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RESEARCH PAPER

Design, synthesis, biological evaluation and molecular docking studies of novel pleuromutilin derivatives containing nitrogen heterocycle and alkylamine groups

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ABSTRACT

A series of pleuromutilin derivatives containing alkylamine and nitrogen heterocycle groups were designed and synthesised under mild conditions. The *in vitro* antibacterial activity of these semisynthetic derivatives against four strains of *Staphylococcus aureus* (MRSA ATCC 43300, *S.aureus* ATCC 29213, *S.aureus* AD3, and *S.aureus* 144) were evaluated by the broth dilution method. Compound **13** was found to have excellent antibacterial activity against MRSA (MIC = 0.0625 μg/mL). Furthermore, compound **13** was further studied by the time-killing kinetics and the post-antibiotic effect approach. In the mouse thigh infection model, compound **13** exhibited superior antibacterial efficacy than that of tiamulin. Meanwhile, compound **13** showed a lower inhibitory effect than that of tiamulin on RAW264.7 and 16HBE cells at the concentration of 10 μg/mL. Molecular docking study revealed that compound **13** can effectively bind to the active site of the 50S ribosome (the binding free energy = −9.66 kcal/mol).

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Pleuromutilin; alkylamine; nitrogen heterocycles; molecular docking; MRSA; antibacterial activity

1. Introduction

The abuse of antibiotics contributes to the emergence of multiple drug-resistant bacteria, including Methicillin-resistant *Staphylococcus aureus* (MRSA)[1]. MRSA can cause a wide variety of clinical diseases, such as skin and soft tissue infections (SSTIs), sepsis, pneumonia, meningitis, infective endocarditis and bacteraemia[2,3]. The prevalence of MRSA infection had become a major public health concern worldwide[4]. Vancomycin is "the last line of defense" against Methicillin-resistant *Staphylococcus aureus* infections[5]. But it has recently been reported that the horizontal transfer of the vanA gene from vancomycin-resistant *E. faecalis* to MRSA, leading the resistance of MRSA to vancomycin[6]. With the prevalence of MRSA, new antimicrobial agents with novel modes of action are urgently needed.

Pleuromutilin (**1**, Figure 1) is a tricyclic diterpene natural product, which was first discovered and isolated from two basidiomycetes, *Pleurotus mutilus* and *P. passeckerianus* in 1951[7]. Pleuromutilin exhibited antibacterial activity towards part of Gram-negative bacteria and most of Gram-positive bacteria, such as *Staphylococcus aureus*[8]. Pleuromutilin exerts antibacterial activity by binding to the V domain of the peptidyl transferase centre (PTC) of the 50S subunit of the bacterial ribosome[9]. Thus, pleuromutilin has aroused considerable research for this unique antibacterial mechanism. The structural optimizations of pleuromutilin on the C-14 side chain prompted the discovery of tiamulin (**2**, Figure 1) and valnemulin (**3**, Figure 1) which were authorised as veterinary medicines in 1979 and 1999, respectively[10]. Retapamulin (**4**, Figure 1) became the first pleuromutilin approved for human use by U.S Food and Drug Administration (FDA) in 200711. Lefamulin (**5**, Figure 1) is the first approved systemic pleuromutilin antibiotic for the treatment of community-acquired bacterial pneumonia (CABP)[12].

Previous work in our group has led to a variety of semisynthetic pleuromutilin derivatives. These derivatives contain 2-aminophenylthiol, piperazine ring, piperidine ring and 1,2,3-triazole as linkers on the C14 side chain of pleuromutilin[13–15]. The bioassay results confirmed that pleuromutilin derivatives using 2-aminobenzenethiol as the linking chain exhibited good antibacterial activity against MRSA[14]. A survey reported that 59% of small-molecule drugs contain nitrogen heterocycles among U.S. FDA approved drugs on the market[16]. These backgrounds inspired us to develop novel pleuromutilin derivatives containing nitrogen heterocycles with 2-aminobenzenethiol as the linking arm. Additionally, in order to enrich the diversity of introduced groups, we also introduced alkylamines on the C14 side chain.

In this study, we designed and synthesised 21 pleuromutilin derivatives with nitrogen heterocycles and alkylamines under mild conditions and evaluated their antibacterial activity against MRSA *in vitro* and *in vivo*. To investigate the possible binding mode of these compounds with 50S ribosomes, the molecular docking experiment was also performed.

2. Results and discussion

2.1. Chemistry

The synthetic course of all pleuromutilin derivatives was depicted in Scheme 1. Compound **6** was synthesised by the reaction of

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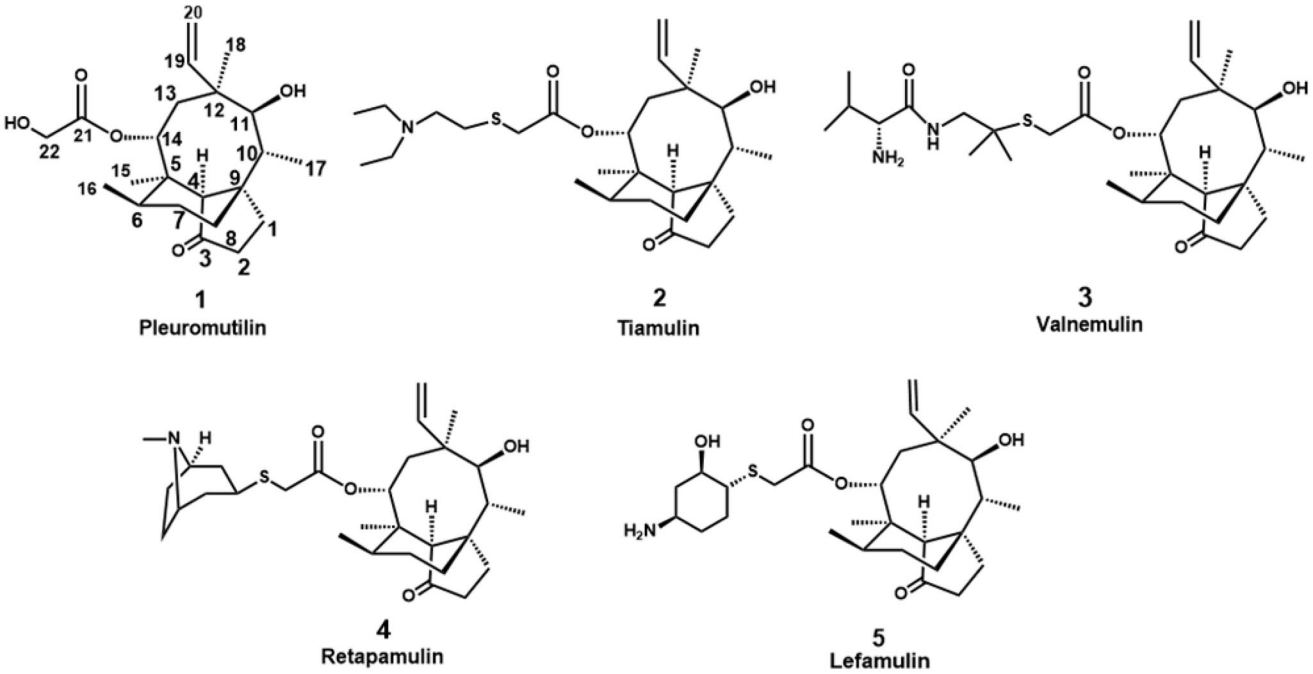
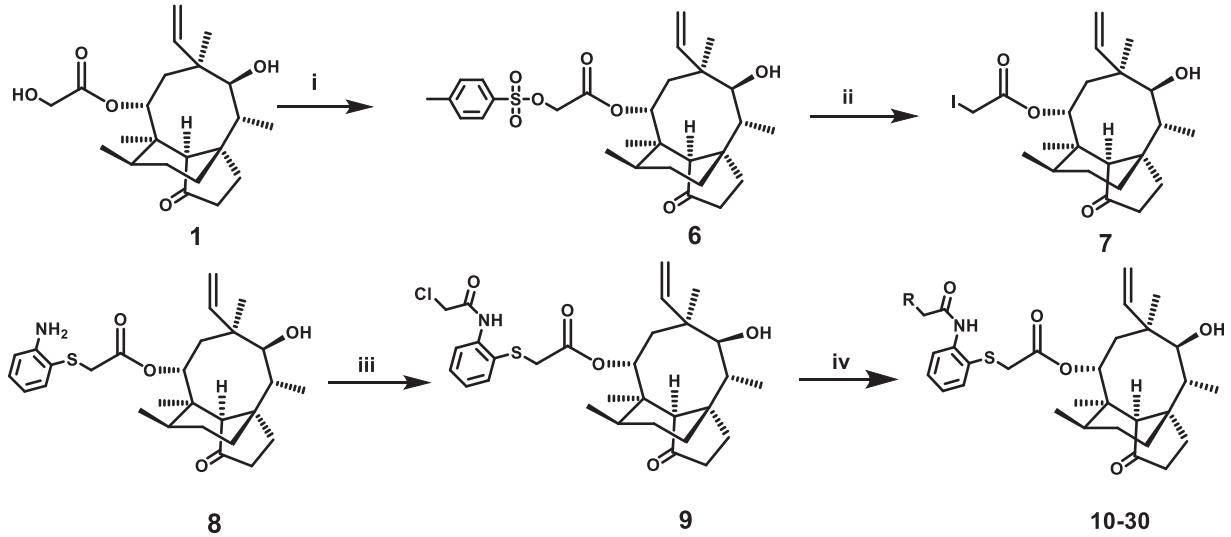


Figure 1. Structure of pleuromutilin (*1*), tiamulin (*2*), valnemulin (*3*), retapamulin (*4*), lefamulin (*5*).



Scheme 1. Reagent and conditions: (i) dichloromethane, 4-tosyl chloride, NaOH, rt, 3 h (ii) ethyl acetate, 2-aminobenzenethiol, NaOH, 70 °C, 3 h; (iii) acetonitrile, chloroacetyl chloride, triethylamine, rt, 4 h; (iv) acetonitrile, secondary amine, K_2CO_3, 78 °C, 12 h.

pleuromutilin with *p*-toluenesulfonyl chloride in the presence of acetonitrile and inorganic base (sodium or potassium hydroxide)[13]. Compound **7** was prepared through the reaction of compound **6** with iodine by a nucleophilic substitution reaction. Compound **8** and the acyl chloride group of chloracetyl chloride were converted into 22–(2-(2-chloroacetamido)phenyl)thioacetyl-l-yl-22-deoxy pleuromutilin (compound **9**) through condensation reaction. The target = compounds containing alkylamine (compounds **10**–**14**) and nitrogen heterocycle (compounds **15**–**30**) were synthesised by secondary amine intermediate and compound **9** under alkaline condition. All compounds are > 95% pure by HPLC analysis.

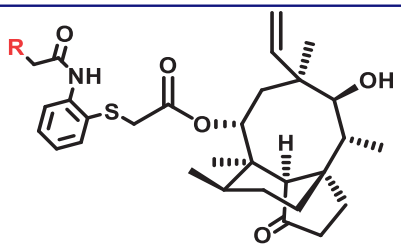
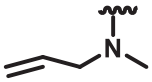
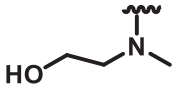
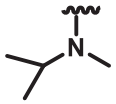
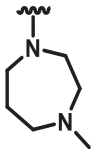
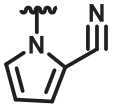
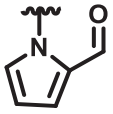
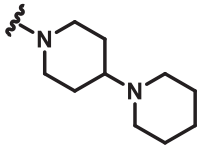
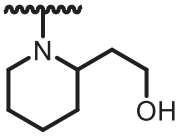
2.2. In vitro *antibacterial activity*

The *in vitro* antibacterial activity of these pleuromutilin derivatives was evaluated against MRSA (ATCC 43300), *S. aureus* (ATCC 29213), *S. aureus* (AD3), *S. aureus* (144) in accordance with the Clinical and Laboratory Standards Institute (CLSI)[17]. The MICs and MBCs of the 21 new pleuromutilin derivatives were determined by the broth micro dilution method. Tiamulin (**2**, Figure 1), valnemulin (**3**, Figure 1), retapamulin (**4**, Figure 1) and vancomycin were used as the reference antibacterial drugs. Results of MICs and MBCs were displayed in Table 1.

As presented in Table 1, the MIC values of most of synthesised compounds against MRSA (ATCC 43300), *S. aureus* (ATCC 29213), *S. aureus* (AD3) and *S. aureus* (144) ranged from 0.25 to 0.0625 μg/mL, 0.5 to 0.0625 μg/mL, 0.5 to 0.0625 and 1 to 0.125 μg/mL, respectively. The results showed that most of the tested compounds displayed stronger antibacterial activity than tiamulin against those four Gram-positive bacteria.

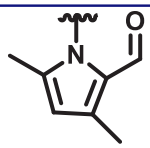
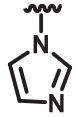
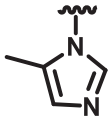
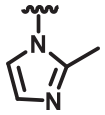
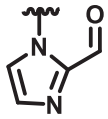
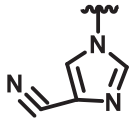
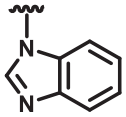
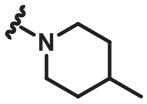
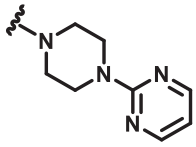
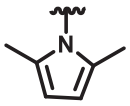
Compound **10** (MIC = 0.0625 μg/mL) exhibited superior antibacterial activity against MRSA than compound **11** (MIC = 0. 25 μg/

Table 1. MIC and MBC (μg/mL) values of compounds **10–30,** tiamulin, valnemulin, vancomycin, and retapamulin against MRSA (ATCC 43300), *S. aureus* (ATCC 29213), *S. aureus* (AD3) and *S. aureus* (144).

		MIC/MBC(μg/mL)			
Compound No	R	MRSA ATCC 43300	*S.aureus* ATCC 29213	*S.aureus* AD3	*S.aureus* 144
					
10		0.0625/0.125	0.0625/0.25	0.0625/0.5	0.0625/0.25
11		0.25/0.25	0.25/1	0.5/2	0.5/2
12		0.125/0.25	0.125/0.25	0.125/2	0.25/0.5
13		0.0625/0.125	0.0625/0.25	0.0625/0.5	0.125/0.5
14		0.125/0.25	0.125/0.25	0.125/2	0.25/0.5
15		0.25/0.5	0.25/0.5	0.25/2	0.25/0.5
16		>16/>32	>16/>32	>16/>32	>16/>32
17		0.125/>16	0.125/>16	0.125/>16	0.125/>16
18		0.0625/0.125	0.0625/0.25	0.0625/0.5	0.125/0.5
19		0.125/0.5	0.125/0.5	0.25/1	0.25/0. 5
20		0.125/0.25	0.0625/0.25	0.125/0.5	0.125/0.5

(*continued*)

Table 1. Continued.

Compound No	R	MIC/MBC(μg/mL) MRSA ATCC 43300	*S.aureus* ATCC 29213	*S.aureus* AD3	*S.aureus* 144
21		>16/>32	>16/>32	>16/>32	>16/>32
22		0.125/0.25	0.125/0.5	0.125/1	0.25/0.5
23		0.25/0.25	0.25/0.25	0.25/2	0.25/0.5
24		0.25/0.5	0.25/0.5	0.5/4	0.5/1
25		0.25/0.5	0.5/1	0.5/2	0.5/1
26		0.25/0.25	0.25/1	0.25/2	0.5/0.5
27		0.125/0.25	0.125/0.25	0.25/2	0.25/1
28		0.125/1	0.5/2	0.5/4	1/4
29		0.125/0.125	0.125/1	0.125/2	0.125/1
30		>16/>32	>16/>32	>16/>32	>16/>32
Tiamulin		0.25/0.5	0.5/1	0.5/2	0.5/2
Valnemulin		0.0625/0.25	0.0625/0.0625	0.0625/0.25	0.0625/0.125
Retapamulin		0.0625/0.125	0.0625/0.125	0.0625/0.25	0.125/0.25
Vancomycin		1/1	1/2	1/1	1/2

mL). Therefore, we hypothesised that the longer side chain of the introduced secondary amine might yield the poorer antibacterial activity, which was consistent with previously reported[18]. When the terminal methyl group in compound **11** was replaced by hydroxyl, compound **13** (MIC = 0.0625 μg/mL) was obtained. We hypothesised that the strong binding ability between hydroxyl and the residue of 50S ribosomes might be the reason for the superior activity of compound **13**. When the hydrogen atom on the C2 position of the pyrrole ring was substituted by nitrile and aldehyde group, compounds **17** and **18** were obtained. The MIC values of compound **17** and **18** against MRSA were 0.125 and 0.0625 μg/mL. The results showed that the compound containing stronger electron-withdrawing ability group relatively displayed better antibacterial activity[19]. In order to compare the difference

of compounds containing different nitrogen heterocycles, compounds **18** and **25** were prepared. As shown in Table 1, compound **18** displayed superior antibacterial activity than compound **25**. The results indicated that the superior antibacterial activity of compound **18** containing pyrrole ring might be related to the strong electron-rich ability, which was consistent with previously reported[20]. Compounds **19**, **20** and **28** exhibited superior antibacterial activity. This result indicated that these compounds containing the piperidinyl group showed a better antibacterial effect[21]. Compound **22** exhibited superior antibacterial activity against MRSA than compounds **23** and **24**. We hypothesised that the introduction of methyl groups at C2 and C5 sites of imidazole increased the steric hindrance of the compounds and might yield poorer antibacterial activity.

According to the previous studies[22], compounds were considered bactericidal when MBC/MIC ratio $\leq$ 4 and bacteriostatic when MBC/MIC ratio $>$ 4. The MBC/MIC ratios of most pleuromutilin derivatives against MRSA were $\leq$4. Thus, most of these compounds could be considered as prospective bactericidal agents.

Among these derivatives, compounds **10**, **13**, and **18** exhibited a greater bactericidal effect than other pleuromutilin derivatives. Therefore, the time-kill kinetics experiments of these three compounds were performed. Tiamulin and retapamulin were used as positive controls. The results were shown in Figure 2.

Compounds **10** and **18** at 4 $\times$ MIC induced inhibition of MRSA (-1.22 $\log_{10}$ CFU/mL and -1.17 $\log_{10}$ CFU/mL reduction) after 3 h incubation. Compound **13**, tiamulin and retapamulin at 2 $\times$ MIC induced killing of MRSA (-1.2 $\log_{10}$ CFU/mL, -0.96 $\log_{10}$ CFU/mL and -1.08 $\log_{10}$ CFU/mL reduction) after 3 h of incubation. 99.9% of MRSA was killed by 4 $\times$ MIC of compounds **10** and **18** at 24 h. After exposure for 24 h, compound **13** killed 99.9% of MRSA at 2 $\times$ MIC concentration[23]. The results indicated that compounds **10**, **13** and **18** against MRSA were time-dependent agents. For time-dependent antibacterial agents, multiple or continuous intravenous administration may achieve better therapeutic effects[23,24].

In order to explore the potential of these drugs for continuous inhibition of bacteria and provide rational dosing regimen, we performed the post-antibiotic effect (PAE) tests on compounds **10**, **13** and **18**. The bacterial growth kinetics curves and the PAE exhaustive results were presented in Figure 3 and Table 2, respectively. According to previous work of our team, the PAE of tiamulin at the concentrations of 2 $\times$ MIC and 4 $\times$ MIC for 1 h were 1.53 h and 1.90 h; after 2 h exposure, the PAE results for the same concentrations were 1.65 h and 2.04 h, respectively[14]. The results suggested that a similar post-antibacterial effect was observed between compound **10** and tiamulin ($P > 0.05$). The results also suggested that compound **13** displayed similar post-antibacterial effects to tiamulin ($P > 0.05$), while compound **18** exhibited a longer PAE than tiamulin. Thus, compound **18** could be administered at longer intervals than tiamulin in clinical use.

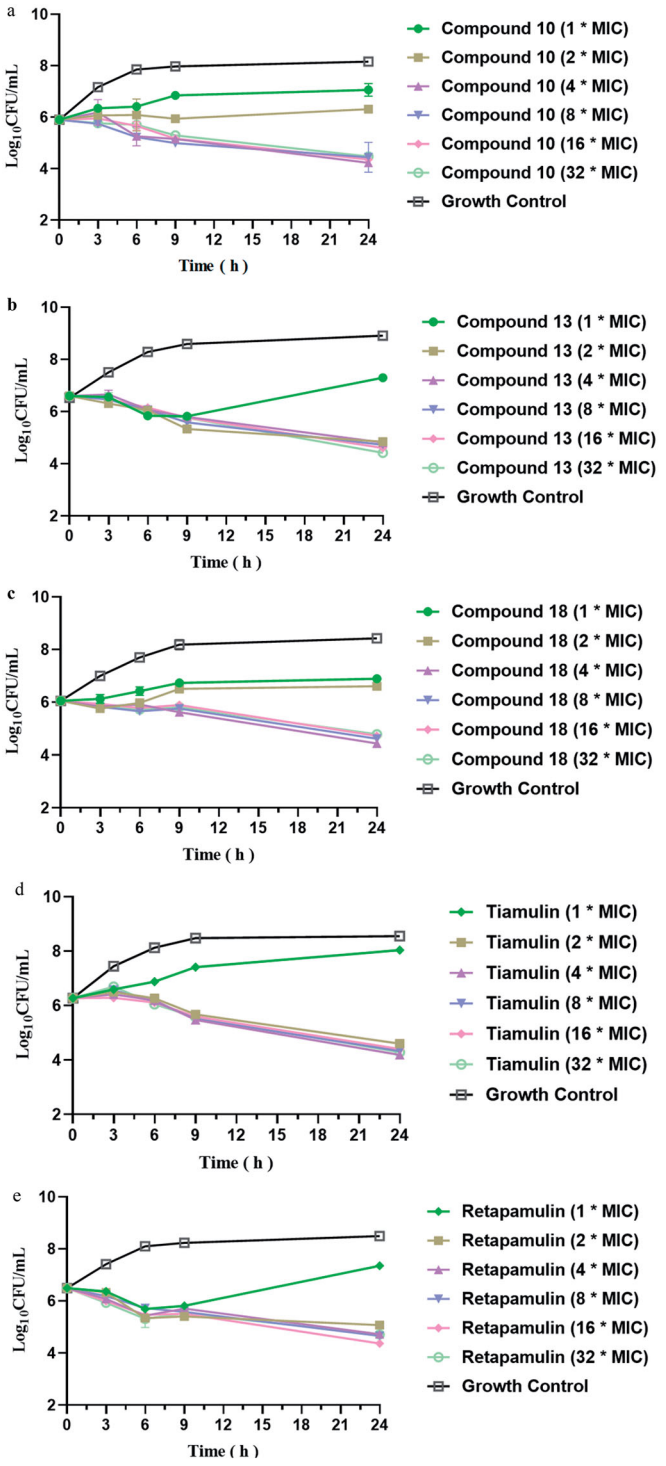


Figure 2. Time-kill curves for MRSA ATCC 43300 with different concentrations of compounds ***10*** (a), ***13*** (b), ***18*** (c), tiamulin (d), retapamulin (e).

2.3. In vivo *antibacterial activity*

Since compound **13** showed excellent *in vitro* antibacterial activity against MRSA, the *in vivo* antibacterial activity of compound **13** was further investigated by neutropenic murine thigh infection model. Tiamulin and vancomycin were used as positive controls, and physiological saline was used as a negative control. The results were shown in Figure 4.

Compared with no-drug control group, the experimental groups reduced the bacterial load in thigh muscle of mice -1.25 $\log_{10}$ CFU/mL (tiamulin), -1.84 $\log_{10}$ CFU/mL (vancomycin), -1.78 $\log_{10}$ CFU/mL (compound **13**), respectively. The significant statistically difference between tiamulin and compound **13** were observed ($P < 0.001$, $n = 6$/group). Compound **13** exhibited a significant treatment effect against MRSA compared to tiamulin (-0.53 $\log_{10}$ CFU/mL). No statistical difference was observed between the compound **13** and vancomycin in the infection model. The results showed that compound **13** could be used to treat MRSA infection *in vivo* and was more effective than tiamulin in reducing the MRSA load of thigh infected mice. Compound **13** exhibited a similar level of efficacy to vancomycin in reducing the MRSA load of thigh infected mice.

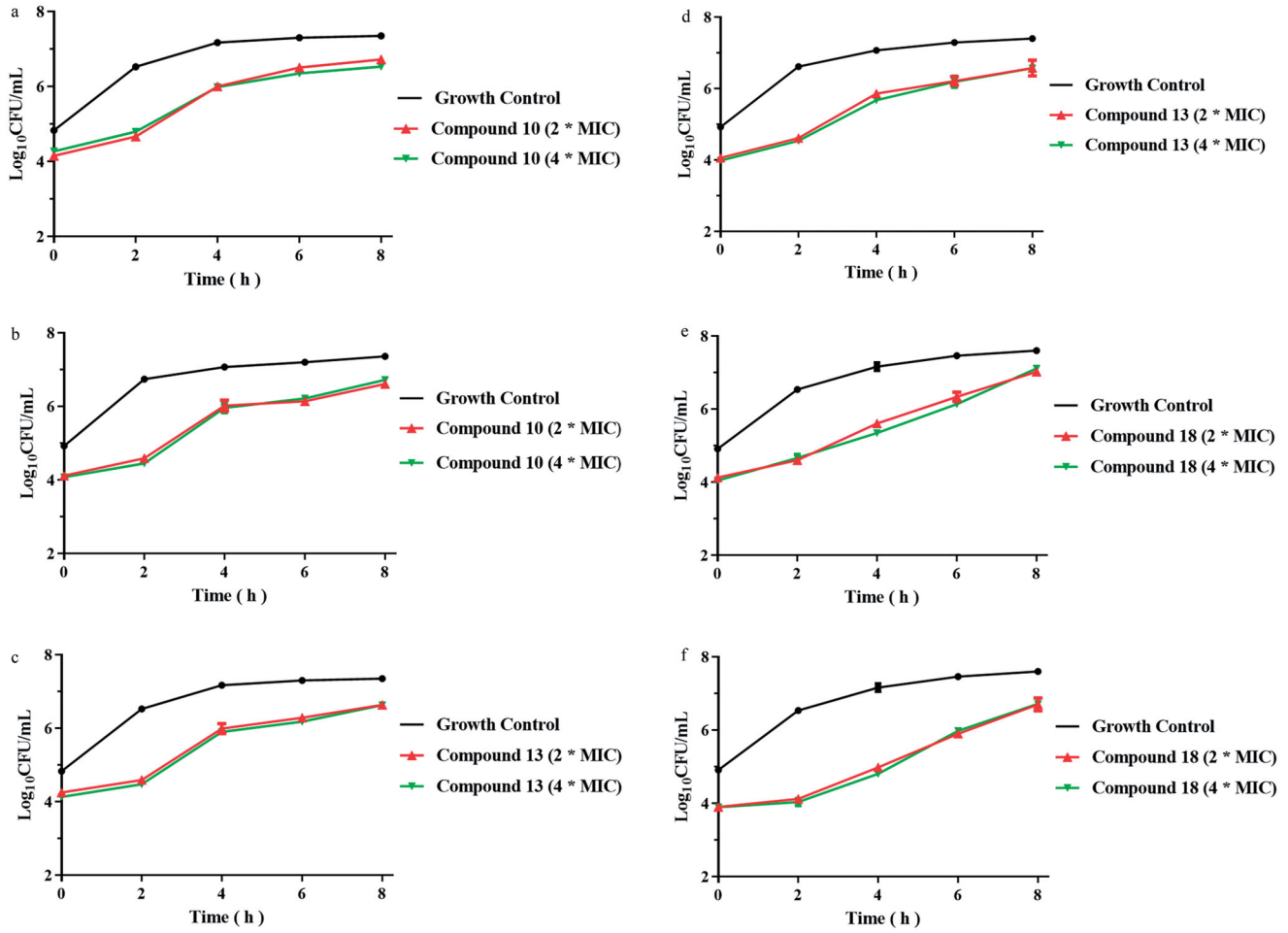


Figure 3. The bacterial growth kinetic curves for MRSA ATCC 43300 exposed to compound ***10*** (a), compound ***13*** (c) and compound ***18*** (e) for 1 h, compound ***10*** (b), compound ***13*** (d) and compound ***18*** (f) for 2 h.

Table 2. The PAEs values of compounds **10**, **13** and **18** against MRSA ATCC 43300.

		PAE (h)	
Compound	Concentration	Exposure for 1 h	Exposure for 2 h
Compound **10**	2 × MIC	1.55	1.63
	4 × MIC	1.62	1.73
Compound **13**	2 × MIC	1.54	1.76
	4 × MIC	1.61	1.90
Compound **18**	2 × MIC	1.81	2.58
	4 × MIC	1.90	3.00

2.4. Cytotoxicity assay

The cytotoxicity of compounds **10, 13**, **18** and tiamulin to RAW264.7 and human cell lines 16HBE (non-cancer cell line) cells were evaluated by MTT assay. The results were presented in Figure 5(a,b). At the concentration of 5 μg/mL, compounds **10**, **13**, **18** had no significant inhibitory effect on RAW264.7 cells. Under the same experimental conditions, we evaluated the cytotoxicity of these compounds and tiamulin at the concentration of 10 μg/mL. RAW264.7 cells treated with compounds **10** and **18** have a statistical difference compared with the control group. However, no statistical difference was observed between control group and compound **13** treated group. At the concentration of 5 μg/mL, compounds **13** and tiamulin had no significant inhibitory effect on 16HBE cells. At the concentration of 10 μg/mL, lower inhibitory effect was observed on compound **13** treated group. The result indicated that compound **13** was less cytotoxic than tiamulin on RAW264.7 cells and 16HBE cells.

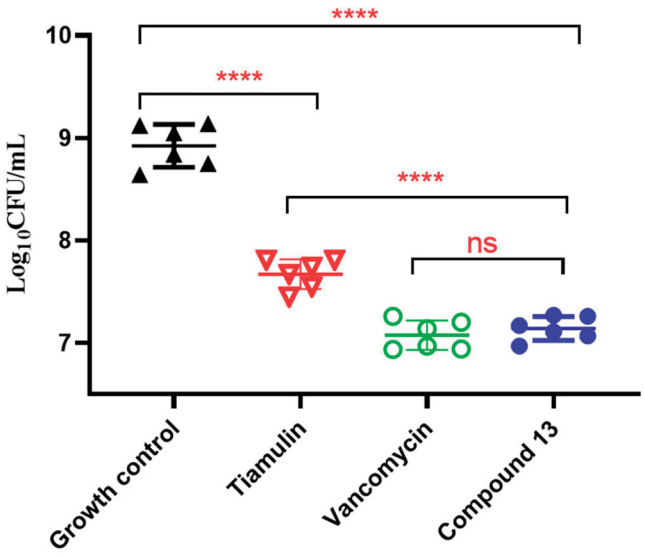


Figure 4. Efficacy of tiamulin, vancomycin, compound ***13*** against MRSA ATCC 43300 in murine neutropenic thigh models: black triangle: growth control; red triangle: tiamulin (20 mg/kg); green circular: vancomycin (20 mg/kg), blue circular: compound ***13*** (20 mg/kg).

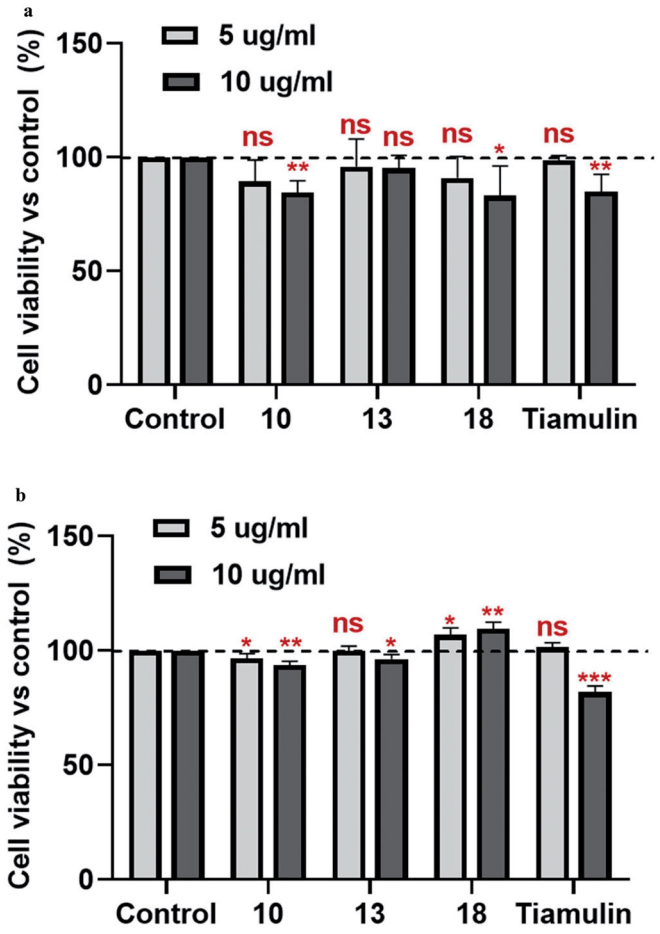


Figure 5. The cytotoxicity assay of compounds ***10***, ***13***, ***18*** and tiamulin to RAW264.7 (**a**) and 16HBE (**b**) cells at the concentration of 5 μg/mL and 10 μg/mL.

Compound **13** might exhibit better safety profile as compared to that of tiamulin.

2.5. Molecular docking study

A molecular docking study was performed to predict the possible binding mode of compound **13** with 50S ribosomes (PDB: 1XBP)[25]. Through Ledock software, the redocking of tiamulin into 1XBP placed the ligand in the same conformation as that in the X-ray structure (RMSD = 0.728). The binding free energy of compound **13** with the 50S ribosome was calculated to be −9.66 kcal/mol. The docking results of compound **13** show similar binding mode (Figure 6(a)) to tiamulin. The docking results presented a superimposition mode of 50S ribosomes and compound **13**.

Three hydrogen bonds played an important role on the binding of compound **13** to 50S ribosome. One hydrogen bond (distance: 2.5 Å) was formed between the ketone on the C3 atom of compound **13** (pleuromutilin core) and the residue of A-2482. One hydrogen bond (distance: 2.0 Å) was constituted by the amide side chain on the compound **13** and the residue of U-2564. The third hydrogen bond was constituted by the hydroxyl side chain on the compound **13** and the residues of C-2420 (distance: 2.2 Å). The superior docking mode of compound **13** demonstrated that it might has a stronger affinity with 50S ribosomes.

3. Conclusions

In this study, a series of novel pleuromutilin derivatives embracing alkylamine and nitrogen heterocycles were synthesised and evaluated for their *in vitro* and *in vivo* antibacterial activity against MRSA. Most of tested compounds exhibited superior antibacterial activities against MRSA and *S. aureus* than tiamulin. The time-kill curve results suggested that compounds **13** were in a time-dependent manner against MRSA. The PAE study indicated that compound **13** had a similar PAE time against MRSA compared with tiamulin. Compound **13** was more effective than tiamulin in the neutropenic murine thigh infection model, and exhibited a similar treatment effect compared to the vancomycin. Compound **13** formed three strong hydrogen bonds with residues of 50S ribosome. This study indicated that compound **13** could be served as a potential candidate against MRSA infections for further optimisation and discovery.

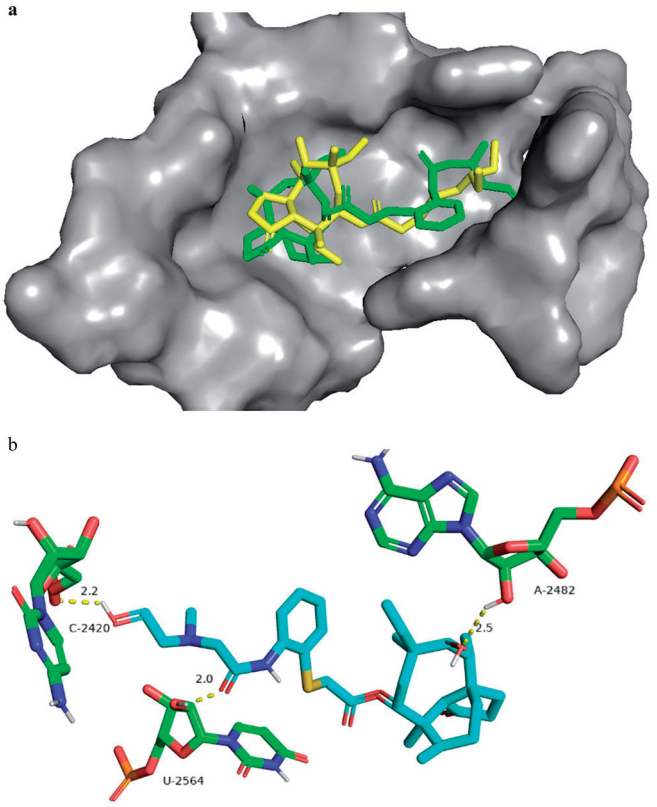


Figure 6. Docking mode of compound **13** to 1XBP (a). Docking mode of tiamulin (yellow) and compound **13** (green) to 1XBP. (b) 3 D representation of docking poses for compound **13** in the 50S ribosome residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

4. Experimental

4.1. Materials

Pleuromutilin (>90% pure) was purchased from Great Enjoyhood Biochemical Co. Ltd., (Daying, China). The aprotic solvent used in this experiment were purchased from TiTan Technology Co. Ltd., (Shanghai, China). The other analytical grade solvents were purchased from Guangzhou General Reagent Factory (Guangzhou, China). The target compounds were purified by silica gel column chromatography (200–300 mesh, Branch of Qingdao Haiyang Chemical Co. Ltd., Shandong, China). Melting Point Apparatus were purchased from Gongyi Yuhua Instrument Co., Ltd. The optical rotations were measured on an Autopol III automatic polarimeter (Rudolph Research analytical).

4.2. Synthesis

General method for the synthesis of novel pleuromutilin derivatives based on compound **1** were illustrated in Scheme 1. Compound 22–(2-(2-chloroacetamido)phenyl)thioacety-l-yl-22-deoxypleuromutilin(compound **9**) and a variety of secondary amine were prepared according to the literature. All those pleuromutilin derivatives were purified by silica column chromatography. These synthesised target compounds were characterised by ^{1}H NMR, ^{13}C NMR and high-resolution mass spectral (HR-MS) analysis. ^{1}H NMR and ^{13}C NMR spectra were processed with Bruker AV-400 and AV-600 spectrometer in chloroform-*d* or DMSO-d_6 using trimethylsilane as an internal standard. Chemical shift values (δ) were reported in ppm. High-resolution mass spectra were recorded by Thermo Scientific Q Exactive Focus Orbitrap LC-MS/MS with an electrospray ionisation (ESI) source. HPLC was performed using a Waters e2695 liquid chromatography column (Phe-nomenex 4.6 × 250 mm, 5 μm, mobile phase A: 0.1% formic acid in water; mobile phase B: methanol; mobile phase C: acetonitrile). The results confirmed that the chemical structures of pleuromutilin derivatives were consistent with the expected structures.

4.2.1. 22–(2-Aminophenylsulfanyl)-22-deoxy pleuromutilin (compound 8)

Compound **6** (1 g, 1.87 mmol) and iodine (0.48 g, 1.87 mmol) were dissolved in ethyl acetate (30 ml). The mixture was refluxed at 70 °C for 3 h until the reaction was completed. Then 2-amino thiophenol (0.25 g, 2.04 mmol) and 10 N sodium hydroxide (0.75 g, 18.75 mmol) were added into the above solution. The mixture was refluxed at 78 °C for 3 h and the reaction was monitored by TLC (dichloromethane/meoh = 200:1 (v/v)). Upon completion, the mixture was washed with brine (30 ml × 3) and dichloromethane (50 ml × 3), dried with over anhydrous Na_2SO_4. The organic layer was removed *in vacuo* to yield pale yellow crude product. The crude product was purified with flash column chromatography to obtain the pure solid.

4.2.2. 22–(2-(2-Chloroacetamido)phenyl)thioacetyl–l-yl-22-deoxypleuromutilin (compound 9)

Compound **8** (1 g, 2.06 mmol) and chloroacetyl chloride (0.26 g, 2.27 mmol) were dissolved in acetonitrile (30 ml). 2–3 drops of triethylamine were dripped slowly into the above solution. The resulting reaction mixture was stirred at room temperature for 4 h until reaction was completed. A mixture of the reaction was washed with brine (30 ML) and ethyl acetate (50 ml) for three times, dried with over anhydrous Na_2SO_4.

Then, the organic layer was removed *in vacuo* to yield pure product.

4.2.3. General procedure for the synthesis of compounds 10–30

Compound **9** (2 g, 3.56 mmol) was dissolved in acetonitrile (20 ml). Then added K_2CO_3 (1.48 g, 10.68 mmol) and derivatives which contain alkylamine or nitrogen heterocycles groups (3.92 mmol) to the solution and stirred for 12 h at 78 °C until reaction was completed. The mixture was washed with dichloromethane (30 ml) and water (50 ml) for 3 times. The organic phase was dried over anhydrous Na_2SO_4 and evaporated in vacuum. The crude production was purified by silica gel column chromatography (dichloromethane: methanol = 60:1 or ethyl acetate: petroleum ether =1.5:1).

4.2.4. 22–(2-(2-(Dimethylamino)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 10)

White powder; yield: 75.2%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.23 (1H, s), 8.37 (1H, d, J = 8.1 Hz), 7.51 (1H, dd, J = 7.8, 1.5 Hz), 7.32 (1H, t, J = 7.1 Hz), 7.04–7.00 (1H, m), 6.40 (1H, dd, J = 17.4, 11.0 Hz, H19), 5.68 (1H, d, J = 8.5 Hz, H14), 5.32–5.29 (1H, m, H20), 5.16 (1H, dd, J = 17.4, 1.5 Hz, H20), 3.51–3.44 (2H, m), 3.34–3.30 (1H, m), 13.21 (2H, s, H22), 2.48 (6H, s), 2.29 (1H, d, J = 6.9 Hz), 2.25–2.23 (1H, m), 2.21 (1H, d, J = 3.8 Hz, 11-OH), 2.20–2.14 (1H, m), 2.05 (1H, s, H8), 1.96 (1H, dd, J = 16.0, 8.6 Hz, H10), 1.78–1.73 (1H, m, H6), 1.67–1.63 (1H, m, H2), 1.62–1.59 (1H, m, H2), 1.52 (1H, dd, J = 13.7, 3.3 Hz, H4), 1.44 (2H, d, J = 2.6 Hz, H7), 1.38 (3H, s, H15), 1.36–1.30 (2H, m, H8, H13), 1.11 (3H, s, H18), 0.87 (3H, d, J = 7.0 Hz, H17), 0.62 (3H, d, J = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-d_6) δ 217.57 (C3), 168.97, 167.79 (C21), 141.17, 138.51 (C19), 133.45, 129.06, 124.68, 123.91, 121.02, 115.57 (C20), 73.01 (C11), 70.37 (C14), 63.88, 57.62 (C4), 46.17, 45.38 (C9), 44.44 (C13), 43.99 (C12), 41.89 (C5), 40.54, 37.30 (C6), 36.82 (C10), 36.74 (C2), 34.44 (C22), 30.54 (C8), 28.90 (C7), 27.02 (C18), 24.90 (C1), 16.42 (C16), 14.92 (C15), 11.97 (C17); HR-MS (ESI): Calcd for $C_{32}H_{46}N_2O_5S$ ($M+H^+$): 571.3201; Found: 571.3187; Melting point: 73–76 °C; $[\alpha]_D^{26} = 2.5°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: Retention Time (RT) = 10.93 min, purity = 99.40%.

4.2.5. 22–(2-(2-(Methyl(propyl)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 11)

White powder; yield: 50.8%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.34 (1H, s), 8.43 (1H, dd, J = 8.3, 1.2 Hz), 7.50 (1H, dd, J = 7.8, 1.5 Hz), 7.34–7.30 (1H, m), 7.00 (1H, td, J = 7.6, 1.4 Hz), 6.41 (1H, dd, J = 17.4, 11.0 Hz, H19), 5.69 (1H, d, J = 8.5 Hz, H14), 5.31 (1H, dd, J = 9.6, 1.4 Hz, H20), 5.16 (1H, dd, J = 17.4, 1.5 Hz, H20), 3.49–3.38 (2H, m), 3.34–3.30 (1H, m, H3), 3.17 (2H, s), 2.53–2.48 (2H, m), 2.39 (3H, s), 2.29 (1H, d, J = 6.9 Hz), 2.24 (1H, d, J = 2.3 Hz,), 2.21 (1H, d, J = 4.1 Hz, 11-OH), 2.05 (1H, s, H8), 1.96 (1H, dd, J = 16.0, 8.6 Hz, H10), 1.75 (1H, d, J = 11.6 Hz), 1.62–1.58 (3H, m), 1.56 (1H, d, J = 7.4 Hz), 1.49 (1H, d, J = 8.6 Hz), 1.44 (1H, d, J = 3.5 Hz), 1.38 (3H, s, H15), 1.35–1.33 (1H, m), 1.32–1.29 (1H, m), 1.16 (1H, dd, J = 12.7, 5.2 Hz, H13), 1.12 (3H, s, H15), 1.07 (1H, d, J = 4.8 Hz, H13), 0.96 (3H, t, J = 7.4 Hz, H18), 0.86 (3H, d, J = 7.0 Hz, H17), 0.62 (3H, d, J = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-d_6) δ 217.56 (C3), 169.47, 167.70 (C21), 141.17, 138.63 (C19), 133.61, 129.14, 124.53, 123.35, 120.61, 115.56 (C20), 73.12 (C11), 73.01, 70.37 (C14), 62.33, 59.94 (C4), 57.62, 45.38 (C9), 44.43 (C13), 43.33 (C12), 41.88 (C5), 37.37 (C6), 36.82 (C10), 36.73 (C2), 34.44 (C22), 30.54 (C8), 28.89 (C7), 27.02 (C18), 24.91 (C1), 20.80, 16.39 (C16), 14.91 (C15), 12.10, 11.97 (C17); HR-MS (ESI): Calcd for $C_{34}H_{50}N_2O_5S$ ($M+H^+$): 599.3514; Found: 599.3499; Melting point: 54–57 °C; $[\alpha]_D^{26} = 2.6°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 9.44 min, purity = 97.84%.

4.2.6. 22–(2-(2-(n-Allylmethylamine)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 12)

White powder; yield: 31.4%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.33 (1H, s), 8.41–8.38 (1H, m), 7.54–7.50 (1H, m), 7.31 (1H, t, J = 7.1 Hz), 7.05–7.00 (1H, m), 6.35 (1H, dd, J = 17.5, 11.0 Hz, H19), 5.98–5.91 (1H, m), 5.67 (1H,d, J = 8.4 Hz), 5.30–5.25 (2H, m), 5.23–5.21 (1H, m, H20), 5.19 (1H, d, J = 1.4 Hz), 5.15–5.10 (1H, m, H20), 3.51–3.41 (2H, m), 3.35–3.33 (1H, m), 3.20 (5H, d, J = 5.2 Hz), 2.60 (2H, s), 2.42 (3H, s), 2.35 (1H, d, J = 9.3 Hz), 2.24 (1H,d, J = 8.4 Hz), 2.19 (1H, d, J = 9.2 Hz,11-OH), 2.08 (1H, s, H8), 1.94 (1H, dd, J = 16.0, 8.6 Hz, H10), 1.77–1.73 (1H, m, H6), 1.64–1.62 (1H, m, H2), 1.60 (1H, d, J = 3.2 Hz, H7), 1.47 (1H, dd, J = 5.9, 3.0 Hz, H4),

1.44 (1H, d, $J = 3.4$ Hz, H15), 1.37 (3H, s, H8, H13, H15), 1.11 (3H, s, H18), 0.88 (3H, d, $J = 7.0$ Hz, H17), 0.60 (3H, d, $J = 6.9$ Hz, H16). ^{13}C NMR (151 MHz, DMSO- d_6) δ 217.56 (C3), 169.18, 167.82 (C21), 141.16, 138.63 (C19), 135.79, 133.66, 129.14, 124.63, 123.62, 120.80, 118.66 (C20), 115.56, 73.01 (C11), 70.39 (C14), 61.31, 60.75, 57.62 (C4), 45.38 (C9), 44.43, 44.02 (C13), 43.19 (C12), 41.88 (C5), 37.46 (C6), 36.82 (C10), 36.73 (C2), 34.44 (C22), 30.54 (C8), 28.89 (C7), 27.02 (C18), 24.91 (C1), 16.39 (C16), 14.93 (C15), 11.97 (C17); HR-MS (ESI): Calcd for $C_{34}H_{48}N_2O_5S$ ($M + Na^+$): 619.3177; Found: 619.3160; Melting point: 58–61 °C; $[\alpha]_D^{26} = 3.3°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 10.52 min, purity = 97.57%.

4.2.7. 22–(2-(2-(n-Methylethanolamine)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 13)

White powder; yield: 78.9%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.41 (1H, s), 8.37–8.34 (1H, m), 7.54–7.52 (1H, m), 7.33 (1H, t, $J = 7.1$ Hz), 7.05–7.01 (1H, m), 6.38 (1H, dd, $J = 17.4$, 11.0 Hz, H19), 5.62 (1H, d, $J = 8.5$ Hz, H14), 5.29–5.26 (1H, m, H20), 5.17–5.12 (1H, m, H20), 3.76 (3H, s), 3.54–3.41 (2H, m), 3.34–3.22 (4H, m), 3.01 (1H, s), 2.80 (2H, d, $J = 5.2$ Hz), 2.47 (3H, s), 2.22 (1H, d, $J = 3.3$ Hz, 11-OH), 2.20–2.19 (1H, m), 2.01 (1H, s, H8), 1.89 (1H, dd, $J = 16.0$, 8.6 Hz, H10), 1.75 (1H, s, H6), 1.73–1.70 (1H, m, H6), 1.64–1.62 (1H, m, H2), 1.61–1.59 (2H, m, H7), 1.51–1.44 (3H, m, H4, H8, H13), 1.34 (3H, s, H15), 1.08 (3H, s, H18), 0.85 (3H, d, $J = 7.0$ Hz, H17), 0.50 (3H, d, $J = 7.0$ Hz, H16). ^{13}C NMR (151 MHz, DMSO- d_6) δ 217.59 (C3), 169.58, 167.89 (C21), 141.18, 138.54 (C19), 133.42, 129.03, 124.69, 124.27, 121.22, 115.61 (C20), 73.03 (C11), 70.35 (C14), 62.52, 60.08, 59.74, 57.64 (C4), 45.39 (C9), 44.44 (C13), 44.03, 43.96 (C12), 41.88 (C5), 37.21 (C6), 36.82 (C10), 36.75 (C2), 34.45 (C22), 30.55 (C8), 28.89 (C7), 27.03 (C18), 24.91 (C1), 16.44 (C16), 14.93 (C15), 11.97 (C17); HR-MS (ESI): Calcd for $C_{33}H_{48}N_2O_6S$ ($M\text{-}H^-$): 599.3160; Found: 599.3162; Melting point: 73–76 °C; $[\alpha]_D^{26} = 3.0°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 5.84 min, purity = 96.80%.

4.2.8. 22–(2-(2-(n-Isopropylmethylamine)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 14)

White powder; yield: 54.7%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.54 (1H, s), 8.44 (1H, d, $J = 8.2$ Hz), 7.51–7.49 (1H, m), 7.32 (1H, t, $J = 7.2$ Hz), 7.00 (1H, t, $J = 7.0$ Hz), 6.41 (1H, dd, $J = 17.4$, 11.0 Hz, H19), 5.69 (1H, d, $J = 8.5$ Hz, H14), 5.33–5.28 (1H, m, H20), 5.20–5.14 (1H,m), 3.43 (2H, d, $J = 4.8$ Hz), 3.35–3.30 (1H, m), 3.17 (2H, s, H22), 3.01–2.93 (1H, m), 2.37 (3H, s), 2.29 (1H, d, $J = 7.0$ Hz), 2.24 (1H, d, $J = 2.5$ Hz,), 2.05 (1H, s, H8), 1.95 (1H, dd, $J = 16.0$, 8.6 Hz, H10), 1.75 (2H, dd, $J = 14.5$, 2.7 Hz, H16), 1.62 (1H, s, H12), 1.49–1.45 (3H, m, H4, H8), 1.44 (d, $J = 3.3$ Hz, H14), 1.37 (3H, s, H15), 1.30 (1H, d, $J = 10.0$ Hz, H15), 1.25 (1H, t, $J = 5.2$ Hz), 1.11 (10H, d, $J = 6.8$ Hz, H8), 0.86 (3H, d, $J = 7.0$ Hz, H17), 0.62 (3H, d, $J = 7.0$ Hz, H16).^{13}C NMR (151 MHz, DMSO-d_6) δ 217.56 (C3), 169.95, 167.69 (C21), 141.18, 138.74 (C19), 133.73, 129.21, 124.38, 123.02, 120.23, 115.56 (C20), 73.01 (C11), 70.35 (C14), 58.12 (C4), 57.61, 54.12, 45.38 (C9), 44.41 (C13), 44.02 (C12), 41.87 (C5), 38.58, 37.28 (C6), 36.81 (C10), 36.72 (C2), 34.44 (C22), 30.54 (C8), 28.87 (C7), 27.02 (C18), 24.90 (C1), 18.68, 18.62, 16.40 (C16), 14.92 (C15), 11.96 (C17); HR-MS (ESI): Calcd for $C_{34}H_{50}N_2O_5S$ ($M\text{-}H^-$): 597.3367; Found: 597.3371; Melting point: 71–73 °C; $[\alpha]_D^{26} = 3.2°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 16.41 min, purity = 99.46%.

4.2.9. 22–(2-(2-(Azetidine)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 15)

White powder; yield: 57%; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 10.14 (1H, s), 8.37 (1H, d, $J = 8.0$ Hz), 7.51 (1H, dd, $J = 7.8$, 1.2 Hz), 7.31 (1H, t, $J = 7.2$ Hz), 7.03–7.00 (1H, m), 6.41 (1H, dd, $J = 17.4$, 11.0 Hz, H19), 5.69 (1H, d, $J = 8.5$ Hz, H14), 5.31–5.29 (1H, m, H20), 5.17–5.14 (1H, m), 3.54–3.44 (6H, m), 3.34–3.27 (4H, m), 2.28 (1H, d, $J = 6.9$ Hz), 2.25–2.20 (1H, m), 2.20–2.16 (3H, m, 11-OH, H22), 2.05 (1H, s, H8), 1.96 (1H, dd, $J = 16.0$, 8.6 Hz, H10), 1.75–1.73 (1H, m, H6), 1.64 (1H, d, $J = 3.3$ Hz), 1.62 (1H, s, H2), 1.49–1.43 (3H, m), 1.38 (3H, s, H15), 1.35–1.32 (1H, m, H8), 1.11 (3H, s, H18), 1.06 (1H, d, $J = 16.1$ Hz, H8), 0.86 (3H, d, $J = 7.0$ Hz, H17), 0.64 (3H, d, $J = 7.1$ Hz, H16). ^{13}C NMR (151 MHz, DMSO-d_6) δ 217.58 (C3), 168.65, 167.84 (C21), 141.16, 138.46 (C19), 133.39, 128.99, 124.72, 123.94, 121.05, 115.54 (C20), 73.01 (C11), 70.39 (C14), 63.24, 57.63 (C4), 55.80, 45.38 (C9), 44.45 (C13), 43.97 (C12), 41.89 (C5), 40.55, 37.43, 36.85 (C6), 36.74 (C10), 34.44 (C2), 30.55 (C8), 28.91 (C7), 27.02 (C18), 24.91 (C1), 17.59, 16.41 (C16), 14.88 (C15), 11.98 (C17).; HR-MS (ESI): Calcd for $C_{33}H_{46}N_2O_5S$ ($M + H^+$): 583.3201; Found: 583.3190; Melting point: 80–83 °C; $[\alpha]_D^{26} = 3.8°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 9.76 min, purity = 98.60%.

4.2.10. 22–(2-(2-(n-Methylhomopiperazinyl)acetamido))phenyl) thioacety-l-yl-22-deoxy pleuromutilin (compound 16)

White powder; yield: 73.2%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.37 (1H, s), 8.46–8.43 (1H, m), 7.53–7.50 (1H, m), 7.32 (1H, t, $J = 7.2$ Hz), 7.04–7.00 (1H, m), 6.38 (1H, dd, $J = 17.4$, 11.0 Hz, H19), 5.65 (1H, d, $J = 8.5$ Hz, H14), 5.29–5.26 (1H, m), 5.18–5.13 (1H, m, H20), 3.53–3.44 (2H, m), 3.34 (2H, s), 2.95 (4H, d, $J = 5.0$ Hz), 2.94–2.91 (3H, m), 2.88 (4H, d, $J = 5.3$ Hz), 2.48 (3H, s), 2.24 (1H, s), 2.21 (1H, d, $J = 3.8$ Hz, 11-OH), 2.04 (1H, s, H8), 2.00 (2H, d, $J = 5.7$ Hz), 1.94–1.88 (1H, m, H10), 1.77–1.71 (2H, m), 1.62 (2H, t, $J = 8.3$ Hz, H2), 1.49–1.43 (3H, m, H13), 1.36 (3H, s, H18), 1.32–1.28 (1H,m, H8), 1.10 (3H, s, H18), 0.86 (3H,d, $J = 7.0$ Hz, H17), 0.60 (3H, d, $J = 7.0$ Hz, H16).^{13}C NMR (151 MHz, DMSO-d_6) δ 217.57(C3), 169.44, 167.83(C21), 141.19, 138.68(C19), 133.59, 129.13, 124.61, 123.50, 120.72, 115.57(C20), 73.00(C11), 70.42(C14), 62.99, 57.63(C4), 57.61, 56.28, 55.11, 46.61, 45.38(C9), 44.44(C13), 43.99(C12), 41.88(C5), 37.47(C6), 36.83(C10), 36.73(C2), 34.44(C22), 30.54(C8), 28.91(C7), 27.03(C18), 24.90(C1), 16.45(C16), 14.95(C15), 11.98(C17).HR-MS (ESI): Calcd for $C_{36}H_{53}N_3O_5S$ ($M + H^+$): 640.3779; Found: 640.3766; Melting point: 118–124 °C; $[\alpha]_D^{26} = 2.9°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 4.65 min, purity = 99.53%.

4.2.11. 22–(2-(2-(Pyrrole-2-nitrile)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 17)

White powder; yield: 40.7%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.18 (1H, s), 8.32 (1H, d, $J = 8.1$ Hz), 7.55–7.52 (1H, m), 7.35 (1H, t, $J = 7.1$ Hz), 7.04 (3H, dd, $J = 4.4$, 1.4 Hz), 6.92 (1H, dd, $J = 4.0$, 1.5 Hz), 6.43 (1H, dd, $J = 17.4$, 11.0 Hz, H19), 6.35–6.33 (1H, m), 5.68 (1H, d, $J = 8.5$ Hz, H14), 5.28–5.25 (1H, m), 5.17–5.11 (1H, m, H20), 4.99 (2H, d, $J = 2.4$ Hz), 3.39–3.29 (2H, m), 2.29 (1H, d, $J = 6.8$ Hz), 2.22–2.20 (1H, m, 11-OH), 2.20–2.14 (1H, m), 2.04 (1H, s, H8), 1.99 (1H, dd, $J = 16.0$, 8.6 Hz, H10), 1.77 (1H, d, $J = 11.6$ Hz, H6), 1.69–1.63 (1H, m), 1.62–1.61 (1H, m, H12), 1.61–1.58 (1H, m), 1.52 (1H, dd, $J = 10.8$, 2.8 Hz, H4), 1.47 (2H, d, $J = 3.0$) , 1.37 (3H, s, H15), 1.33–1.24 (2H, m, H13), 1.11 (3H, s, H18), 0.89 (3H, d, $J = 7.0$ Hz, H17), 0.52 (3H, d, $J = 7.0$ Hz, H16). ^{13}C NMR (151 MHz, DMSO-d_6) δ 217.60 (C3), 168.17 (C21), 165.94, 141.22 (C19), 136.59, 131.00, 129.84, 129.18, 127.75, 126.41, 125.31, 120.79, 115.69 (C20), 114.09, 109.67, 104.17, 73.06 (C11), 70.48 (C14), 57.69 (C4), 51.21, 45.41 (C9), 44.50 (C13), 44.11 (C12), 41.94 (C5), 36.86 (C6), 36.79 (C10), 36.42 (C2), 34.46 (C22), 30.57 (C8), 28.99 (C7), 27.04 (C18), 24.93 (C1), 16.51 (C16), 14.56 (C15), 12.01 (C17); HR-MS (ESI): Calcd for $C_{35}H_{43}N_3O_5S$ ($M + Na^+$): 640.2816; Found: 640.2795; Melting

point: 85–88 °C; $[\alpha]_D^{27} = 1.7°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 8.34 min, purity =98.21%.

4.2.12. 22–(2-(2-(Pyrrole-2-carboxaldehyde)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 18)

White powder; yield: 68.8%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.59 (1H, d, *J* = 1.0 Hz), 9.00 (1H, s), 8.31 (1H, d, *J* = 7.8 Hz), 7.53–7.49 (1H, m), 7.31 (1H, t, *J* = 7.1 Hz), 7.11–7.10 (1H, m), 7.07–7.05 (1H, m), 7.03–6.99 (1H, m), 6.44 (1H, dd, *J* = 17.4, 11.0 Hz), 6.39–6.37 (1H, m), 5.68 (1H, d, *J* = 8.5 Hz), 5.33–5.11 (5H, m), 3.33 (2H, d, *J* = 9.4 Hz), 2.32–2.28 (1H, m), 2.25 (1H, dd, *J* = 8.7, 2.6 Hz, 11-OH), 2.21–2.16 (1H, m, H8), 2.05 (2H, d, *J* = 4.1 Hz), 1.98 (1H, dd, *J* = 16.0, 8.6 Hz, H10), 1.78–1.73 (1H, m, H6), 1.66–1.64 (1H, m, H2), 1.62 (1H, d, *J* = 2.5 Hz), 1.61–1.58 (1H, m), 1.51 (1H, d, *J* = 3.5 Hz, H4), 1.49–1.46 (2H, m, H15), 1.44 (1H, d, *J* = 3.6 Hz, H15), 1.38 (3H, s, H8, H13), 1.12 (3H, s, H18), 0.88 (3H, d, *J* = 7.0 Hz, H17), 0.56 (3H, d, *J* = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 217.60 (C3), 180.01, 168.17, 166.88 (C21), 141.21, 137.30 (C19), 133.75, 131.97, 131.71, 128.05, 125.90, 124.67, 115.69 (C20), 110.03, 73.07 (C11), 70.43 (C14), 60.23, 57.69 (C4), 51.83, 45.41 (C9), 44.49 (C13), 44.11 (C12), 41.93 (C5), 36.85 (C6), 36.78 (C10), 36.62 (C2), 34.46 (C22), 30.57 (C8), 28.96 (C7), 27.04 (C18), 24.93 (C1), 21.24, 16.50 (C16), 14.56 (C15), 12.0 (C17); HR-MS (ESI): Calcd for $C_{35}H_{44}N_2O_6S$ ($M + Na^+$): 643.2813; Found: 643.2790; Melting point: 85–88 °C; $[\alpha]_D^{27} = 1.2°$ (c = 1.00, CH_2Cl_2);Analytical HPLC: RT =10.35 min, purity = 99.73%.

4.2.13. 22–(2-(2–(1,4′-Bipiperidin)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 19)

White powder; yield: 64%; ^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 10.16 (1H, s), 8.20 (1H, d, *J* = 8.1 Hz), 7.56 (1H, d, *J* = 7.7 Hz), 7.29 (1H,t, *J* = 7.7 Hz), 7.06 (1H, t, *J* = 7.5 Hz), 6.03 (1H, dd, *J* = 17.1, 11.8 Hz, H19), 5.47 (1H, d, *J* = 8.3 Hz, H14), 4.93 (1H, s, H20), 4.91 (1H, d, *J* = 5.6 Hz, H20), 4.48 (1H, d, *J* = 6.1 Hz), 3.86–3.72 (2H, m), 3.38 (1H, d, *J* = 6.0 Hz, H11), 3.33 (2H, s, H22), 3.15–3.06 (2H, m), 2.94 (1H, d, *J* = 3.2 Hz), 2.50 (2H, s), 2.48 (3H, s, H2, H4), 2.35 (1H, s, 11-OH), 2.23 (2H, d, *J* = 11.0 Hz), 2.18–2.14 (1H, m, H10), 2.01 (1H, d, *J* = 6.2 Hz, H13), 1.95 (1H, dd, *J* = 15.8, 8.4 Hz, H13), 1.69 (5H, d, *J* = 11.8 Hz), 1.61 (2H, dd, *J* = 19.6, 12.3 Hz, H7), 1.47 (5H, d, *J* = 16.9 Hz), 1.38 (3H, s), 1.29 (3H, s, H15), 1.28–1.22 (2H, m, H8, H13), 0.98 (3H, s, H18), 0.79 (3H, d, *J* = 7.0 Hz, H17), 0.51 (3H, d, *J* = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 217.55 (C3), 168.96 (C21), 141.17, 138.50 (C19), 133.30, 129.02, 124.55, 124.29, 123.32, 120.48, 115.53 (C20), 73.01 (C11), 70.23 (C14), 62.20, 57.60 (C4), 57.42, 55.38, 55.34, 53.83, 50.16 (C9), 45.38 (C13), 44.0 (C12)1, 43.66, 41.86 (C5), 41.77, 37.38, 37.19, 36.82 (C10), 36.72 (C2), 34.44 (C22), 34.35, 30.55 (C8), 28.86 (C7), 28.13, 27.04, 26.57 (C18), 24.90 (C1), 16.42 (C16), 14.90 (C15), 11.96 (C17); HR-MS (ESI): Calcd for $C_{40}H_{59}N_3O_5S$ ($M + H^+$): 694.4249; Found: 694.4207; Melting point: 93–97 °C; $[\alpha]_D^{27} = 2.4°$ (c = 1.00, CH_2Cl_2);Analytical HPLC: RT = 4.17 min, purity = 95.67%.

4.2.14. 22–(2–(2-(2-Piperidineethanol)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 20)

White powder; yield: 67.7%; ^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 10.39 (1H, s), 8.27 (1H, d, *J* = 7.8 Hz), 7.55 (1H, d, *J* = 7.7 Hz), 7.29 (1H, t, *J* = 7.7 Hz), 7.05 (1H, t, *J* = 7.5 Hz), 6.03 (1H, dt, *J* = 17.4, 11.4 Hz, H19), 5.48 (1H, d, *J* = 8.2 Hz, H20), 4.97–4.88 (2H, m), 4.48 (1H, d, *J* = 6.0 Hz), 4.40 (1H, t, *J* = 4.8 Hz), 3.86–3.69 (2H, m), 3.47 (1H, dq, *J* = 10.9, 5.3 Hz), 3.42–3.40 (1H, m), 3.38 (2H, t, *J* = 6.0 Hz), 3.29 (1H, d, *J* = 17.1 Hz), 3.05 (1H, d, *J* = 17.2 Hz), 2.83 (1H, d, *J* = 6.1 Hz), 2.57 (1H, s), 2.50 (1H, s), 2.44–2.39 (1H, m, H3), 2.35 (1H, s, H7), 2.17 (1H, dd, *J* = 18.9, 11.0 Hz, H13), 2.08–2.01 (2H, m), 1.98–1.93 (1H, m, H10), 1.76 (1H, d, *J* = 10.2 Hz, H2), 1.70–1.66 (1H, m, H2), 1.60 (5H, d, *J* = 11.1 Hz), 1.54 (1H, dd, *J* = 13.7, 7.4 Hz, H7), 1.43 (1H, dd, *J* = 7.5, 3.6 Hz, H7), 1.39 (1H, d, *J* = 14.0 Hz), 1.34 (2H, d, *J* = 13.6 Hz, H7), 1.29 (3H, d, *J* = 12.7 Hz), 1.26–1.19 (2H, m, H15), 1.04 (1H, dd, *J* = 15.9, 6.0 Hz, H15), 0.99 (3H, d, *J* = 2.8 Hz, H18), 0.79 (3H, d, *J* = 6.9 Hz, H17), 0.50 (3H, t, *J* = 7.6 Hz, H16). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 217.58 (C3), 170.06, 167.76 (C21), 167.65, 141.19, 141.14, 138.83 (C19), 133.90, 133.80, 129.26, 129.22, 124.34, 122.79, 120.09, 120.05, 115.56 (C20), 73.02 (C14), 70.38 (C14), 58.65, 57.63 (C4), 45.39 (C9), 44.42 (C13), 44.05, 44.01 (C12), 41.88 (C5), 37.42 (C6), 36.82 (C10), 36.73 (C22), 34.44 (C22), 30.56 (C8), 28.88 (C7), 27.02 (C18), 24.91 (C1), 22.57, 16.40 (C16), 14.92 (C15), 11.97 (C17); HR-MS (ESI): Calcd for $C_{34}H_{48}N_2O_7S_2$ ($M + H^+$): 655.3776; Found: 655.3735; Melting point: 89–95 °C; $[\alpha]_D^{27} = 2.5°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 8.86 min, purity = 99.40%.

4.2.15. 22–(2-(2–(3,5-Dimethyl-1H-pyrrole-2-carboxaldehyde)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 21)

White powder; yield: 16.4%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.62 (1H, s), 9.00 (1H, s), 8.30 (1H, d, *J* = 7.7 Hz), 7.49 (1H, dd, *J* = 7.8, 1.5 Hz), 7.30 (1H, t, *J* = 7.1 Hz), 7.02–6.98 (1H, m), 6.43 (1H, dd, *J* = 17.4, 11.0 Hz, H19), 5.95 (1H, s), 5.68 (1H, d, *J* = 8.5 Hz, H14), 5.32–5.29 (1H, m, H20), 5.23 (1H, d, *J* = 16.1 Hz), 5.18 (1H, s), 5.13 (1H, d, *J* = 1.5 Hz), 3.38–3.28 (2H, m), 2.35 (3H, s), 2.32 (3H, s), 2.25–2.19 (2H, m), 2.05 (2H, d, *J* = 4.0 Hz, H8), 1.97 (1H, dd, *J* = 16.0, 8.6 Hz, H10), 1.75 (1H, d, *J* = 11.7 Hz), 1.68–1.64 (1H, m), 1.64–1.60 (2H, m, H2), 1.59 (1H, d, *J* = 5.8 Hz), 1.49 (3H, dd, *J* = 13.9, 3.5 Hz, H4), 1.43 (2H, dd, *J* = 8.7, 4.8 Hz, H7), 1.37 (3H, s, H15), 1.11 (3H, s, H18), 0.87 (3H, d, *J* = 7.0 Hz, H17), 0.58 (3H, d, *J* = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 217.60 (C3), 177.28, 168.12 (C21), 166.87, 141.20, 137.25 (C19), 131.64, 128.03, 127.89, 126.01, 124.72, 115.67 (C20), 112.06, 73.05 (C11), 70.41 (C14), 60.23, 57.68 (C4), 48.26, 45.41 (C9), 44.48 (C13), 44.08 (C12), 41.92 (C5), 40.54, 36.85 (C6), 36.77 (C10), 36.66 (C2), 34.46 (C22), 30.56 (C8), 28.97 (C7), 27.04 (C18), 24.93 (C1), 21.24, 16.46 (C16), 14.98, 14.56 (C15), 12.01, 11.13 (C17); HR-MS (ESI): Calcd for $C_{37}H_{48}N_2O_6S$ ($M + Na^+$): 671.3126; Found: 671.3110; Melting point: 91–94 °C; $[\alpha]_D^{27} = 1.7°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 7.63 min, purity = 98.06%.

4.2.16. 22–(2-(2-(Imidazole acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 22)

White powder; yield: 27.6%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.16 (1H, s), 8.31 (1H,d, *J* = 8.1 Hz), 7.69 (1H, s), 7.51 (1H, dd, *J* = 7.8, 1.4 Hz), 7.34 (1H, t, *J* = 7.2 Hz), 7.19 (s, 1H), 7.14 (1H, s), 7.07–7.04 (1H, m), 6.38 (1H, dd, *J* = 17.5, 11.0 Hz, H19), 5.63 (1H, d, *J* = 8.5 Hz, H14) , 5.26 (1H, dd, *J* = 11.0, 1.1 Hz), 5.15 (1H, dd, *J* = 17.5, 1.2 Hz, H20), 4.90 (2H, s, H22), 3.42–3.24 (4H, m), 2.27 (1H, d, *J* = 7.1 Hz), 2.23 (1H, d, *J* = 3.7 Hz, 11-OH), 2.22–2.20 (1H, m), 2.04 (1H, s, H8), 1.97 (1H, d, *J* = 7.4 Hz, H10), 1.78–1.73 (3H, m), 1.59 (1H, d, *J* = 4.3 Hz, H2), 1.45 (1H, d, *J* = 3.3 Hz, H7), 1.42 (1H, s, H7), 1.36 (3H, s, H15), 1.34–1.28 (2H, m, H8, H13), 1.12 (3H, s, H18), 0.90 (3H, d, *J* = 7.0 Hz, H17), 0.48 (3H, d, *J* = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 217.61 (C3), 168.16, 166.56 (C21), 141.23, 138.73 (C19), 136.74, 131.10, 128.70, 127.82, 126.30, 125.03, 120.97, 115.68 (C20), 73.05 (C11), 70.48 (C14), 57.69 (C4), 55.38, 49.58, 45.42 (C9), 44.51 (C13), 44.10 (C12), 41.94 (C5), 36.87 (C6), 36.78 (C10), 36.51 (C2), 34.46 (C22), 30.56 (C8), 29.00 (C7), 27.04

(C18), 24.93 (C1), 16.51 (C16), 15.01 (C15), 12.01 (C17); HR-MS (ESI): Calcd for $C_{33}H_{43}N_3O_5S$ (M-H$^-$): 592.2850; Found: 592.2853; Melting point: 104–107 °C; $[\alpha]_D^{27} = 1.4°$ (c = 1.00, CH_2Cl_2);Analytical HPLC: RT = 4.28 min, purity = 99.44%.

4.2.17. 22–(2-(2–(4-Methylimidazole)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 23)

White powder; yield: 35.4%; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 9.05 (1H, d, *J* = 17.8 Hz), 8.32 (1H, t, *J* = 8.1 Hz), 7.59 (1H, d, *J* = 35.0 Hz), 7.51 (1H, d, *J* = 7.8 Hz), 7.36–7.33 (1H, m), 7.06–7.03 (1H, m), 6.86 (1H, d, *J* = 47.2 Hz), 6.40–6.36 (1H, m, H19), 5.65–5.62 (1H, m, H14), 5.32–5.25 (2H, m), 5.18–5.14 (1H, m, H20), 4.80 (2H, d, *J* = 14.5 Hz), 3.34–3.27 (3H, m), 2.27 (3H, d, *J* = 5.1 Hz,11-OH), 2.24 (2H, dd, *J* = 15.9, 8.2 Hz), 2.16 (1H, dd, *J* = 19.5, 9.4 Hz), 2.04 (1H, s, H8), 1.97–1.94 (1H, m, H10), 1.74 (1H, s, H4), 1.62 (3H, td, *J* = 10.5, 9.2, 3.7 Hz), 1.44 (4H, td, *J* = 10.7, 10.0, 4.7 Hz, H7, H3), 1.36 (3H, s, H8, H13), 1.11 (3H, s, H18), 0.89 (3H, t, *J* = 3.5 Hz, H17), 0.49 (3H, dd, *J* = 14.3, 7.1 Hz, H16). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 217.60 (C3), 168.14, 166.68 (C21), 141.23, 138.47 (C19), 131.17, 130.75, 126.25, 124.91, 117.14 (C20), 115.68, 73.05 (C17), 70.47 (C14), 57.68 (C4), 49.57, 47.46, 45.41 (C9), 44.51 (C13), 44.10 (C12), 41.94 (C5), 36.87, 36.78 (C6), 36.49 (C10), 34.46 (C2), 30.56 (C8), 28.99 (C7), 27.04 (C18), 24.93 (C1), 16.51, 16.48 (C16), 15.00 (C15), 14.09, 12.01 (C17), 9.20; HR-MS (ESI): Calcd for $C_{34}H_{45}N_3O_5S$ (M + H$^+$): 608.3153; Found: 608.3143; Melting point: 109–112 °C; $[\alpha]_D^{27} = 1.7°$ (c = 1.00, CH_2Cl_2);Analytical HPLC: RT = 4.54 min, purity = 99.22%.

4.2.18. 22–(2–(2-(2-Methylimidazole)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 24)

White powder; yield: 19.2%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.04 (1H, s), 8.31 (1H, d, *J* = 8.2 Hz), 7.51 (1H, dd, *J* = 7.8, 1.5 Hz), 7.34 (1H, t, *J* = 7.1 Hz), 7.04 (3H, dd, *J* = 4.2, 2.9 Hz), 6.37 (1H, dd, *J* = 17.5, 11.0 Hz, H19), 5.64 (1H, d, *J* = 8.5 Hz, H14), 5.28–5.25 (1H, m), 5.14 (1H, dd, *J* = 17.5, 1.4 Hz, H20), 4.79 (2H, s), 3.37–3.26 (3H, m), 2.48 (3H, s), 2.28 (1H, d, *J* = 6.3 Hz,), 2.26–2.22 (2H, m), 2.20 (1H, d, *J* = 3.7 Hz, 11-OH), 2.04 (1H, s, H8), 2.00–1.93 (1H, m, H10), 1.75 (dd, *J* = 14.4, 2.8 Hz, 2H), 1.61 (1H, s, H2), 1.58 (1H, dd, *J* = 7.0, 3.7 Hz), 1.46 (1H, d, *J* = 5.3 Hz), 1.44 (1H, d, *J* = 3.9 Hz, H4), 1.42–1.39 (1H, m, H7), 1.36 (3H, s, H15), 1.34–1.31 (1H, m, H7), 1.11 (3H, s, H18), 0.89 (3H, d, *J* = 7.0 Hz, H17), 0.48 (3H, d, *J* = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 217.61 (C3), 168.16, 166.51 (C21), 145.09, 141.22 (C19), 130.95, 127.75, 126.57, 126.42, 125.26, 121.45, 115.68 (C20), 73.04 (C11), 70.48 (C14), 57.68 (C4), 48.93, 45.41 (C9), 44.51 (C13), 44.09 (C12), 41.94 (C5), 40.54, 36.88 (C6), 36.77 (C10), 36.50 (C2), 34.46 (C22), 30.56 (C8), 29.02 (C7), 27.03 (C18), 24.93 (C1), 16.47 (C16), 15.01 (C15), 13.07, 12.01 (C17); HR-MS (ESI): Calcd for $C_{34}H_{45}N_3O_5S$ (M + H$^+$): 608.3153; Found: 608.3441; Melting point: 108–111 °C; $[\alpha]_D^{27} = 1.2°$ (c = 1.00, CH_2Cl_2);Analytical HPLC: RT = 4.83 min, purity = 99.56%.

4.2.19. 22–(2-(2-(Imidazole-2-carboxaldehyde)acetamido)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 25)

White powder; yield: 13.6%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.85 (1H, s), 9.36 (1H, s), 8.25 (1H, d, *J* = 8.2 Hz), 7.54 (1H, d, *J* = 7.7 Hz), 7.39 (1H, s), 7.35–7.31 (2H, m), 7.05 (1H, t, *J* = 7.5 Hz), 6.42 (1H, dd, *J* = 17.3, 11.0 Hz, H19), 5.69 (1H, d, *J* = 8.5 Hz, H14), 5.47–5.29 (2H, m), 5.18 (2H, dd, *J* = 48.6, 14.2 Hz, H20), 3.47 (1H, d, *J* = 16.2 Hz), 3.44–3.27 (2H, m, H22), 2.20 (2H,t, *J* = 8.1 Hz, 11-OH, H8), 2.02 (4H, dq, *J* = 24.6, 8.6, 7.6 Hz), 1.77 (1H, d, *J* = 12.6 Hz, H6), 1.61 (3H, dd, *J* = 13.8, 6.5 Hz, H4, H2), 1.46 (2H, d, *J* = 12.2 Hz, H7), 1.38 (3H, s, H15), 1.32–1.25 (2H, m, H8, H13), 1.11 (3H, s, H18), 0.89 (3H, d, *J* = 6.8 Hz, H17), 0.51 (3H, d, *J* = 6.8 Hz, H16); ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 217.61 (C3), 182.27, 168.22 (C21), 165.95, 141.22 (C19), 136.73, 131.17, 130.93, 129.26, 127.66, 126.28, 125.51, 118.90 (C20), 115.69, 73.07 (C11), 70.46 (C14), 57.70 (C4), 50.39, 45.42 (C9), 44.49 (C13), 44.12 (C12), 41.94 (C5), 40.54, 36.85 (C6), 36.79 (C10), 36.31 (C2), 34.46 (C22), 30.57 (C8), 28.97 (C7), 27.04 (C18), 24.93 (C1), 16.52 (C16), 14.56 (C15), 12.01 (C17); HR-MS (ESI): Calcd for $C_{34}H_{43}N_3O_6S$ (M-H$^-$): 620.2799; Found: 620.2802; Melting point: 98–101 °C; $[\alpha]_D^{27} = 2.1°$ (c = 1.00, CH_2Cl_2);Analytical HPLC: RT =5.12 min, purity = 98.23%.

4.2.20. 22–(2-(2–(1 h-imidazole-4-carbonitrile)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 26)

White powder; yield: 45.8%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.60 (1H, s), 8.25 (1H, d, *J* = 8.3 Hz), 7.73 (2H, s), 7.57 (1H, d, *J* = 7.7 Hz), 7.37 (1H,t, *J* = 7.8 Hz), 7.10 (1H, t, *J* = 7.5 Hz), 6.35 (1H, dd, *J* = 17.5, 11.1 Hz, H19), 5.62 (1H, *J* = 8.5 Hz, H14), 5.30 (1H, d, *J* = 1.1 Hz, H20), 5.22–5.11 (2H, m, H20), 4.97 (2H, d, *J* = 2.5 Hz), 3.57–3.38 (2H, m), 3.35 (1H, d, *J* = 5.5 Hz), 2.26 (2H, d, *J* = 20.7 Hz), 2.21 (2H, d, *J* = 7.7 Hz, H4), 2.05 (1H, s, H8), 2.03–1.97 (1H, m), 1.82–1.65 (2H, m), 1.60 (3H. d, *J* = 9.1 Hz, H15), 1.50–1.44 (2H, m, H8, H13), 1.37 (3H, s, H15), 1.12 (3H, s, H18), 0.92 (3H, d, *J* = 6.9 Hz, H17), 0.46 (3H, d, *J* = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 217.60 (C3), 168.17 (C21), 165.74, 141.26, 141.22 (C19), 136.25, 131.26, 130.45, 127.48, 126.55, 125.67, 116.06 (C20), 115.68, 112.19, 73.05 (C11), 70.49 (C14), 57.69 (C4), 49.90, 45.42 (C9), 44.51 (C13), 44.11 (C12), 41.94 (C5), 40.54, 36.87 (C6), 36.79 (C10), 36.20 (C2), 34.46 (C22), 30.56 (C8), 29.00 (C7), 27.04 (C18), 24.93 (C1), 16.53 (C16), 15.01 (C15), 12.01 (C17); HR-MS (ESI): Calcd for $C_{34}H_{42}N_4O_5S$ (M-H$^-$): 617.2803; Found: 617.2808; Melting point: 105–108 °C; $[\alpha]_D^{27} = -1.9°$ (c = 1.00, CH_2Cl_2);Analytical HPLC: RT = 5.34 min, purity = 97.36%.

4.2.21. 22–(2-(2-Benzimidazolylphenyl)thioacety-l-yl-22-deoxypleuromutilin (compound 27)

White powder; yield: 36.72%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.20 (1H, S), 8.21 (2H, d, *J* = 7.9 Hz), 7.80 (1H, s), 7.44 (1H, d, *J* = 6.3 Hz), 7.39 (1H, dd, *J* = 7.7, 1.4 Hz), 7.30 (1H, d, *J* = 7.1 Hz,), 7.26 (2H, d, *J* = 5.5 Hz), 7.25–7.21 (1H, m,), 6.94 (1H, t, *J* = 7.2 Hz), 6.25 (1H, dd, *J* = 17.4, 11.0 Hz, H19), 5.48 (1H, d, *J* = 8.5 Hz, H14), 5.16 (1H, d, *J* = 11.0 Hz, H20), 5.07 (2H, d, *J* = 2.8 Hz), 5.04–5.01 (1H, m), 3.23 (1H, d, *J* = 6.4 Hz, H22), 2.92 (2H, q, *J* = 16.2 Hz), 2.15–2.11 (2H, m), 1.95 (1H, s, H10), 1.90–1.85 (1H, m, H6), 1.72–1.66 (2H, m, H2), 1.51 (2H, dd, *J* = 11.0, 4.9 Hz, H7), 1.39 (1H, d, *J* = 2.9 Hz), 1.33 (1H, d, *J* = 2.3 Hz,), 1.27 (3H, s, H15), 1.24–1.15 (2H, m, H8, H13), 1.02 (3H, s, H18), 0.81 (3H, d, *J* = 7.0 Hz, H17), 0.39 (3H, d, *J* = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 168.16 (C3), 166.37 (C2), 145.40, 143.73, 141.22 (C19), 136.55, 134.76, 130.80, 129.50, 127.67, 126.48, 125.42, 122.90, 122.06, 119.88, 115.69 (C20), 110.75, 73.05 (C11), 70.48 (C14), 57.69 (C4), 47.61, 45.42 (C9), 44.52 (C13), 44.12 (C12), 41.95 (C5), 40.54, 36.87 (C6), 36.79 (C10), 36.38 (C2), 34.47 (C22), 30.57 (C8), 29.01 (C7), 27.05 (C18), 24.94 (C1), 16.52 (C16), 15.02 (C15), 12.02 (C17); HR-MS (ESI): Calcd for $C_{37}H_{45}N_3O_5S$ (M-H$^-$): 642.3007; Found: 642.3009; Melting point: 110–113 °C; $[\alpha]_D^{27} = 1.3°$ (c = 1.00, CH_2Cl_2);Analytical HPLC: RT = 6.23 min, purity =96.51%.

4.2.22. 22–(2-(2–(4-Methylpiperidine)phenyl)thioacety-l-yl-22-deoxypleuromutilin (compound 28)

White powder; yield: 67%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.16 (1H, s), 8.20 (1H, d, J = 8.1 Hz), 7.56 (1H, d, J = 7.8 Hz), 7.29 (1H, t, J = 7.7 Hz), 7.06 (1H, t, J = 7.6 Hz), 6.01 (1H, dd, J = 17.6, 11.4 Hz, H19), 5.46 (1H, d, J = 8.3 Hz, H14), 4.92–4.89 (2H, m, H20), 4.49 (1H, d, J = 6.1 Hz), 3.86–3.72 (2H, m), 3.37 (1H, t, J = 6.0 Hz,), 3.10 (2H, d, J = 5.7 Hz, H22), 2.87 (1H, s), 2.50 (2H, s), 2.35 (1H, s), 2.21 (3H, q, J = 16.5, 13.7 Hz,), 2.15 (1H, d, J = 11.0 Hz, 11-OH), 2.09–2.02 (2H, m), 1.95–1.90 (1H, m), 1.61 (5H, t, J = 15.5 Hz), 1.38–1.35 (4H, m, H7, H4, H2), 1.28 (3H, s, H15), 1.07–1.00 (2H, m, H8, H13), 0.98 (3H, s), 0.93 (3H, d, J = 5.4 Hz, H18), 0.79 (3H, d, J = 7.0 Hz, H17), 0.51 (3H, d, J = 7.1 Hz, H16). ^{13}C NMR (151 MHz, DMSO-d_6) δ 217.58 (C3), 169.08, 167.68 (C21), 141.16, 138.60 (C19), 133.43, 129.05, 124.54, 123.30, 120.55, 115.53 (C20), 73.00 (C11), 70.37 (C14), 62.59, 57.61 (C4), 54.18, 45.38 (C9), 44.44 (C13), 43.95 (C12), 41.88 (C5), 40.54, 37.28 (C6), 36.84 (C10), 36.73 (C2), 34.60 (C22), 34.56, 34.43, 30.54 (C8), 30.16, 28.92 (C7), 27.03 (C18), 24.91 (C1), 22.22, 16.44 (C16), 14.93 (C15), 11.98 (C17); HR-MS (ESI): Calcd for $C_{36}H_{52}N_2O_5S$ ($M+H^+$): 625.3670; Found: 625.3652; Melting point: 87–91 °C; $[\alpha]_D^{27} = 3.6°$ (c = 1.00, CH_2Cl_2);Analytical HPLC: RT = 12.23 min, purity = 95.23%.

4.2.23. 22–(2–(2-(2-(1-Piperazinyl)pyrimidine)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 29)

White powder; yield: 40.7%; ^{1}H NMR (400 MHz, Chloroform-d) δ 10.46 (1H, s), 8.43 (1H, s), 8.34 (2H,d, J = 4.7 Hz), 7.53 (1H, dd, J = 7.8, 1.3 Hz), 7.36–7.32 (1H, m), 7.06 (1H, d, J = 7.5 Hz), 6.54 (1H, t, J = 4.7 Hz), 6.38 (1H, dd, J = 17.4, 11.0 Hz, H19), 5.65 (1H, d, J = 8.5 Hz), 5.28 (1H, d, J = 11.1 Hz), 5.14 (1H, dd, J = 17.4, 1.3 Hz, H20), 4.00 (4H, s), 3.54–3.43 (2H, m), 3.31 (1H, d, J = 3.9 Hz), 3.29 (1H, s), 2.76 (4H, s), 2.28 (1H, d, J = 7.0 Hz, 11-OH), 2.25–2.23 (1H, m), 2.22–2.20 (1H, m), 2.03 (1H, s, H8), 1.92 (1H, dd, J = 16.0, 8.6 Hz, H10), 1.77–1.74 (1H, m, H2), 1.74–1.71 (1H, m, H2), 1.63–1.58 (2H, m), 1.48 (1H, s), 1.47–1.43 (3H, m, H7, H4), 1.36 (3H, s, H15), 1.34–1.27 (2H, m, H8, H13), 1.10 (3H, s, H18), 0.87 (3H, d, J = 7.0 Hz, H17), 0.58 (3H, d, J = 7.0 Hz, H16). ^{13}C NMR (151 MHz, DMSO-d_6) δ 217.54 (C3), 168.62, 167.92 (C21), 161.65, 158.39, 141.11, 138.83 (C19), 133.96, 129.24, 124.64, 123.45, 120.64, 115.52 (C20), 110.65, 73.00 (C11), 70.41 (C14), 62.09, 57.61 (C4), 55.38, 53.13, 45.37 (C9), 44.45 (C13), 43.97 (C12), 41.86 (C5), 40.54, 37.75 (C6), 36.81 (C10), 36.70 (C2), 34.43 (C22), 30.52 (C8), 28.89 (C7), 26.99 (C18), 24.90 (C1), 16.40 (C16), 14.94 (C15), 11.97 (C17). HR-MS (ESI): Calcd for $C_{38}H_{51}N_5O_5S$ ($M+Na^+$): 712.3504; Found: 712.3491; Melting point: 106–109 °C; $[\alpha]_D^{27} = 2.8°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 11.48 min, purity = 98.03%.

4.2.24. 22–(2–(2-(2-(2,5-Dimethyl-1H-pyrrol)pyrimidine)phenyl)thioacety-l-yl-22-deoxy pleuromutilin (compound 30)

White powder; yield: 36.5%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.67 (1H, s), 8.18 (1H, d, J = 8.1 Hz), 7.56 (1H, d, J = 7.8 Hz), 7.33–7.29 (1H, m), 7.08 (1H, t, J = 7.6 Hz), 6.14 (1H, t, J = 5.4 Hz, H19), 6.02 (1H, dd, J = 17.7, 11.2 Hz), 5.46 (1H, d, J = 8.4 Hz, H14), 4.96 (1H, d, J = 1.3 Hz), 4.94–4.93 (1H, m), 4.90 (1H, d, J = 1.5 Hz), 4.48 (1H, d, J = 6.1 Hz), 4.03–4.01 (3H, m), 3.81–3.69 (2H, m), 3.38 (1H, d, J = 6.1 Hz), 2.50 (2H, d, J = 1.6 Hz), 2.35 (1H, s), 2.17 (2H, dd, J = 18.6, 10.9 Hz, H4), 2.07 (1H d, J = 9.3 Hz, H8), 1.99 (2H, s), 1.94 (1H, dd, J = 15.8, 8.4 Hz), 1.66–1.60 (3H, m, H15), 1.43 (2H, dd, J = 10.4, 7.1, 3.4 Hz, H8, H13), 1.37 (1H, t, J = 4.1 Hz), 1.31 (1H, s), 1.27 (3H, s, H15), 1.18 (2H,t, J = 7.1 Hz), 0.99 (3H, s, H18), 0.81 (3H, d, J = 7.5 Hz, H17), 0.50 (3H, d, J = 7.1 Hz, H16). ^{13}C NMR (151 MHz, DMSO-d_6) δ 217.60 (C3), 177.28, 168.12 (C21), 166.87, 141.20 (C19), 137.25, 131.64, 128.03, 127.89, 126.01, 124.72, 115.67 (C20), 112.06, 73.05 (C11), 70.41 (C14), 57.68 (C4), 48.26 (C9), 45.41 (C13), 44.48 (C12), 44.08, 41.92 (C5), 40.54, 36.85 (C6), 36.77 (C10), 36.66 (C2), 34.46 (C22), 30.56 (C8), 28.97 (C7), 27.04 (C18), 24.93 (C1), 21.24, 16.46 (C16), 14.98, 14.56 (C15), 12.01 (C17), 11.13; HR-MS (ESI): Calcd for $C_{36}H_{48}N_2O_5S$ ($M\text{-}H^-$): 619.3211; Found: 619.3163; Melting point: 80–88 °C; $[\alpha]_D^{27} = 1.6°$ (c = 1.00, CH_2Cl_2); Analytical HPLC: RT = 7.09 min, purity = 95.04%.

4.3. In vitro *efficacy of pleuromutilin derivatives*

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

The antimicrobial activity of the newly designed pleuromutilin derivatives against MRSA (ATCC 43300), *S. aureus* (ATCC 29213), *S. aureus* (AD3), and *S. aureus* (144) were evaluated using tiamulin, valnemulin, retapamulin, and vancomycin as positive controls. The MIC value was recorded as the lowest concentration of the sample that completely inhibited the visible growth of bacteria after incubating in the 96-well plate for 18–24 h at 37 °C. Stock solutions of these derivatives were prepared by 90% ultrapure-water, 5% dimethyl sulfoxide (DMSO) and 5% Tween 80 at the concentration of 320μg/mL. According to the broth micro dilution methods, a series of two-fold dilution were prepared in Mueller-Hinton broth medium (MHB), and the amount of inoculation in each well was 5×10^5 CFU/mL.

Each concentration of these compounds and positive controls was conducted for three parallel experiments. After the MIC results were observed, the 96-well plate were kept in 37 °C in 5% CO_2 atmosphere for 24 h, and the MBC was determined by inoculating the borehole bacterial solution from no obvious bacterial growth on Mueller-Hinton agar plate (MHA)[26]. The MBC was recorded as the lowest concentration of these tested compounds that was able to produce a 99.9% decrease in viable bacterial cells on the agar plates.

4.3.2. Constant concentration time-kill curves

To investigate the antibacterial effect of compound **10**, compound **13**, and compound **18** at different drug concentrations against MRSA (ATCC 43300) *in vitro*, the time-kill cure experiment was performed. MRSA (ATCC 43300) was collected in logarithmic period then diluted to 1×10^6 CFU/mL. Bacterial solutions were treated with compound **10**, compound **13**, compound **18**, tiamulin, and retapamulin at concentrations of 2 × MIC, 4 × MIC, 8 × MIC, 16 × MIC, 32 × MIC, respectively. Mixtures were placed in 37 °C constant temperature shaking incubator. Samples(100 μL) were diluted in sterile saline and plated on MH agar plates at different time points (0, 3, 6, 9 and 24 h), respectively. These results were recorded after 18–24 h of incubation. Time-kill curves were established by plotting bacteria counts ($\log_{10}$ CFU/mL) versus time.

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

The post-antibiotic effect *in vitro* (PAE) of compound **10**, compound **13**, compound **18** and tiamulin against MRSA was performed by MH broth dilution method. A logarithmic-phase broth culture solution of MRSA was diluted in MH broth to a final count of approximately 1×10^6 CFU/mL. Bacterial solutions were treated with compounds **10**, **13**, **18**, and tiamulin at final concentrations of 2 × MIC, 4 × MIC, 8 × MIC, 16 × MIC, 32 × MIC, respectively. Two groups of mixtures were incubated at a 37 °C constant

temperature vibration incubator for 1 h and 2 h. After that, the drugs were removed from the mixtures by diluting 1000 times with the preheated MH broth. Two groups of samples were collected after the completion of dilution and were incubated under the condition of 37 °C constant temperature incubator, a 95% humidified atmosphere and 5% CO_2. Samples (100 μL) were diluted in sterile saline and plated on MH agar plates at the following time points (0, 2, 4, 6, and 8 h), respectively. Finally, these plates were incubated for 24 h, the number of colonies were counted manually. The PAE was calculated using the following equation: PAE = T_A - T_C (where T_A is the time required for the increase of 1 $\log_{10}$ CFU/mL from the removal of the antimicrobial, and T_C represents the time required for bacterial cultures not treated with an antimicrobial to increase 1 $\log_{10}$ CFU/mL) and expressed in an hour.

4.4. In vivo *efficacy of pleuromutilin derivatives*

4.4.1. Neutropenic murine thigh infection model

Six-week-old, specific pathogen-free (SPF), female ICR/Swiss mice weighing 22–28 g were used for a thigh infection model experiment. Neutropenia was induced by cyclophosphamide administration at 4 day (150 mg/kg) and 1 day (100 mg/kg) prior to infection by intraperitoneal injection. A logarithmic-phase culture solution of MRSA was diluted in MH broth to a final count of approximately 1×10^7 CFU/mL. After dilution, the concentration of 10^7 CFU/mL (0.1 ml/thigh) were injected into each thigh of the mice to produce a thigh infection model caused by MRSA. 2 h after infection, the thigh infection model was established. After two hours, mice were randomly divided into 3 groups (3 per group) and injected intraperitoneally with normal saline (control group), compound **13** (20 mg/kg), and tiamulin (20 mg/kg) as a control group and two experimental groups. Mice that received intravenous injections were euthanized 24 h, thigh tissue was aseptically collected, placed into pre-weighed tubes (containing 1 ml of iced saline), weighed, and homogenised. Ten-fold serial dilutions of thigh tissue homogenates were plated onto MH agar plates and the number of colonies were counted manually. This experiment protocols were reviewed and approved by the Committee on the Ethics of Animals of South China Agricultural University (Approval number: 2021C061).

4.5. Cytotoxicity assay

To assessed the effects of tiamulin and the target compounds on the viability of RAW264.7 macrophages and human cell lines 16HBE (non-cancer cell line), the MTT assay was performed as described in references[27]. RAW264.7 cells and 16HBE cells were seeded in a 96-well plate at a density of 1.0×10^5 cells per well.

Cells were treated with the target compounds (5 μg/mL and 10 μg/mL) and were cultured at 37 °C in the atmosphere of 5% CO_2 for 24 h. After incubation, 0.5 mg/mL MTT were add to per well for 4 h at 37 °C. After the MTT solution was discarded, the DMSO (150 μL) was added to each well to dissolve all crystals. The absorbance at 490 nm was recorded using a microplate spectrophotometer (BIO-TEK Instrument Inc, USA).

4.6. Molecular modeling

Docking studies revealed the binding modes of pleuromutilin derivatives analogs which based on the crystal structure of *S. aureus* 50S ribosomal in complex with tiamulin (PDB ID code: 1XBP). The peptidyl transferase centre (PTC), consisting primarily of all residues within 40 Å around the tiamulin in 1XBP. The binding site of tiamulin in 1XBP was set to the docking position. All compounds were prepared with Avogadro 1.1.1[28], with a 5000 steps Steepest Descent as well as 1000 steps Conjugate Gradients geometry optimisation using MMFF94 force field. Docking experiments were performed using LeDock Vina and Pymol[29]

Disclosure statement

No potential conflict of interest was reported by the author(s).

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