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PII: S0223-5234(20)30707-8

DOI: https://doi.org/10.1016/j.ejmech.2020.112735

Reference: EJMECH 112735

To appear in: *European Journal of Medicinal Chemistry*

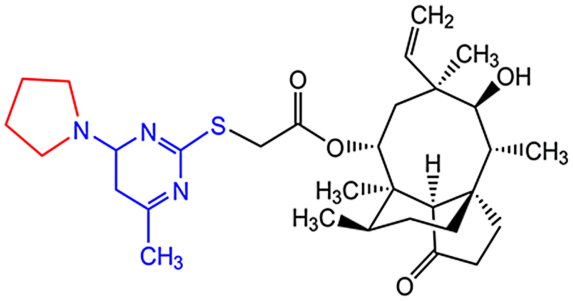
Received Date: 1 June 2020

Revised Date: 15 July 2020

Accepted Date: 5 August 2020

Please cite this article as: Y. Fan, Y. Liu, H. Wang, T. Shi, F. Cheng, B. Hao, Y. Yi, R. Shang, Novel pleuromutilin derivatives with substituted 6-methylpyrimidine: Design, synthesis and antibacterial evaluation, *European Journal of Medicinal Chemistry*, https://doi.org/10.1016/j.ejmech.2020.112735.

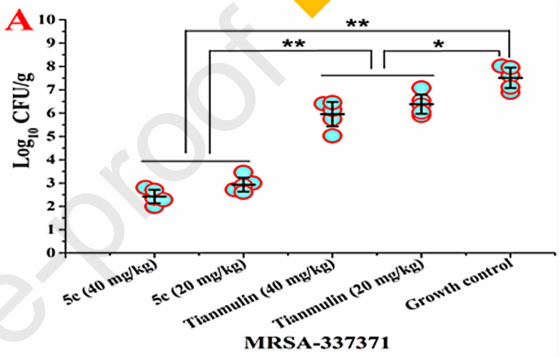
Structures of compounds **5c** and it antibacterial activities



5c

MIC = 0.0562 μM (*S. aureus*)
MIC = 0.1124 μM (MRSE)
MIC = 0.0562 μM (MRSA)
MIC = 0.0562 μM (*S. dysgalactiae*)
MIC = 0.0562 μM (*S. agalactiae*)

ED_{50} = 18.02 mg/kg (MRSA)



Novel pleuromutilin derivatives with substituted 6-methylpyrimidine: Design, synthesis and antibacterial evaluation

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Abstract: A series of novel pleuromutilin derivatives with substituted 6-methylpyrimidine moieties was designed, synthesized, and evaluated for their antibacterial activities. Most of the tested compounds exhibited potent antibacterial activities against *Staphylococcus aureus* ATCC 25923 (*S. aureus*-25923), methicillin-resistant *Staphylococcus epidermidis* ATCC 51625 (MRSE-51625), methicillin-resistant *Staphylococcus aureus* BNCC 337371 (MRSA-337371), *Streptococcus dysgalactiae* (*S. dysgalactiae*) and *Streptococcus agalactiae* (*S. agalactiae*). Compounds **5c** and **5g** were the most active and displayed bacteriostatic activities against MRSA. *In vivo* mouse systemic infection experiment showed that **5c** significantly improved the survival rate of mice (ED_{50} = 18.02 mg/kg), reduced the bacterial load and alleviated the pathological changes in the lungs of the affected mice.

Keywords: Pleuromutilin derivatives, Synthesis, Antibacterial activity, *In vivo* efficacy.

1. Introduction

A major challenge to global health and wealth has been caused by the increasing bacterial resistance to currently used antibiotics [1,2]. Multidrug resistant (MDR) bacteria, especially methicillin-resistant *Staphylococcus aureus* (MRSA), are a growing concern internationally [3]. Moreover, most antibiotics, including vancomycin which is indicated for the treatment of life-threatening infections by Gram-positive bacteria, are losing their efficiency because of the wide-spreading bacterial resistance [4]. Therefore, there is an urgent need for novel antimicrobial agents against the resistant bacteria without cross resistance to currently used antibiotics.

Pleuromutilin (**1**) (Fig. 1), a natural compound isolated from *Pleurotus mutilus* and *P. passeckerianus* in 1951 [5], is characterized as the glycolic ester of the diterpene (+)-mutilin [6]. Pleuromutilin derivatives selectively inhibited bacterial protein synthesis through interaction with prokaryotic ribosomes and preventing the peptidyl transferase reaction [7,8]. The crystallography data, utilizing a structure of 50S ribosomal subunit from *Deinococcus radiodurans* in complex with tiamulin, further confirmed this interaction mode in which the tricyclic core of the tiamulin are mediated through hydrophobic interactions and hydrogen bond formed mainly by the nucleotides of domain V [9,10]. Preliminary studies showed that the modifications of pleuromutilin side chain improved its biological activity [11,12]. Furthermore, the special action mode of pleuromutilin encourages researchers to modify it for new antibiotics to reduce cross-resistance in clinical use [13]. Such modifications successfully prompted the approvals of tiamulin, valnemulin, retapamulin and lefamulin (Fig. 1) [14-17].

It is well known that heterocyclic ring bearing polar groups at the C-14 side chain of pleuromutilin derivatives raise their antibacterial activities [18]. A series of novel pleuromutilin derivatives with pyrimidine moieties was reported in our lab and further confirmed that these derivatives presented improved activities against resistant Gram-positive bacterial strains [18,19]. In view of the above findings, we now report the design, synthesis, and antibacterial activities of novel pleuromutilin derivatives with substituted 6-methylpyrimidine moieties.

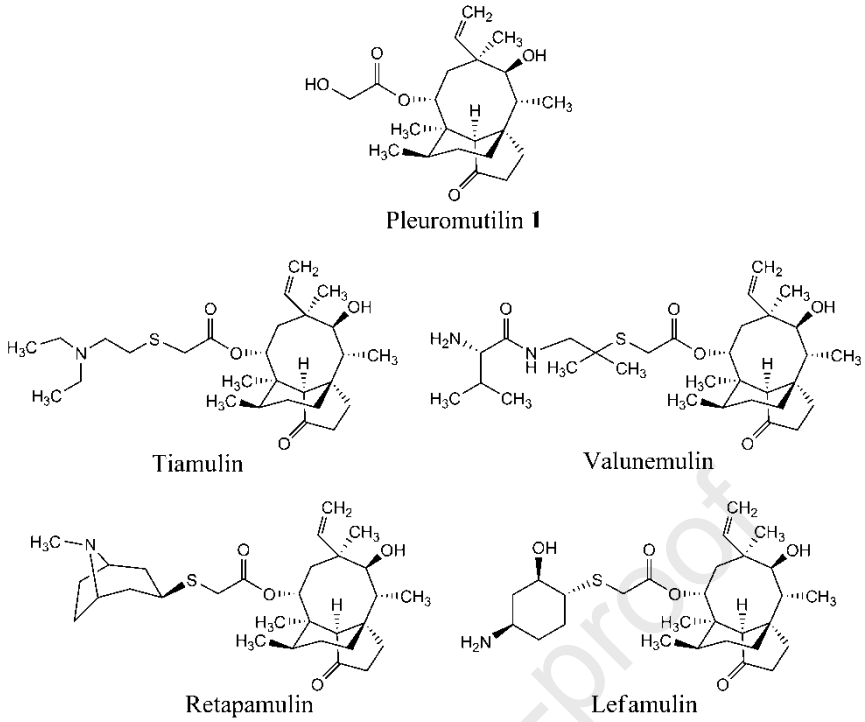
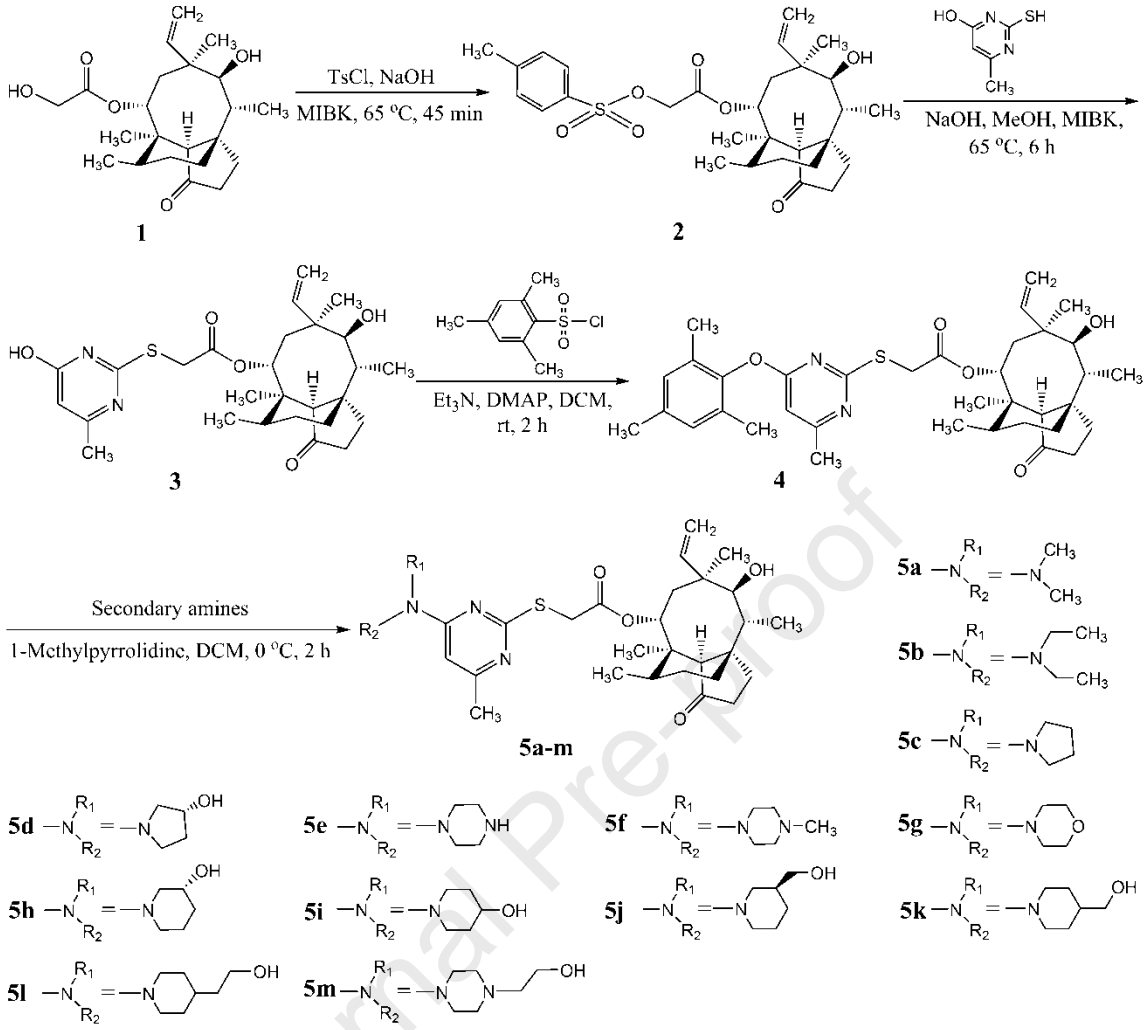


Fig. 1. Structural formulas of pleuromutilin, tiamulin, valnemulin, retapamulin and lefamulin.

2. Results and Discussion

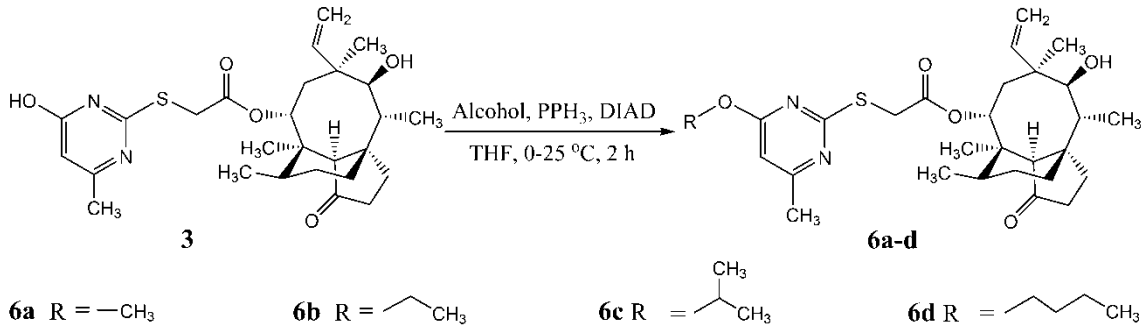
2.1. Chemistry

The synthetic approaches for the preparation of target pleuromutilin derivatives **5a-m** and their intermediates were illustrated in Scheme 1. The pleuromutilin **1** was converted into 22-O-tosylpleuromutilin **2**, a key intermediate used for synthesizing almost all pleuromutilin derivatives. Compound 14-O-[(4-hydroxy-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**3**) was prepared by nucleophilic substitution of **2** with 4-hydroxy-2-mercapto-6-methylpyrimidine under basic conditions. However, we used the one pot method to synthesize **3** from **1** in 76% yield. The target pleuromutilin derivatives (**5a-m)** were directly prepared with one pot in 41-66% yield using compound **3**, the commercially available 2,4,6-trimethylbenzenesulphonyl chloride and various secondary amines in the presence of triethylamine and 1-methylpyrrolidine.

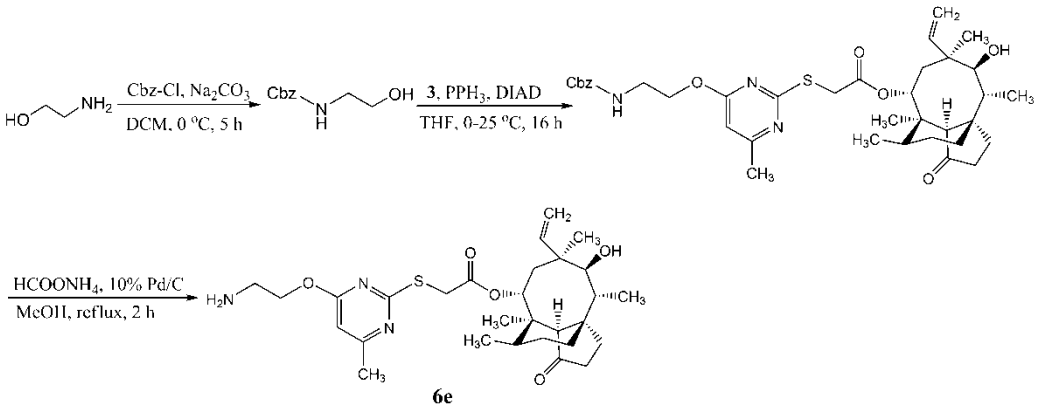


Scheme 1. General synthetic scheme for the pleuromutilin derivatives **5a-m**.

O-Alkylation of compound **3** with alcohols using Mitsunobu reaction directly afforded the target pleuromutilin derivatives **6a-d** (Scheme 2). However, **6e** was obtained in three steps, including the amino protection of 2-aminoethanol with benzyl carbonochloridate (CbzCl), O-alkylation and deprotection employing catalytic hydrogenolysis (Scheme 3).



Scheme 2. Synthesis of pleuromutilin derivatives **6a-d**.



Scheme 3. Preparation of pleuromutilin derivative **6e**.

All the structures of the synthesized compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR (Supplementary data) and HRMS spectra. Furthermore, a crystal of compound **3** was obtained as a colorless, block-like from a solution of ethanol and acetone by slow evaporation method at room temperature (Fig. 2).

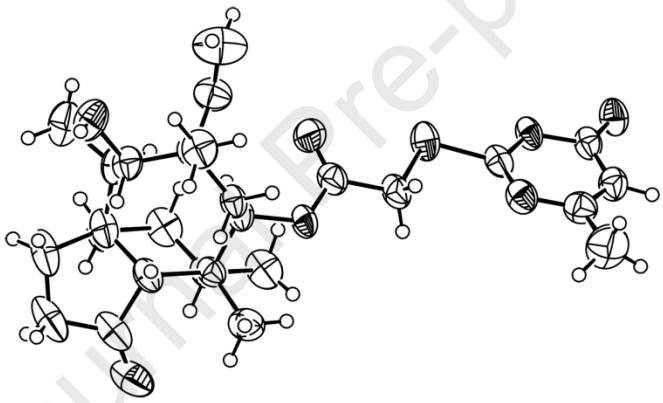


Fig. 2. ORTEP diagram for compound **3** with ellipsoids set at 75% probability.

2.2. Antibacterial testing of pleuromutilin derivatives

The minimum inhibitory concentrations (MICs) of the synthesized pleuromutilin derivatives, as well as tiamulin fumarate and retapamulin used as reference drugs, were assessed against a panel of predominantly Gram-positive bacteria, including *Staphylococcus aureus* ATCC 25923 (*S. aureus*-25923), methicillin-resistant *Staphylococcus epidermidis* ATCC 51625 (MRSE-51625), methicillin-resistant *Staphylococcus aureus* BNCC 337371 (MRSA-337371), *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Streptococcus agalactiae* (*S. agalactiae*) and *Enterococcus faecalis* (*E. faecalis*), as well as *Pasteurella multocida* CVCC 1659 (*P. multocida*-1659) and *Escherichia coli* CICC 10389 (*E. coli*-10389) used as representative Gram-negative strains. The results of MICs for all screened compounds were listed in Table 1. In general, all the compounds showed higher antibacterial activities against *S. aureus*-25923, MRSE-51625, MRSA-337371, *S. dysgalactiae* and *S. agalactiae* than that against *E. faecalis*, *P. multocida*-1659 and *E. coli*-10389. Compounds with N-alkylation of 6-methylpyrimidine (**5a-m**) and **3**

exhibited excellent antibacterial efficiency against *S. aureus*-25923, MRSE-51625, MRSA-337371, *S. dysgalactiae* and *S. agalactiae* with MIC values ranging from 0.05 to 0.84 μM. However, Compounds with O-alkylation of 6-methylpyrimidine (**6a-e**) showed comparatively lower antibacterial efficiency against these strains with MIC values ranging from 0.12 to 3.58 μM. For *S. aureus*-25923, MRSE-51625, MRSA-337371, *S. dysgalactiae* and *S. agalactiae*, compounds **5c** and **5g** showed the highest antibacterial activities than the other synthesized compounds and tiamulin fumarate, but comparable antibacterial activities to that of retapamulin. The MIC results indicated that the introduction of amine groups into the C-14 glycolic acid side chain of pleuromutilin could enhance antibacterial activities, which was consistent with previous reported [18,20].

TABLE 1. Antibacterial activities of the synthesized pleuromutilin derivatives.

Compounds	MIC (μM)							
	S. aureus	MRSE	MRSA	*S. dysgalactiae*	*S. agalactiae*	*E. faecalis*	*P. multocida*	*E. coli*
3	0.25	0.25	0.25	0.12	0.12	15.96	7.95	> 63.84
5a	0.12	0.24	0.24	0.12	0.12	15.11	30.23	> 60.46
5b	0.22	0.45	0.45	0.22	0.11	28.71	57.42	> 57.42
5c	0.06	0.11	0.06	0.06	0.06	14.41	14.41	> 57.63
5d	0.11	0.11	0.22	0.22	0.11	28.00	14.00	> 56.01
5e	0.11	0.22	0.11	0.05	0.11	14.03	28.05	> 56.11
5f	0.43	0.43	0.43	0.43	0.11	54.76	54.76	> 54.76
5g	0.11	0.11	0.11	0.05	0.05	14.00	14.00	> 56.01
5h	0.21	0.21	0.21	0.11	0.05	27.34	27.34	> 54.67
5i	0.21	0.21	0.21	0.11	0.11	13.67	27.34	> 54.67
5j	0.10	0.21	0.21	0.05	0.05	26.74	26.74	> 53.48
5k	0.21	0.42	0.84	0.42	0.84	53.48	53.48	> 53.48
5l	0.10	0.20	0.20	0.05	0.05	26.09	52.17	> 52.17
5m	0.10	0.20	0.41	0.10	0.10	26.04	26.04	> 52.09
6a	0.24	0.24	0.24	0.12	0.24	30.99	61.98	> 61.98
6b	0.12	0.24	0.12	0.12	0.24	15.09	> 60.35	> 60.35
6c	0.46	0.46	0.23	0.92	0.23	58.79	> 58.79	> 58.79
6d	1.79	1.79	3.58	0.45	0.90	57.32	> 57.32	> 57.32
6e	0.92	0.92	0.92	0.23	0.46	58.68	> 58.68	> 58.68
Tiamulin	0.82	0.82	0.82	0.20	0.20	52.48	3.28	52.48
Retapamulin	0.24	0.12	0.12	0.06	0.06	15.45	0.97	30.90

Compounds **5c** and **5g** displayed promising antibacterial activities and therefore were further evaluated for their *in vitro* time-kill assay. The time-kill curves of different concentrations of **5c** and **5g**, as well as tiamulin fumarate used as reference drug, against MRSA-337371 displayed concentration-dependent bacteriostatic effects (Fig. 3). Although 0.5 × and 1 × MIC of compound **5c**, **5g** and tiamulin fumarate slowed bacterial propagation when compared to control, their higher concentrations (2 × and 4 × MIC) showed relatively bacteriostatic kinetics against MRSA. Treated with **5c** and **5g**, living cell counts of MRSA were reduced by at least 2 $\log_{10}$ at 2× and 4 × MIC nearly within 8 h (Fig. 3A and B). However, the bacteriostatic effect of tiamulin reduced living cell count only 1 $\log_{10}$ under the same conditions (Fig. 3C).

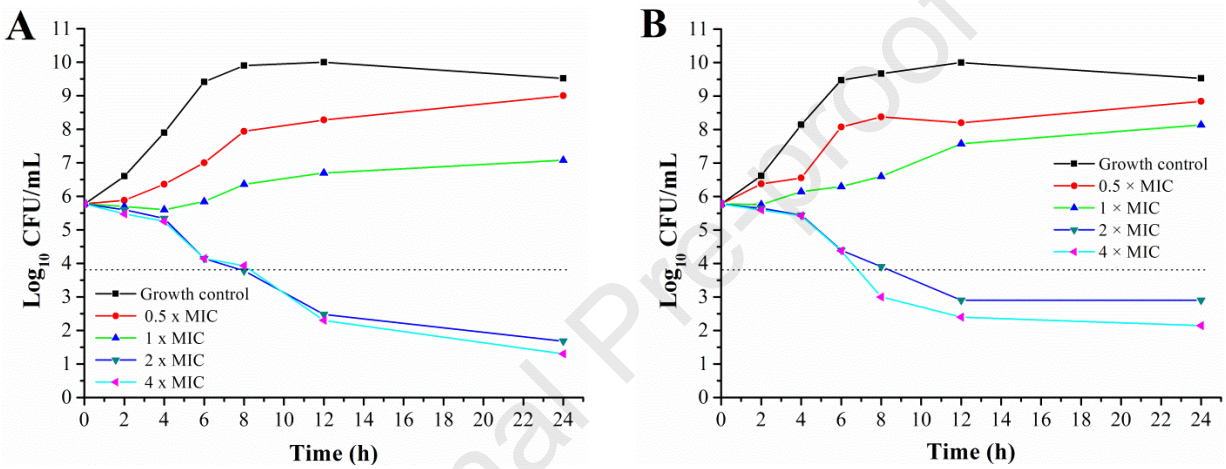


Fig. 3. Time-kill analysis of compounds **5c** (A), **5g** (B) and tiamulin fumarate (C) against MRSA-337371.

2.3. In vivo efficacy in mouse model

Bearing the excellent *in vitro* antibacterial activities, compound **5c** was assessed for its *in vivo* efficacy by measuring the survival of mice after a single lethal challenge of MRSA-337371 (3×10^8 CFU in 0.1 mL saline), and tiamulin fumarate used as the reference drug. After being infected with MRSA, mice were intraperitoneally treated with different doses of **5c** dissolved in vehicle. Mice injected with vehicle alone showed 100% mortality in this model. Treatment with **5c** and tiamulin fumarate displayed dose-dependent protection and led to the survival of the mice (Fig. 4), with ED_{50} of 18.02 and 25.85 mg/kg body weight, respectively.

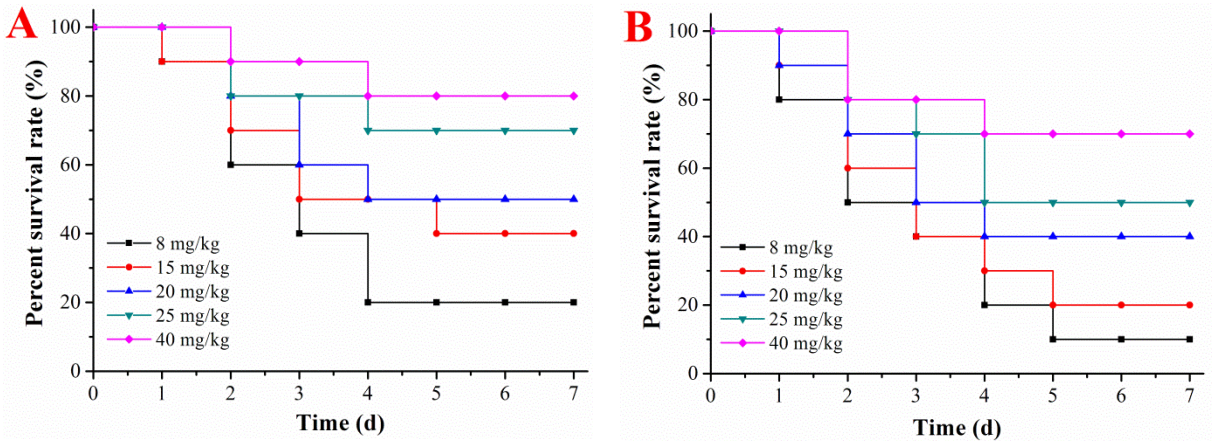
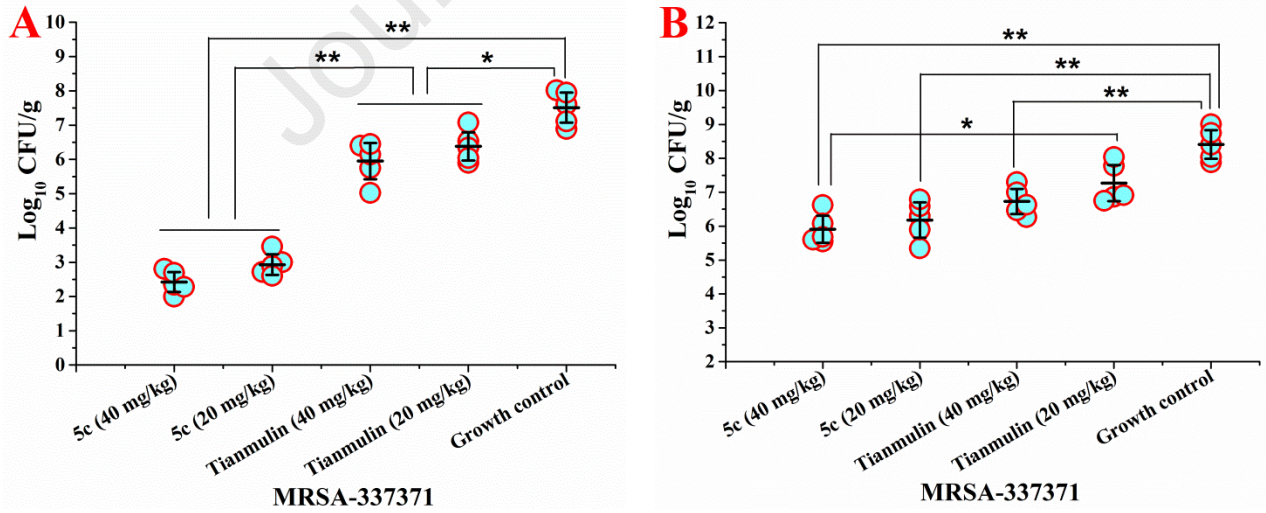


Fig. 4. Efficacy of compound **5c** (A) and tiamulin fumarate (B) in mouse systemic infection model.

For further evaluating the *in vivo* efficacy of **5c**, the lung, kidney and liver of infection model mice were examined for their MRSA load after treatment for 2 d. Treatment with **5c** at 40 and 20 mg/kg doses extremely significantly reduced MRSA load in lung compared to that in the control group (~ 4.8 $\log_{10}$ CFU/mL, $p < 0.01$) and the tiamulin groups (~3.5 $\log_{10}$ CFU/mL, $p < 0.01$). Tiamulin at 40 and 20 mg/kg showed significantly treatment effects (1.1-1.6 $\log_{10}$ CFU/mL) against MRSA in lung compared to that in the control group ($p < 0.05$) (Fig. 5A). In the kidney, MRSA load in the two **5c** groups and tiamulin group (40 mg/kg) were extremely significantly lower than that in the control group (Fig. 5B). However, no significant difference of MRSA load in liver was observed among the five groups (Fig. 5C).



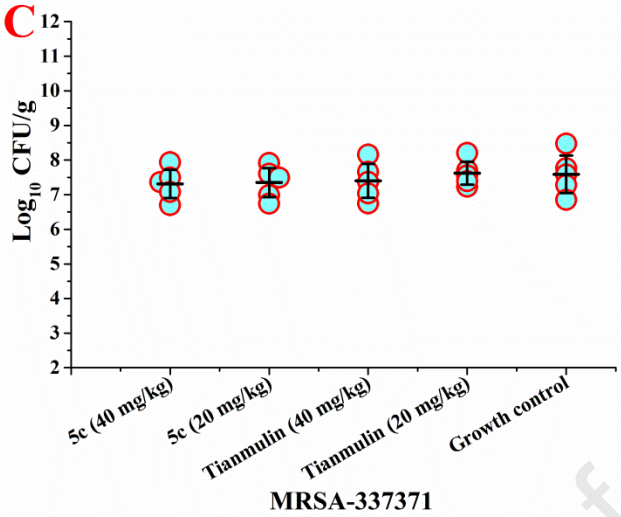
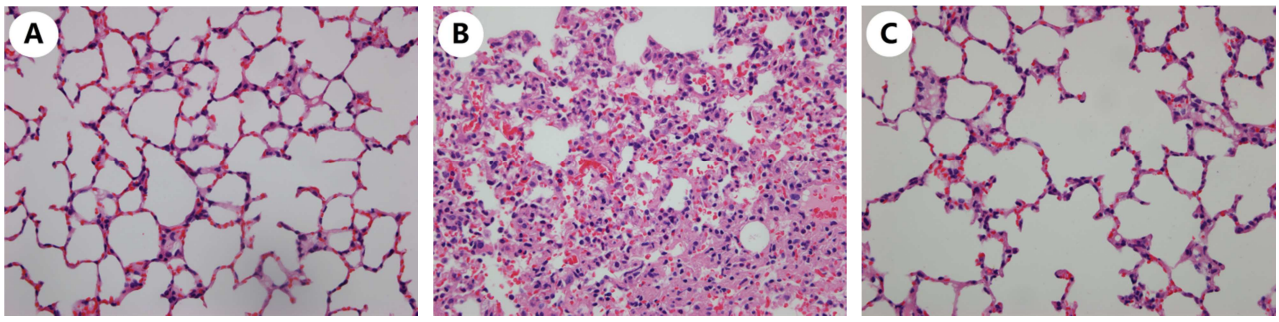


Figure 5. The bacterial load of compound **5c** and tiamulin in lung (A), kidney (B) and liver (C) after challenge of MRSA.

To assess whether compound **5c** attenuates lung tissue damage caused by MRSA, histopathological changes from the non-treatment and treatment groups were assessed by light microscopy. Compared to the normal control group (Fig. 6A), the predominant changes in the lungs of mice in the control group were congestion, edema, diffuse infiltration of neutrophils cells within the alveolar septa and alveolar lumen, fibrous tissue hyperplasia and alveolar wall thickening (Fig. 6B). It is worth noting that the lungs of mice at the group treated with 40 mg/kg of **5c** were histologically normal with no significant inflammatory cell infiltration in pulmonary interstitium (Fig. 6C). Infiltration of a few inflammatory cells, slightly thickening of alveolar septum and wall were found in the lungs of mice treated with 20 mg/kg of **5c** (Fig. 6D). However, lungs of mice at the tiamulin groups (40 and 20 mg/kg) showed mild to moderate infiltration of neutrophils cells and local congestion (Fig. 6E and F).



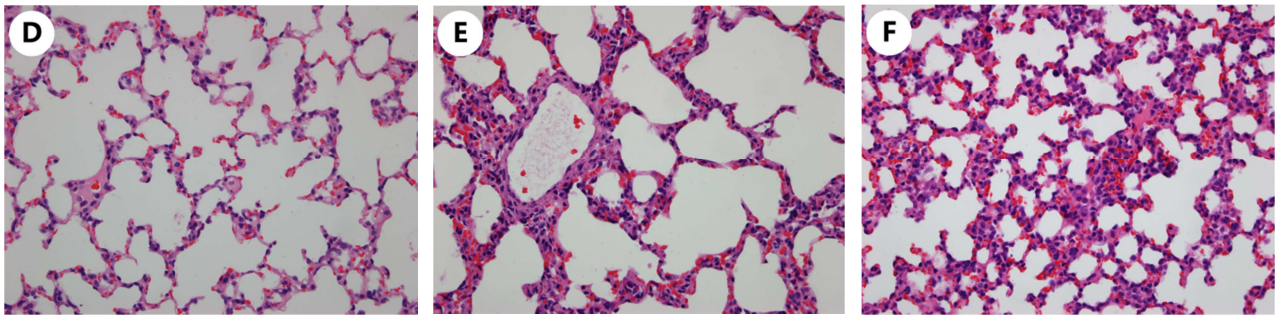


Fig. 6. Representative lung histology of mice in normal control group (A), control group (B), **5c** treated group with 40 mg/kg (C), **5c** treated group with 20 mg/kg (D), tiamulin treated group with 40 mg/kg (E), and tiamulin treated group with 20 mg/kg (F) treatment.

3. Conclusion

In summary, we have synthesized a series of novel pleuromutilin derivatives possessing 6-methylpyrimidine moieties. These derivatives were initially evaluated for their *in vitro* antibacterial activities against *S. aureus*-25923, MRSE-51625, MRSA-337371, *S. dysgalactiae*, *S. agalactiae*, *E. faecalis*, *P. multocida*-1659 and *E. coli*-1659. The MIC values demonstrated that all the synthesized derivatives possessed potent antibacterial activity properties. Compounds **5c** and **5g** were the most active antibacterial agents. In time-to-kill assays, **5c** and **5g** showed relatively bacteriostatic kinetics against MRSA. In further *in vivo* antibacterial study, compound **5c** displayed potent efficacy to improve the survival rate of mice, reduce the bacterial load and alleviate the pathological damage in the lungs of the infected mice. The antibacterial effect of **5c** found in this study suggested that it merited further drug development as a potential agent against MRSA infections.

4. Materials and methods

4.1. General

All reactions were performed using standard laboratory equipment and glassware. Reagents and solvents were obtained commercially and used without further purification. The progress of all reactions and purity of the compounds were monitored by thin layer chromatography (TLC) on silica gel plates (GF254; Qingdao Haiyang Chemical Co., Ltd, Shandong, China), followed by visualization after spraying with a 0.05% $KMnO_4$ aqueous solution or under UV illumination directly. Infrared (IR) spectra were obtained on a Thermo Nicolet NEXUS-670 spectrometer using KBr thin films, and the absorptions are reported in cm^{-1}. All ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker 400 MHz and 100 MHz spectrometers in $CDCl_3$, respectively. The chemical shifts (δ)

were reported in parts per million (ppm) relative to tetramethylsilane. High-resolution mass spectra (HRMS) were obtained with a Bruker Daltonics APEX II 47e mass spectrometer equipped with an electrospray ion source.

4.2. Chemistry

*4.2.1. 14-O-[(4-ol-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**3**)*

A 10 mL of NaOH aqueous solution (10 M) was added dropwise to a mixture of pleuromutilin (18.93 g, 50 mmol) and *p*-toluenesulfonyl chloride (10.49 g, 55 mmol) in methyl isobutyl ketone (150 mL). The mixture was vigorously stirred for 45 min at 65 ◦C, followed by washing with 60 mL water for 10 min and separation. The organic layer was added to a solution of sodium salt of 2-mercapto-6-methylpyrimidin-4-ol which was obtained by 2-mercapto-6-methylpyrimidin-4-ol (7.11, 50 mmol) and 5.5 mL NaOH aqueous solution (10 M) in 50 mL methanol. The mixture was stirred for 6 h at 65 ^{o}C and evaporated under reduced pressure to dryness. The crude product was extracted with 100 mL ethyl acetate and then cooled on an ice bath for 2 h. The target compound **3** was then precipitated without further purification to yield 19.12 g (76%). IR (KBr): 3489, 2985, 2932, 2864, 1718, 1657, 1582, 1541, 1460, 1363, 1284, 1180, 1118, 1019, 914, 869 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.09 (s, 1H), 6.47 (dd, *J* = 17.4, 11.0 Hz, 1H), 6.07 (d, *J* = 1.1 Hz, 1H), 5.76 (dd, *J* = 8.6, 3.2 Hz, 1H), 5.34 (dd, *J* = 11.0, 1.6 Hz, 1H), 5.23 – 5.13 (m, 1H), 3.95 – 3.81 (m, 2H), 3.37 (d, *J* = 6.3 Hz, 1H), 2.38 – 2.23 (m, 2H), 2.21 (d, *J* = 2.8 Hz, 4H), 2.07 (dq, *J* = 16.2, 2.9 Hz, 2H), 1.77 (dt, *J* = 14.4, 3.1 Hz, 1H), 1.69 – 1.61 (m, 2H), 1.56 – 1.38 (m, 6H), 1.36 – 1.23 (m, 2H), 1.16 (d, *J* = 2.9 Hz, 4H), 0.87 (dd, *J* = 7.2, 2.5 Hz, 3H), 0.75 (dd, *J* = 7.1, 2.8 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 216.9, 166.8, 165., 165.1, 158.8, 138.9, 117.3, 108.6, 74.5, 70.1, 58.1, 45.4, 44.5, 43.9, 41.9, 36.7, 36.0, 34.4, 33.2, 30.4, 26.8, 26.3, 24.8, 24.1, 16.8, 14.8, 11.5; HRMS (ES) calcd $[M + H]^{+}$ for $C_{27}H_{38}N_2O_5S$ 503.2576, found 503. 2569.

*4.2.2. General procedure for the synthesis of compounds **5a-m***

To a solution of compound **3** (0.75 g, 1.5 mmol) in 20 mL dry DCM, triethylamine (0.73 g, 7.2 mmol), 4-dimethylaminopyridine (0.01 g, 0.1 mmol) and mesitylene-2-sulfonyl chloride (0.50 g, 2.3 mmol) were added and stirred at room temperature for 2-3 h. Then, amine (7.5 mmol) and 1-methylpyrrolidine (1.27 g, 15 mmol) were added in one portion and the reaction was stirred at 0 ^{o}C for 1-2 h. The mixture was washed with 5% citric acid, followed by drying with Na_2SO_4 overnight and rotary evaporation to dryness. The crude residue obtained was purified by silica gel column chromatography (petroleum ether: ethyl acetate 1:1-1:10 v/v) to afford the desired compounds.

*4.2.2.1. 14-O-[(4-(Dimethylamino)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5a**)*

White solid, 0.32 g (yield 41%); IR (KBr): 3448, 2930, 2864, 1733, 1671, 1591, 1500, 1459, 1407, 1371, 1292,

1153, 1117, 1031, 916, 807 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 6.50 (ddd, *J* = 17.0, 11.0, 5.7 Hz, 1H), 5.82 (s, 1H), 5.71 (d, *J* = 8.4 Hz, 1H), 5.30 (dt, *J* = 11.0, 1.4 Hz, 1H), 5.16 (dd, *J* = 17.4, 1.7 Hz, 1H), 3.93 – 3.75 (m, 2H), 3.58 (t, *J* = 6.1 Hz, 1H), 3.33 (dd, *J* = 10.7, 6.5 Hz, 1H), 3.05 (s, 2H), 2.29 (dd, *J* = 11.0, 5.4 Hz, 4H), 2.24 – 2.12 (m, 2H), 2.07 (s, 1H), 1.97 (t, *J* = 8.1 Hz, 2H), 1.76 – 1.70 (m, 1H), 1.66 – 1.58 (m, 2H), 1.55 – 1.35 (m, 7H), 1.25 (td, *J* = 8.2, 7.7, 2.8 Hz, 3H), 1.11 (d, *J* = 2.4 Hz, 4H), 0.85 (d, *J* = 7.0 Hz, 3H), 0.71 (dd, *J* = 6.8, 1.4 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 216.0, 167.4, 158.6, 138.2, 115.9, 97.1, 73.6, 68.3, 57.1, 57.14, 45.4, 44.4, 43.4, 42.9, 40.9, 36.2, 35.8, 34.9, 33.5, 33.0, 29.4, 25.9, 25.2, 23.8, 15.8, 13.9, 13.2, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{29}H_{43}N_3O_4S$ 530.3044, found 530.3047.

*4.2.2.2. 14-O-[(4-(Diethylamino)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5b**)*

White solid, 0.49 g (yield 59%); IR (KBr): 3448, 3082, 2930, 2864, 1732, 1 1582, 1499, 1459, 1416, 1371, 1292, 1153, 1117, 1017, 915, 807, 547 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 6.44 (dd, *J* = 17.3, 11.1 Hz, 1H), 5.75 (s, 1H), 5.64 (d, *J* = 8.5 Hz, 1H), 5.23 (dd, *J* = 10.9, 1.6 Hz, 1H), 5.09 (dd, *J* = 17.5, 1.7 Hz, 1H), 3.88 – 3.68 (m, 2H), 3.50 (d, *J* = 6.3 Hz, 1H), 3.26 (dd, *J* = 10.8, 6.6 Hz, 2H), 2.27 – 2.16 (m, 4H), 2.16 – 2.07 (m, 2H), 1.99 (d, *J* = 12.2 Hz, 3H), 1.91 (dd, *J* = 14.9, 7.6 Hz, 4H), 1.70 – 1.65 (m, 1H), 1.56 (ddt, *J* = 11.2, 8.1, 4.1 Hz, 2H), 1.48 – 1.27 (m, 7H), 1.22 – 1.14 (m, 3H), 1.04 (d, *J* = 2.9 Hz, 4H), 0.78 (d, *J* = 7.0 Hz, 3H), 0.65 (dd, *J* = 6.9, 4.1 Hz, 3H)·; ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.0, 167.5, 158.8, 138.2, 115.9, 97.0, 73.5, 68.2, 59.4, 57.1, 45.3, 44.4, 43.4, 42.9, 40.9, 35.8, 34.9, 33.5, 33.1, 33.0, 29.4, 25.9, 25.3, 23.8, 22.8, 15.8, 13.9, 13.2, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{31}H_{47}N_3O_4S$ 558.3319, found 558.3326.

*4.2.2.3. 14-O-[(4-(Pyrrolidine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5c**)*

White solid, 0.45 g (yield 54%); IR (KBr): 3448, 2928, 2864, 1734, 1592, 1499, 1459, 1416, 1370, 1293, 1153, 1117, 1030, 981, 915 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 6.51 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.82 (s, 1H), 5.71 (d, *J* = 8.5 Hz, 1H), 5.30 (dd, *J* = 10.9, 1.7 Hz, 1H), 5.16 (dd, *J* = 17.4, 1.7 Hz, 1H), 3.94 – 3.75 (m, 2H), 3.54 (s, 2H), 3.33 (dd, *J* = 10.7, 6.4 Hz, 2H), 2.33 – 2.24 (m, 4H), 2.22 – 2.11 (m, 2H), 2.06 (d, *J* = 13.1 Hz, 2H), 1.99 – 1.92 (m, 3H), 1.75 (dd, *J* = 14.6, 3.2 Hz, 1H), 1.62 (td, *J* = 11.0, 9.1, 4.6 Hz, 2H), 1.55 – 1.37 (m, 7H), 1.32 – 1.23 (m, 3H), 1.11 (s, 4H), 0.85 (d, *J* = 7.0 Hz, 3H), 0.72 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.0, 167.5, 158.9, 138.3, 115.9, 97.1, 73.7, 68.4, 59.4, 57.2, 45.3, 44.5, 43.5, 43.0, 41.0, 35.9, 35.0, 33.5, 33.1, 29.5, 26.0, 25.4, 23.9, 22.8, 20.1, 15. 9, 14.0, 13.3, 10.5; HRMS (ES) calcd $[M + H]^+$ for $C_{31}H_{45}N_3O_4S$ 556.3218, found 556.3223.

*4.2.2.4. 14-O-[(4-(2-(3S)-3-Hydroxypyrrolidine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5d**)*

White solid, 0.49 g (yield 57%); IR (KBr): 3386, 2928, 2863, 1718, 1592, 1498, 1456, 1417, 1370, 1294, 1220, 1153, 1117, 1019, 969, 915, 806 cm^{-1};^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.55 – 6.42 (m, 1H), 5.85 (s, 1H), 5.74 – 5.65 (m, 1H), 5.36 – 5.27 (m, 1H), 5.16 (dd, *J* = 17.4, 1.7 Hz, 1H), 3.94 – 3.80 (m, 2H), 3.73 (t, *J* = 7.0 Hz, 2H), 3.58 (s, 2H), 3.34 (s, 1H), 2.28 (s, 4H), 2.20 (dt, *J* = 9.3, 4.5 Hz, 2H), 2.10 – 2.05 (m, 2H), 1.75 (d, *J* = 12.7 Hz, 1H), 1.66 – 1.60 (m, 2H), 1.53 – 1.36 (m, 7H), 1.24 (t, *J* = 7.0 Hz, 5H), 1.11 (d, *J* = 2.6 Hz, 4H), 0.86 (d, *J* = 7.0 Hz, 3H), 0.72 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.1, 167.4, 159.0, 138.4, 116.0, 97.2, 73.6, 69.4, 68.5, 57.2, 57.2, 53.8, 44.5, 43.6, 43.0, 43.0, 35.8, 35.1, 33.5, 33.1, 32.8, 32.8, 29.5, 26.0, 25.4, 23.9, 22.5, 17.5, 15.9, 14.0, 10.5; HRMS (ES) calcd $[M + H]^+$ for $C_{31}H_{45}N_3O_5S$ 572.3131, found 572.3136.

*4.2.2.5. 14-O-[(4-(Piperazine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5e**)*

White solid, 0.38 g (yield 45%); IR (KBr): 3448, 2926, 2863, 1734, 1591, 1499, 1459, 1416, 1372, 1291, 1226, 1153, 1117, 1018, 981, 915, 809 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.44 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.75 (s, 1H), 5.64 (d, *J* = 8.5 Hz, 1H), 5.29 – 5.22 (m, 1H), 5.09 (dd, *J* = 17.4, 1.7 Hz, 1H), 3.85 – 3.69 (m, 2H), 3.49 (s, 2H), 3.26 (dd, *J* = 10.6, 6.6 Hz, 2H), 2.26 – 2.17 (m, 4H), 2.13 (t, *J* = 7.5 Hz, 2H), 1.99 (d, *J* = 10.4 Hz, 2H), 1.91 (dd, *J* = 15.2, 7.9 Hz, 4H), 1.68 (dd, *J* = 14.7, 3.2 Hz, 2H), 1.61 – 1.54 (m, 2H), 1.46 – 1.28 (m, 7H), 1.19 (dd, *J* = 9.2, 6.9 Hz, 3H), 1.04 (s, 4H), 0.78 (d, *J* = 7.0 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 216.0, 167.5, 158.7, 138.2, 115.9, 97.1, 73.6, 68.3, 60.9, 57.1, 45.3, 44.4, 43.4, 42.9, 40.9, 37.0, 35.8, 34.9, 33.5, 33.0, 29.4, 28.7, 25.9, 25.2, 23.8, 22.7, 15.8, 13.9, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{31}H_{46}N_4O_4S$ 571.3208, found 571.302.

*4.2.2.6. 14-O-[(4-(4-Methylpiperazine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5f**)*

White solid, 0.37 g (yield 42%); IR (KBr): 3547, 2937, 2865, 1735, 1671, 1591, 1499, 1459, 1416, 1373, 1291, 1222, 1148, 1117, 1018, 980, 914, 814, 700 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.45 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.75 (s, 1H), 5.64 (d, *J* = 8.5 Hz, 1H), 5.27 – 5.19 (m, 2H), 5.09 (dd, *J* = 17.5, 1.7 Hz, 1H), 3.87 – 3.69 (m, 2H), 3.50 (s, 2H), 3.26 (dd, *J* = 10.9, 6.5 Hz, 3H), 2.29 – 2.10 (m, 6H), 1.99 (d, *J* = 9.6 Hz, 2H), 1.93 – 1.80 (m, 4H), 1.68 (dd, *J* = 14.6, 3.2 Hz, 1H), 1.56 (td, *J* = 10.5, 9.8, 3.5 Hz, 2H), 1.37 (d, *J* = 7.4 Hz, 7H), 1.23 – 1.12 (m, 3H), 1.04 (s, 4H), 0.78 (d, *J* = 7.0 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.1, 167.5, 158.7, 138.2, 115.9, 97.0, 73.5, 68.2, 57.1, 52.4, 47.5, 45.2, 44.4, 43.4, 42.9, 40.9, 35.8, 34.9, 33.5, 33.0, 29.4, 28.7, 25.9, 25.2, 24.3, 23.8, 22.8, 15.8, 13.9, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{32}H_{48}N_4O_4S$ 585.3448, found 585.3502.

*4.2.2.7. 14-O-[(4- (Morpholine-4-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5g**)*

White solid, 0.48 g (yield 56%); IR (KBr): 3547, 2927, 2861, 1732, 1584, 1490, 1448, 1417, 1372, 1307, 1274, 1151, 1117, 1018, 984, 917, 810, 552 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.97 (s, 1H), 5.64 (d, *J* = 8.5 Hz, 1H), 5.25 (dd, *J* = 11.0, 1.6 Hz, 1H), 5.11 (dd, *J* = 17.5, 1.6 Hz, 1H), 3.75 (d, *J* = 1.9 Hz, 2H), 3.68 (t, *J* = 4.9 Hz, 3H), 3.51 (q, *J* = 4.8 Hz, 3H), 3.27 (dd, *J* = 10.7, 6.5 Hz, 1H), 2.22 (d, *J* = 6.1 Hz, 4H), 2.18 – 2.07 (m, 2H), 1.99 (d, *J* = 14.3 Hz, 2H), 1.95 – 1.88 (m, 1H), 1.68 (dd, *J* = 14.4, 3.2 Hz, 1H), 1.56 (td, *J* = 10.4, 10.0, 5.1 Hz, 2H), 1.38 (d, *J* = 16.0 Hz, 7H), 1.25 – 1.17 (m, 3H), 1.06 (s, 4H), 0.79 (d, *J* = 7.0 Hz, 3H), 0.64 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 216.0, 167.3, 160.9, 138.1, 116.1, 96.4, 73.5, 68.5, 65.5, 59.4, 57.1, 44.4, 43.5, 43.2, 42.9, 40.9, 35.7, 34.9, 33.5, 33.0, 29.4, 25.9, 25.3, 23.8, 20.0, 15.8, 13.9, 13.2, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{31}H_{45}N_3O_5S$ 572.3169, found 572.3163.

*4.2.2.8. 14-O-[(4-(3S)-3-Hydroxypiperidine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5h**)*

White solid, 0.54 g (yield 62%); IR (KBr): 3442, 2933, 2862, 1732, 1584, 1494, 1447, 1417, 1373, 1308, 1274, 1151, 1117, 1018, 980, 917, 807, 550 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.52 – 6.38 (m, 1H), 6.08 (d, *J* = 1.8 Hz, 1H), 5.68 (d, *J* = 8.3 Hz, 1H), 5.30 (d, *J* = 6.5 Hz, 2H), 5.15 (ddd, *J* = 17.4, 5.9, 1.6 Hz, 1H), 3.87 – 3.75 (m, 3H), 3.67 (d, *J* = 10.4 Hz, 1H), 3.48 (d, *J* = 27.7 Hz, 1H), 3.40 – 3.27 (m, 2H), 2.25 (s, 4H), 2.19 (dd, *J* = 10.5, 6.8 Hz, 2H), 2.06 (d, *J* = 15.9 Hz, 2H), 2.02 – 1.90 (m, 2H), 1.88 – 1.79 (m, 1H), 1.75 (s, 1H), 1.67 – 1.61 (m, 2H), 1.54 – 1.39 (m, 7H), 1.30 – 1.20 (m, 3H), 1.12 (s, 4H), 0.86 (dt, *J* = 7.0, 3.3 Hz, 3H), 0.68 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.7, 161.6, 138.9, 116.7, 97.2, 74.3, 71.9, 69.4, 65.9, 57.9, 53.1, 50.7, 45.2, 44.4, 43.7, 41.7, 36.5, 35.7, 34.2, 33.8, 32.3, 30.2, 26.6, 26.1, 24.6, 23.7, 21.9, 16.5, 14.7, 13.9, 11.2; HRMS (ES) calcd $[M + H]^+$ for $C_{32}H_{47}N_3O_5S$ 586.3310, found 586.3316.

*4.2.2.9. 14-O-[(4-(4-Hydroxypiperidine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5i**)*

White solid, 0.55 g (yield 63%); IR (KBr): 3438, 2930, 2863, 1719, 1585, 1497, 1453, 1417, 1370, 1301, 1206, 1153, 1117, 1088, 1026, 980, 915, 808, 556 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 6.01 (s, 1H), 5.64 (d, *J* = 8.5 Hz, 1H), 5.24 (d, *J* = 10.7 Hz, 1H), 5.10 (d, *J* = 17.4 Hz, 1H), 4.03 – 3.85 (m, 3H), 3.77 (s, 2H), 3.28 – 3.17 (m, 2H), 2.21 (d, *J* = 4.7 Hz, 4H), 2.14 (q, *J* = 9.1, 8.6 Hz, 2H), 1.99 (dd, *J* = 15.5, 2.9 Hz, 1H), 1.95 – 1.90 (m, 1H), 1.86 (s, 2H), 1.68 (d, *J* = 14.4 Hz, 1H), 1.57 (d, *J* = 11.7 Hz, 4H), 1.47 – 1.32 (m, 7H), 1.21 (d, *J* = 16.2 Hz, 3H), 1.06 (s, 4H), 0.79 (d, *J* = 7.0 Hz, 3H), 0.64 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 216.1, 167.4, 158.3, 138.1, 116.0, 96.4, 73.6, 68.5, 66.3, 58.0, 57.1, 47.6, 44.4, 43.5, 42.9, 40.9, 40.4, 35.8, 34.9, 33.5, 33.0, 32.7, 31.5, 29.4, 25.9, 25.3, 23.8, 15.8, 13.9, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{32}H_{47}N_3O_5S$ 586.3310, found 586.3316.

*4.2.2.10. 14-O-[(4-((3R)-3-Hydroxymethylpiperidine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5j**)*

White solid, 0.47 g (yield 53%); IR (KBr): 3448, 2931, 2862, 1732, 1585, 1496, 1445, 1418, 1308, 1207, 1179, 1117, 1019, 981, 917, 882, 549 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 6.40 (dt, *J* = 10.9, 5.8 Hz, 1H), 6.00 (d, *J* = 2.1 Hz, 1H), 5.62 (d, *J* = 8.5 Hz, 1H), 5.24 (td, *J* = 6.7, 3.3 Hz, 1H), 5.10 (ddd, *J* = 17.4, 5.8, 1.7 Hz, 1H), 3.79 – 3.67 (m, 2H), 3.52 – 3.44 (m, 1H), 3.45 – 3.36 (m, 1H), 3.26 (d, *J* = 8.3 Hz, 1H), 3.09 – 3.01 (m, 1H), 2.18 (s, 4H), 2.16 – 2.09 (m, 2H), 2.00 (d, *J* = 15.8 Hz, 3H), 1.93 (d, *J* = 7.4 Hz, 1H), 1.79 – 1.68 (m, 3H), 1.65 – 1.60 (m, 1H), 1.58 – 1.51 (m, 2H), 1.37 (s, 7H), 1.30 – 1.24 (m, 2H), 1.23 – 1.18 (m, 3H), 1.05 (s, 4H), 0.79 (d, *J* = 6.9 Hz, 3H), 0.66 – 0.58 (m, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.0, 167.7, 160.3, 138.2, 115.9, 96.4, 73.5, 68.6, 63.78, 59.4, 57.1, 46.2, 44.4, 43.5, 42.9, 42.9, 40.9, 37.3, 35.7, 34.9, 33.5, 29.4, 26.1, 25.9, 25.3, 23.8, 23.0, 22.9, 20.0, 15.8, 13.9, 13.2, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{33}H_{49}N_3O_5S$ 600.3470, found 600.3464.

*4.2.2.11. 14-O-[(4-((3R)-3-Hydroxymethylpiperidine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5k**)*

White solid, 0.40 g (yield 45%); IR (KBr): 3356, 2927, 2863, 1724, 1586, 1496, 1458, 1417, 1371, 1304, 1225, 1153, 1117, 1025, 981, 911, 806, 555 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.99 (s, 1H), 5.64 (d, *J* = 8.5 Hz, 1H), 5.24 (dd, *J* = 10.8, 1.7 Hz, 1H), 5.10 (dd, *J* = 17.5, 1.7 Hz, 1H), 4.32 (s, 1H), 3.77 (s, 2H), 3.45 (d, *J* = 5.8 Hz, 2H), 3.27 (dd, *J* = 10.6, 6.4 Hz, 1H), 2.84 – 2.71 (m, 2H), 2.26 – 2.17 (m, 4H), 2.17 – 2.09 (m, 2H), 1.97 (td, *J* = 16.1, 15.6, 5.0 Hz, 2H), 1.76 – 1.65 (m, 3H), 1.56 (ddd, *J* = 12.5, 7.3, 2.8 Hz, 3H), 1.48 – 1.32 (m, 7H), 1.26 (d, *J* = 19.5 Hz, 2H), 1.19 – 1.10 (m, 3H), 1.06 (s, 4H), 0.79 (d, *J* = 6.9 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.1, 167.4, 160.4, 138.1, 116.0, 96.5, 73.6, 68.4, 66.4, 57.2, 44.4, 43.5, 43.1, 43.1, 42.9, 40.9, 37.8, 35.8, 34.9, 33.5, 33.0, 29.4, 27.3, 25.9, 25.3, 23.8, 23.0, 15.8, 13.9, 13.2, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{33}H_{49}N_3O_5S$ 600.3470, found 600.3464.

*4.2.2.12. 14-O-[(4-(4-Hydroxyethylpiperidine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5l**)*

White solid, 0.61 g (yield 66%); IR (KBr): 3422, 2923, 2857, 1720, 1582, 1495, 1532, 1449, 1422, 1372, 1301, 1223, 1154, 1118, 1048, 1029, 971, 915, 819, 551 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.97 (s, 1H), 5.64 (d, *J* = 8.5 Hz, 1H), 5.24 (dd, *J* = 11.0, 1.6 Hz, 1H), 5.10 (dd, *J* = 17.4, 1.7 Hz, 1H), 4.28 (s, 1H), 3.77 (s, 2H), 3.66 (t, *J* = 6.5 Hz, 2H), 3.27 (dd, *J* = 10.4, 6.4 Hz, 1H), 2.76 (td, *J* = 12.8, 10.8, 4.1 Hz, 2H), 2.29 – 2.17 (m, 4H), 2.16 – 2.06 (m, 2H), 2.01 – 1.89 (m, 2H), 1.69 (dt, *J* = 11.5, 7.5 Hz, 4H), 1.63 – 1.49 (m, 3H), 1.48 – 1.33 (m, 8H), 1.31 – 1.23 (m, 2H), 1.21 – 1.10 (m, 3H), 1.06 (s, 4H), 0.79 (d, *J* = 7.0 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.1, 167.5, 160.4, 138.1, 116.0, 96.4, 73.6, 68.4 59.4, 59.2, 57.2, 44.4, 43.5, 42.9, 40.9, 38.1, 35.8, 34.9, 33.5 , 33.0, 31.7, 30.8, 29.4, 25.9, 25.3, 23.8, 23.0, 20.0, 15.8, 13.9, 13.2, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{34}H_{51}N_3O_5S$ 614.3553, found 614.3559.

*4.2.2.13. 14-O-[(4-(4-Hydroxyethylpiperazine-1-yl)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**5m**)*

White solid, 0.46 g (yield 50%); IR (KBr): 3448, 2929, 2864, 1734, 1591, 1499, 1459, 1415, 1347, 1292, 1225, 1153, 1117, 1018, 981, 915, 807, 547 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.44 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.75 (s, 1H), 5.64 (d, *J* = 8.5 Hz, 1H), 5.23 (dd, *J* = 11.0, 1.7 Hz, 1H), 5.09 (dd, *J* = 17.5, 1.7 Hz, 1H), 3.88 – 3.69 (m, 2H), 3.50 (d, *J* = 6.4 Hz, 2H), 3.24 (td, *J* = 14.4, 12.7, 5.7 Hz, 3H), 2.22 (d, *J* = 14.9 Hz, 4H), 2.18 – 2.11 (m, 2H), 1.99 (d, *J* = 11.2 Hz, 2H), 1.94 – 1.82 (m, 4H), 1.70 – 1.64 (m, 1H), 1.60 – 1.50 (m, 3H), 1.48 – 1.29 (m, 7H), 1.26 – 1.15 (m, 4H), 1.04 (s, 5H), 0.78 (d, *J* = 7.0 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 216.0, 167.5, 158.7, 138.2, 115.9, 97.0, 73.6, 68.3, 59.4, 57.1, 45.3, 44.4, 43.4, 42.9, 42.0, 40.9, 35.8, 34.9, 33.5, 33.0, 29.4, 28.7, 25.9, 25.2, 24.2, 23.8, 20.0, 15.8, 13.9, 13.2, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{33}H_{50}N_4O_5S$ 615.3568, found 615.3563.

*4.2.3. General procedure for the synthesis of compounds **6a-e***

To a solution of compound **3** (0.91 g, 1.8 mmol), alcohols (2 mmol) and triphenylphosphine (0.58 g, 2.2 mmol) in 20 mL dry DCM, triethylamine (0.73 g, 7.2 mmol), diisopropyl azodiformate (0.45 g, 2.2 mmol) was added dropwise and stirred at room temperature for 2-12 h. The mixture was washed with water, followed by drying with Na_2SO_4 overnight and rotary evaporation to dryness. Before the synthesis of compounds **6e**, the amino of monoethanolamine was protected by benzyl carbonochloridate (CbzCl). After treatment with $NaHCO_3$ and brine, the residue was treated with Pd/C (0.1 g) and ammonium formate (1.14 g, 18 mmol) in 20 mL MeOH under reflux for 2 h. The reaction mixture was filter and the filtrate was removed in vacuum. The obtained solid residue was dissolved in DCM and washed with water, dried with anhydrous Na_2SO_4 overnight and rotary evaporated to dryness. The crude residue obtained was purified by silica gel column chromatography (petroleum ether: ethyl acetate 3:1-1:5 v/v) to afford the desired compounds.

*4.2.3.1. 14-O-[(4-Methoxy-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**6a**)*

White solid, 0.32 g (yield 35%); IR (KBr): 3448, 2983, 2884, 1734, 1677, 1503, 1455, 1411, 1365, 1288, 1153, 1118, 1019, 980, 916, 754, 722, 542 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.39 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.97 (s, 1H), 5.69 (d, *J* = 8.5 Hz, 1H), 5.26 (dd, *J* = 11.0, 1.6 Hz, 1H), 5.12 (dd, *J* = 17.4, 1.6 Hz, 1H), 3.91 – 3.76 (m, 2H), 3.43 (s, 3H), 3.29 (dd, *J* = 10.3, 6.4 Hz, 1H), 2.27 – 2.12 (m, 3H), 2.09 – 1.93 (m, 6H), 1.70 (dd, *J* = 14.3, 3.3 Hz, 1H), 1.62 – 1.55 (m, 2H), 1.38 (s, 4H), 1.27 – 1.16 (m, 3H), 1.08 (s, 4H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.67 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 215.9, 165.7, 161.1, 158.8, 137.9, 116.2, 106.8 , 73.5, 69.1, 57.0, 44.4, 43.4, 42.9, 40.9, 35.6, 35.0, 33.8, 33.4, 29.3, 29.0, 25.8, 25.4, 23.8, 22.7, 15.9, 13.8, 13.2, 10.5; HRMS (ES) calcd $[M + H]^+$ for $C_{29}H_{42}N_2O_5S$ 517.2747, found 517.2741.

*4.2.3.2. 14-O-[(4-Ethoxy-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**6b**)*

White solid, 0.30 g (yield 31%); IR (KBr): 3468, 2935, 2860, 1730, 1664, 1493, 1456, 1402, 1372, 1310, 1290, 1201, 1168, 1110, 1015, 924, 846, 563 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.47 (dd, *J* = 17.4, 11.0 Hz, 1H), 6.02 (s, 1H), 5.75 (d, *J* = 8.5 Hz, 1H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.19 (dd, *J* = 17.4, 1.6 Hz, 1H), 4.16 – 4.02 (m, 2H), 3.99 – 3.79 (m, 2H), 3.35 (d, *J* = 6.5 Hz, 1H), 2.31 – 2.20 (m, 2H), 2.09 (d, *J* = 33.8 Hz, 4H), 1.76 (dd, *J* = 14.6, 3.1 Hz, 1H), 1.65 (d, *J* = 11.4 Hz, 2H), 1.45 (s, 4H), 1.34 (t, *J* = 7.1 Hz, 4H), 1.29 – 1.21 (m, 5H), 1.14 (s, 4H), 0.86 (d, *J* = 7.0 Hz, 3H), 0.74 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 215.8, 165.8, 160.9, 158.0, 137.9, 116.3, 107.3, 73.5, 69.1, 57.0, 44.4, 43.4, 42.9, 40.9, 38.6, 35.7, 35.0, 33.7, 33.4, 29.4, 28.7, 25.8, 25.3, 23.8, 22.6, 15.9, 13.8, 11.8, 10.4.; HRMS (ES) calcd $[M + H]^+$ for $C_{29}H_{42}N_2O_5S$ 531.2888, found 531.2884.

*4.2.3.3. 14-O-[(4-Isopropoxy-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**6c**)*

White solid, 0.44 g (yield 45%); IR (KBr): 3448, 2925, 2864, 1733, 1676, 1500, 1452, 1413, 1376, 1362, 1297, 1263, 1176, 1116, 1058, 1019, 916, 731 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.87 (s, 1H), 5.67 (d, *J* = 8.5 Hz, 1H), 5.28 (dd, *J* = 11.0, 1.6 Hz, 1H), 5.13 (dd, *J* = 17.5, 1.6 Hz, 1H), 3.89 – 3.67 (m, 2H), 3.28 (d, *J* = 6.5 Hz, 1H), 2.24 – 2.15 (m, 2H), 2.02 (s, 3H), 1.97 – 1.89 (m, 1H), 1.69 (dd, *J* = 14.4, 3.1 Hz, 1H), 1.54 (dd, *J* = 6.7, 3.6 Hz, 6H), 1.38 (s, 7H), 1.18 (s, 4H), 1.08 (d, *J* = 7.0 Hz, 4H), 0.79 (dd, *J* = 6.9, 3.4 Hz, 3H), 0.66 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 215.9, 165.8, 159.9, 157.9, 137.9, 116.3, 73.5, 68.9, 57.0, 44.4, 43.3, 42.9, 40.9, 35.6, 34.9, 34.3, 33.4, 29.3, 28.7, 25.8, 25.3, 23.8, 22.2, 21.7, 18.2, 15.9, 13.8, 13.1, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{30}H_{44}N_2O_5S$ 545.3009, found 545.3004.

*4.2.3.4. 14-O-[(4-Butoxy-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**6d**)*

White solid, 0.24 g (yield 24%); IR (KBr): 3473, 2957, 2865, 1735, 1498, 1439, 1402, 1374, 1269, 1153, 1118, 1018, 981, 915, 839 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.40 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.94 (s, 1H), 5.68 (d, *J* = 8.5 Hz, 1H), 5.26 (dd, *J* = 11.0, 1.6 Hz, 1H), 5.12 (dd, *J* = 17.4, 1.6 Hz, 1H), 3.93 (td, *J* = 7.5, 5.6 Hz, 2H), 3.84 – 3.73 (m, 2H), 3.28 (d, *J* = 6.3 Hz, 1H), 2.24 – 2.15 (m, 2H), 2.06 (s, 4H), 1.98 (s, 2H), 1.62 (td, *J* = 14.6, 13.8, 9.5 Hz, 5H), 1.37 (d, *J* = 9.1 Hz, 8H), 1.21 – 1.14 (m, 3H), 1.07 (s, 3H), 0.90 (t, *J* = 7.4 Hz, 3H), 0.80 (d, *J* = 7.0 Hz, 3H), 0.67 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 215.9, 165.8, 160.9, 158.1, 137.9, 116.2, 107.2, 73.5, 69.0, 59.4, 57.0, 44.4, 43.4, 42.9, 40.8, 35.6, 35.0, 33.7, 33.4, 29.4, 28.6, 25.8, 25.4, 23.8, 22.6, 19.1, 15.8, 13.8, 13.2, 12.6, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{31}H_{46}N_2O_5S$ 559.3122, found 559.3117.

*4.2.3.5. 14-O-[(4-(2-aminoethoxy)-6-methylpyrimidine-2-yl) thioacetyl] mutilin (**6e**)*

White solid, 036 g (yield 37%); IR (KBr): 3495, 3346, 2930, 2884, 1724, 1583, 1547, 1455, 1343, 1286, 1168, 1117, 1055, 1017, 980, 916, 736, 698 cm^{-1}; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.48 (dd, *J* = 17.4, 11.0 Hz, 1H), 6.23 (s, 1H), 5.72 (d, *J* = 8.5 Hz, 1H), 5.30 (d, *J* = 2.9 Hz, 2H), 5.12 (d, *J* = 10.9 Hz, 3H), 4.39 (q, *J* = 5.9 Hz, 2H),

3.90 – 3.77 (m, 2H), 3.56 (q, *J* = 5.6 Hz, 2H), 3.33 (d, *J* = 6.3 Hz, 1H), 2.34 (s, 2H), 2.31 – 2.17 (m, 3H), 2.09 – 1.98 (m, 2H), 1.75 (dd, *J* = 14.3, 3.1 Hz, 1H), 1.68 – 1.58 (m, 2H), 1.49 – 1.39 (m, 5H), 1.26 (h, *J* = 5.1, 4.4 Hz, 3H), 1.12 (s, 4H), 0.85 (d, *J* = 7.0 Hz, 3H), 0.71 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 215.9, 166.9, 138.0, 127.5, 127.2, 116.1, 101.6, 73.5, 68.7, 65.9, 64.6, 57.1, 52.4, 44.4, 43.6, 42.9, 40.9, 35.7, 34.9, 33.4, 33.1, 29.4, 25.8, 25.3, 23.8, 22.6, 15.8, 13.7, 10.4; HRMS (ES) calcd $[M + H]^+$ for $C_{29}H_{43}N_3O_5S$ 546.2986, found 546.2991.

4.3. Biological evaluation

4.3.1. MIC testing

The MICs of the pleuromutilin derivatives were determined using a modified standard microtiter dilution method. Briefly, compounds, as well as tiamulin fumarate and retapamulin used as reference drugs, were dissolved in 25% DMSO to a solution with concentration of 64 μg/mL. All the solutions were then diluted two-fold with distilled water to provide 11 dilutions (final concentration is 0.0613 μg/mL). Inoculums, including *S. aureus*-25923, MRSE-51625, MRSA-337371, *S. dysgalactiae*, *S. agalactiae*, *E. faecalis*, *P. multocida*-1659 and *E. coli*-1659 were incubated overnight at 37 °C and transferred to new nutrient broth (MHB) until the logarithmic phase of growth. The bacteria were then diluted to 10^5-10^6 colony forming units (CFU)/mL in MHB. A 100 μL volume of bacteria in MHB was mixed with 100 μL of serially diluted aliquots of the compounds in sterile 96-well plates, which resulted in the final concentration of each dilutions decreasing two fold. The mixtures were then incubated at 37 °C for 18-24 h in an incubator. The MIC was determined by optical density (OD) measurements at a wavelength of 492 nm as the lowest concentration of the compounds that resulted in no bacterial growth. The results were expressed as an average of the MICs obtained from three independent experiments.

4.3.2. Bactericidal time-kill kinetics

MRSA-337371 was cultured in MuellereHinton broth at 37 °C for 6 h with shaking and then diluted to approximately 6×10^5 CFU/mL. Test compounds **5c**, **5g** and tiamulin fumarate with the final concentrations of 0.5 × MIC, 1 × MIC, 2 × MIC and 4 × MIC were inoculated with the aliquots of bacteria resuspended in fresh media. After specified time intervals (0, 2, 4, 6, 8, 12 and 24 h), 0.5 mL aliquots were serially diluted to 10^{-1} to 10^{-8} by 10-fold in 0.9% saline. The resulted dilutions were plated on sterile MuellereHinton agar plates and incubated at 37 °C for 24 h. The viable colonies were counted and represented as $\log_{10}$ (CFU/mL). The same procedure was repeated in triplicate.

4.4. Mouse systemic infection model

The *in vivo* efficacy of compound **5c** was determined in 6-8 weeks old Kunming mice (specific pathogen-free,

Laboratory Animal Center of Lanzhou University, Lanzhou, China). All mice were rendered neutropenic upon treatment with 150 mg/kg cyclophosphamide intraperitoneally for four days and with 100 mg/kg for one day prior to inoculation, respectively. The neutropenic mice were anesthetized with isoflurane and then received a 0.5 mL MRSA-337371 inoculum of 10^8 CFU/mL via intraperitoneal injection. Some animals were used for determining the survival and ED_{50} which have been described in our previously work [18, 19]. Briefly, about 1 h after infection, the mice were then intraperitoneal administered compound **5c** dissolved in 0.5 mL vehicle (DMSO:Tween-80:sterile:water = 0.5:0.5:9) at doses of 5, 8, 15, 25, and 40 mg/kg body weight (10 per group). Tiamulin fumarate was used as a reference drug in the same manner at the same doses as **5c**. All animals were observed twice daily for symptoms and mortality for the animal procedures. The survival of the mice at 7 d after infection was used as the end-point, and the ED_{50} was calculated by the Bliss method.

For the determination of MRSA in tissues and histological analyses, the neutropenic mice were euthanized and sacrificed after treatment for 2 d. Then the kidney, liver and a part of lung were cut to two parts: one was weighted and ground to homogenate with sterile saline, followed by 10-fold dilution. The resulted dilutions were plated on sterile MuellereHinton agar plates and incubated at 37 °C for 24 h. The same procedure was repeated in triplicate. The viable colonies were counted (represented as CFU/mL) and the bacterial load was calculated as follow: CFU/g = (the mean CFU × 10 ×volume of homogenate × dilution factor)/weight of tissues. The other part of lung was collected and fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E) and examined under light microscopy [21]. The protocol for this study was reviewed and approved by the Laboratory Animal Ethical Commission of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS.

4.5. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows version 24.0 (SPSS Inc., Chicago, USA). The data were analyzed by One-way analysis of variance (ANOVA), followed by Dunnett's *post-hoc* tests as appropriate. Statistical significant difference was defined as a $p < 0.05$ and the extremely significant difference was defined as a $p < 0.01$.

Acknowledgments

This work was financial supported by the National Natural Science Foundation of China (No. 31873027), “Quancheng Scholar” Construction Project of Jinan (No. 00252019025) and Agricultural Science and Technology

Innovation Program (ASTIP, CAAS-LMY-03).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at:

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Figure Legends

Figure 1. Structural formulas of pleuromutilin, tiamulin, valnemulin, retapamulin and lefamulin.

Figure 2. ORTEP diagram for compound **3** with ellipsoids set at 75% probability.

Figure 3. Time-kill analysis of compounds **5c** (A), **5g** (B) and tiamulin fumarate (C) against MRSA-337371.

Figure 4. Efficacy of compound **5c** (A) and tiamulin fumarate (B) in mouse systemic infection model.

Figure 5. The bacterial load of compound **5c** and tiamulin in lung (A), kidney (B) and liver (C) after challenge of MRSA.

Figure 6. Representative lung histology of mice in normal control group (A), control group (B), **5c** treated group with 40 mg/kg (C), **5c** treated group with 20 mg/kg (D), tiamulin treated group with 40 mg/kg (E), and tiamulin treated group with 20 mg/kg (F) treatment.

Scheme 1. General synthetic scheme for the pleuromutilin derivatives **5a-m**.

Scheme 2. Synthesis of pleuromutilin derivatives **6a-d**.

Scheme 3. Preparation of pleuromutilin derivative **6e**.

• A series of novel pleuromutilin derivatives with substituted 6-methylpyrimidine moieties were synthesized.

• Synthesized compounds were evaluated for their antibacterial activity.

• Compounds **5c** showed excellent antibacterial activity and potent therapeutic efficacy in mouse systemic infection.

Declaration of interests

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

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