/mL) was injected by intramuscular into both sides of the posterior thigh of mice to establish an infection model, respectively. After the thigh of mice were infected 2 h, these mice were randomly divided into 3 groups (3 per group). The mice were intravenously injected with 0.9% saline, compound **60** (20 mg/kg) and tiamulin (20 mg/kg). 24 h after the completion of the drug, the mice were euthanized and their thigh tissue were collected. Then their thigh tissues were weighed and homogenized respectively in 3 mL of iced sterile saline. Tissue homogenate was serially diluted 10-fold and plated on MH agar. The resulting bacterial colonies were determined after incubation for 24 h.

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

The cytotoxicity of all target compounds was evaluated by the traditional MTT assay as described in Ref. [28]. RAW 264.7 cells and HepG2 cells were used in this experiment, respectively. The cells were inoculated into 96-well plates at a density of 1.0×10^5 cells per well. Then, the plates were incubated at 37 °C for 24 h. The cells were treated with these target pleuromutilin derivatives (4 μg/mL) and cultured for 16 h at 37 °C. The cells were incubated with 100 μL/well of MTT (0.5 mg/mL in PBS) and cultured for 4 h under 5% CO_2 37 °C. After the medium was removed, 150 μL DMSO was added to each well to dissolve the cells. After 30 min incubation, absorbance at 490 nm was recorded using a microplate spectrophotometer (BIO-TEK Instrument Inc., USA).

4.6. Development of resistance

The development of resistance test was performed as described in the literature with some modifications [29]. The logarithmic phase culutures of MRSA ATCC 43300 were diluted 1:1000 into fresh MHB media with sub-MIC concentration (1/4 MIC) of compound **60** or tiamulin. After stationary cultured at 37 °C for 24 h, the MIC of culture was determined by section 4.3 method. Meanwhile, the freshly diluted MRSA in the broth medium was cultured in sub-MIC concentration (1/4 MIC) of drugs for next passages. The process was repeated for 8 passages.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113624.

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