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Research paper

Synthesis and evaluation of novel pleuromutilin derivatives with a substituted pyrimidine moiety

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ABSTRACT

A series of novel pleuromutilin derivatives possessing 6-hydroxy pyrimidine moieties were synthesized via acylation reactions under mild conditions. The *in vitro* antibacterial activities of the synthesized derivatives against methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), *Bacillus subtilis* (*B. subtilis*), and *Escherichia coli* (*E. coli*) were tested by the agar dilution method. The majority of the screened compounds displayed potent activities. Compounds **3** and **6a** were found to be the most active antibacterial agents against MRSA and MRSE. Moreover, in the vivo experiment, compound **6a** showed comparable antibacterial activity to that of tiamulin, with ED_{50} of 5.47 mg/kg body weight against MRSA.

1. Introduction

Many hospitals lack the effect way to the late phase of infection as a result of the continuing rise in antibiotic resistance among pathogenic bacterial strains. Therefore, development of new antibacterial agents possessing a novel mechanism of action is of great importance to the medical community [1].

Pleuromutilin (**1**, Fig. 1), a natural antibiotic with antibacterial activities, was isolated in 1951 from two basiomycetes species, *Pleurotus mutilis* and *Pleurotus passeckeranius* [2]. The pleuromutilin class has a unique mode of action, which involves inhibition of protein synthesis by primarily inhibiting ribosomal peptidyl transferase center (PTC) [3,4]. Further studies demonstrated that the interactions of tricyclic core of tiamulin are mediated through hydrophobic interactions and hydrogen bonds, which are formed mainly by the nucleotides of domain V [5,6].

The process of semi-synthesis based on natural products, especially the complex natural products, is to be the predominant avenue to new antibiotics [7]. The chemical modifications of pleuromutilin have been made for an attempt to improve the antimicrobial activities and *in vivo* efficacy after the identification of structure of pleuromutilin. The introduction of a thioether at the C-22 position of pleuromutilin analogues and the presence of a basic group enhance antibacterial activity [8]. Thus, the modifications which have focused on their C-14 side chain have led to three drugs: Tiamulin (Fig. 1.), valnemulin and retapamulin (Fig. 1.). Tiamulin and valnemulin were approved for veterinary, and retapamulin became the first pleuromutilin drug approved for use in humans [8–12].

A range of pleuromutilin derivatives with a heterocyclic ring at the C14 side chain has been recently synthesized and studied [13]. Structure activity relationship (SAR) study showed the synthesized pleuromutilin derivatives with a heterocyclic ring at the C14 position may increase hydrogen bonding and π–π stacking interactions and thereby have strong antibacterial properties [14–16]. In addition, compounds bearing primary amine substituents at pyridine ring incorporated into the C14 side chain exhibited antibacterial activity [16]. The molecular docking results revealed the substituents with hydrogen donators, for example, hydroxy and amino group, at the C14 side chain enhance the binding affinities by hydrogen bondings and thus should be introduced to produce new analogues with higher antibacterial activities [17].

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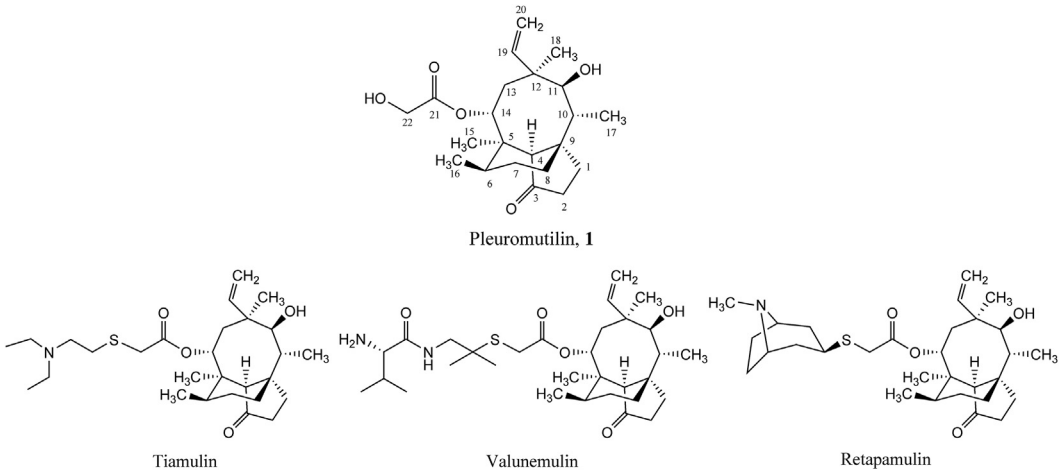


Fig. 1. Structures of pleuromutilin and its drugs.

Previous work in our group has led to the synthesis of pleuromutilin derivatives with a 1,3,4-thiadiazole ring at the C14 position. The antibacterial studies prove that heterocyclic ring bearing polar groups at the C14 side chain of pleuromutilin derivatives may raise their antibacterial activity [17,18]. This conclusion is supported by the work of Ling et al. [16]. They designed and synthesized a serial of pleuromutilin with pyridine ring bearing amino groups which exhibit excellent *in vitro* antibacterial activity against both sensitive and resistant Gram-positive bacterial strains. The compounds with pyrimidine ring were wide found in nature products, such as nucleotides, thiamine (vitaminB1) and alloxan [19]. The pyrimidine system also turned out to be an important pharmacophore, and is found in many synthetic compounds such as antibacterial drug, trimethoprim, barbiturates and the HIV drug, zidovudine [19]. We now report the design, synthesis, and antibacterial studies of novel pleuromutilin derivatives with 6-hydroxy pyrimidine incorporated into the C14 side chain for identification of a higher soluble and more efficacious drug candidate.

2. Results and discussion

2.1. Chemistry

The general synthetic route to build the pleuromutilin derivatives is illustrated in Scheme 1. The lead compound 14-O-[(4-amino-6-hydroxy-pyrimidine-2-yl) thioacetyl] mutilin (**3**) was prepared by nucleophilic substitution of 22-O-tosylpleuromutilin (**2**) with 4-amino-6-hydroxy-2-mercaptopyrimidine monohydrate under basic conditions in 78% yield. Pleuromutilin derivatives **4** and **5** were always simultaneously obtained as isomers from **3** because of the keto–enol equilibrium of the pyrimidinone scaffold [20]. The reaction generally gave **4** and **5** (1.0:2.1 ratio) in the presence of K_2CO_3 as base.

Compounds **6a**–**h** were directly obtained by condensation reactions between the amino group of compound **3** and the carboxyl group of carboxylic acids or amino acids in which amino groups were protected by tert-butoxycarbonyl (BOC) groups. The reactions were performed at room temperature in the presence of 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDCI), 1-hydroxybenzotriazole (HOBt) and N,N-Diisopropylethylamine (DIPEA) as base. The protected amino groups were hydrolysed using a solution of TFA in DCM for 30 min and the compounds **6e**-**h** were obtained in a yield of 41–73%. We also extensively synthesized but obtained with trace the isomers of compounds **6b**-**d**: 14-O-[(4-(2-Chlorobenzamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin, 14-O-[(4-(4-Chlorobenzamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin, 14-O-[(4-(2-Methylbenzamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin, 14-O-[(4-(3-Methylbenzamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin, 14-O-[(4-(2-Methoxylbenzamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin, and 14-O-[(4-(3-Methoxylbenzamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin. The structures of the synthesized derivatives were characterized by IR, ^{1}H NMR, ^{13}C NMR and HRMS spectra (Supplementary data) and further supported by the single crystal X-ray diffraction analysis of compound **4** (Fig. 2).

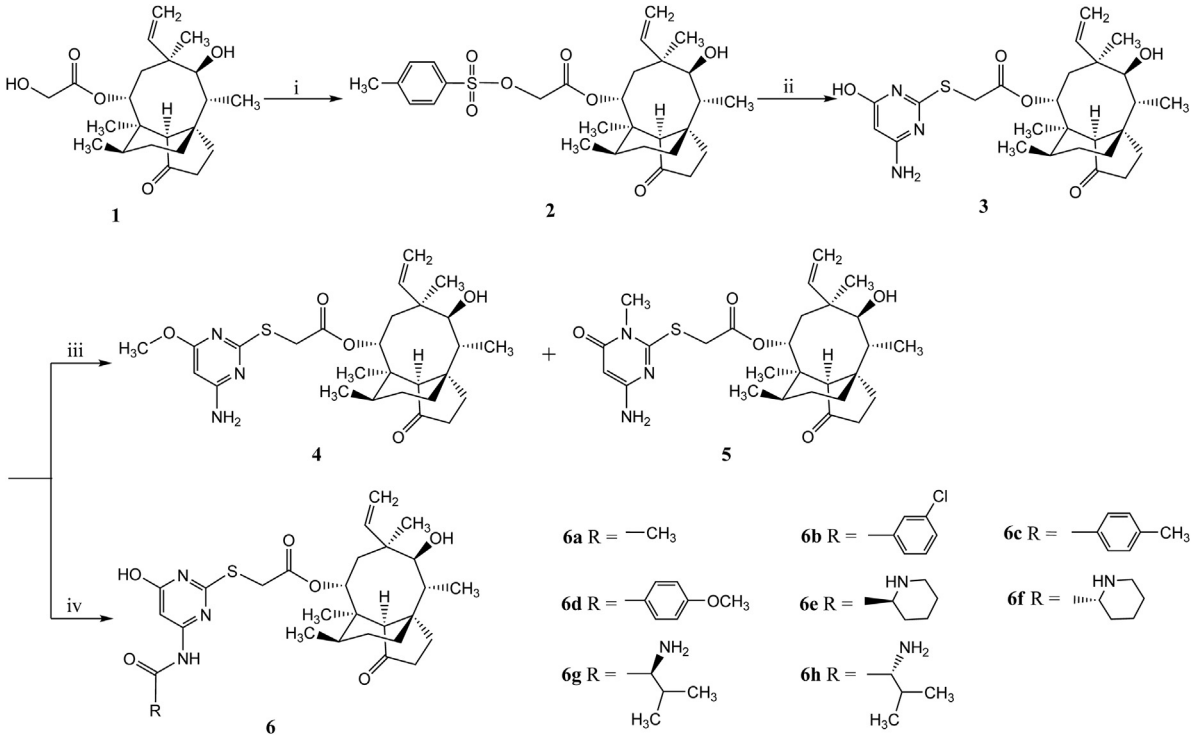
2.2. Antibacterial activity

The minimum inhibitory concentrations (MIC) of the obtained compounds **3**, **4**, **5** and **6a**-**h** in comparison to tiamulin fumarate were tested against drug-resistant microorganisms, including three Gram (+), methicillin-resistant *Staphylococcus aureus* ATCC 29213 (MRSA), methicillin-resistant *Staphylococcus epidermidis* ATCC 35984 (MRSE) and *Bacillus subtilis* ATCC 11778 (*B. subtilis*), and one Gram (−), *Escherichia coli* ATCC 25922 (*E. coli*) by the agar dilution methods. The results are given in Table 1. Compounds **3** and **6a** showed the most antibacterial activities which were superior or comparable antibacterial activities to that of tiamulin fumarate, with MIC values both in the 32–0.125 μg/mL range. Compounds **4**, **5**, **6g** and **6h** showed slightly less activity against MRSA and MRSE and lower potent against *B. subtilis* when compared to that of tiamulin fumarate. However, all the compounds except **6a** displayed lower antibacterial activities against *E. coli* in comparison to that of tiamulin fumarate.

Compounds **3**, **4**, **5**, **6g** and **6h** with a primary amine on the terminal C-14 glycolic acid side chain presented improved activity against MRSA and MRSE compared with the compounds **6b**-**f**. To our surprise, compound **6a** with an acetyl group on the C-14 side chain displayed highest activity than the other compounds. The results of MICs indicated that the introduction of the primary amine into the C-14 glycolic acid side chain could enhance antibacterial activity, which was consistent with previous reported [17,18].

2.3. In vivo efficacy in mouse model

Compound **6a** was evaluated for *in vivo* efficacy by measuring the survival of mice after a lethal challenge of MRSA (1×10^9 CFU in



Scheme 1. Reagent and condition: (i) TsCl, NaOH, H_2O, t-butyl methyl ether, reflux, 1 h; (ii) 4-Amino-6-hydroxy-2-mercaptopyrimidine monohydrate, NaOH, CH_3OH, DCM, H_2O, rt, 48 h; (iii) CH_3I, K_2CO_3, DMF, 80 °C, 4 h; (iv) Acid derivatives, EDCI, HOBt, DIPEA, DCM, rt, 36–48 h.

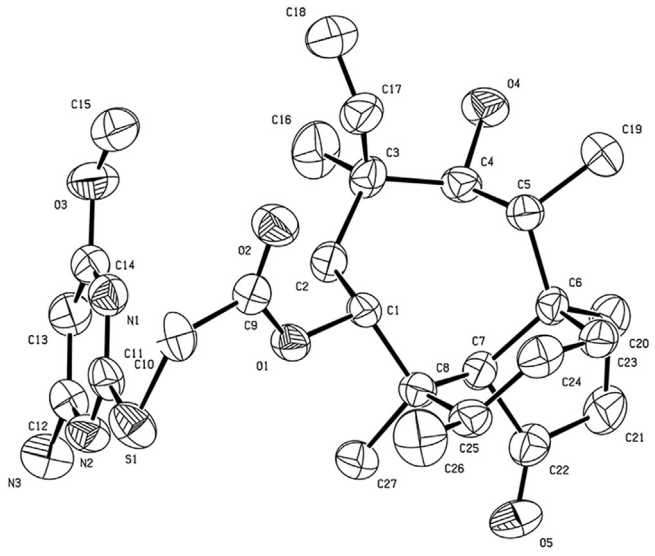


Fig. 2. ORTEP diagram for compound **4** with ellipsoids set at 75% probability (hydrogen atoms were omitted for clarity).

0.1 mL saline), and tiamulin fumarate was used as the reference drug. After being infected with MRSA, the mice were intravenously treated with different doses of **6a** dissolved in 3.0% soybean lecithin solution used as a vehicle. Mice injected with vehicle alone showed 100% mortality in this model. Treatment with **6a** and tiamulin fumarate displayed dose-dependent protection and led to the survival of the mice infected with MRSA (see Fig. 3), with ED_{50} of 5.47 and 5.95 mg/kg body weight, respectively. Thus, **6a** showed comparable antibacterial activity to that of tiamulin against MRSA in the mouse systemic model. The result of *in vivo* efficacy showed that **6a** was able to protect animals in a dose-dependent fashion and might act as a potent antibacterial drug.

3. Conclusion

In conclusion, a series of novel pleuromutilin derivatives bearing 6-hydroxy pyrimidine were synthesized and characterized by IR, ^1H NMR, ^{13}C NMR and HRMS. These derivatives were initially evaluated for their *in vitro* antibacterial activities against three Gram-positive strains (MRSA, MRSE and *Bacillus subtilis*) and a Gram-negative strain *Escherichia coli*. Compound **6a** was chosen for further evaluation *in vivo* activity against MRSA using systemic infection mode in mice. Our results demonstrate that most synthesized compounds have considerable *in vitro* antibacterial activity. Especially compounds with a primary amine on the terminal C-14 glycolic acid side chain showed improved activity against MRSA and MRSE. Compounds **3** and **6a** exhibited superior or comparable antibacterial activities to that of tiamulin fumarate against MRSA and MRSE. It is important to note that **6a** showed comparable efficacy to that of tiamulin fumarate against MRSA in the mouse systemic model.

4. Experimental protocols

4.1. General

All the starting materials were of reagent grade. The solvents used for the isolation/purification of the compounds were purified prior to use unless noted otherwise. All reactions were monitored by thin-layer chromatography (TLC) using 0.2-mm-thick silica gel GF254 pre-coated plates (Qingdao Haiyang Chemical Co., Ltd, Shandong, China). After elution, the plates were visualized under UV illumination at 254 nm for UV active materials. Further visualization was achieved by staining with a 0.05% $KMnO_4$ aqueous

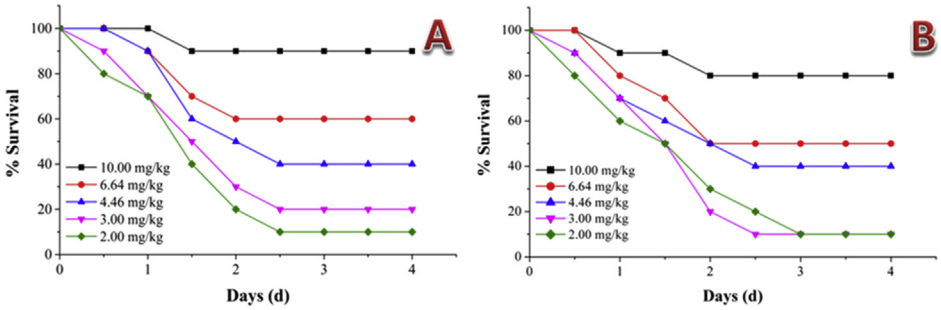


Fig. 3. Efficacy of compound **6a** (A) and tiamulin fumarate (B) in mouse systemic infection model.

Table 1
In vitro antibacterial activities of pleuromutilin derivatives.

Compounds	MICs (μg/mL)			
	MRSA	MRSE	*B. subtilis*	*E. coli*
3	0.25	0.125	32	16
4	1	0.5	16	32
5	2	1	16	32
6a	0.125	0.125	32	8
6b	32	16	64	16
6c	4	2	32	32
6d	4	1	32	16
6e	4	1	16	16
6f	8	2	16	32
6g	2	0.5	16	32
6h	1	0.125	8	64
Tiamulin fumarate	0.5	0.25	32	8

solution. All column chromatography purifications were carried out on a 200–300 mesh silica gel (Qingdao Haiyang Chemical Co., Ltd, Shandong, China) with conventional methods. IR spectra were obtained on a NEXUS-670 spectrometer (Nicolet Thermo, Edina, MN, USA) using KBr thin films, and the absorptions are reported in cm^{-1}. ^1H NMR and ^{13}C NMR spectra were recorded using Bruker-400 MHz spectrometer (Bruker BioSpin, Zürich, Zürich State, Switzerland). 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-Amino-6-hydroxy -pyrimidine-2-yl) thioacetyl] mutilin (*3*)

To a solution of 4-amino-6-hydroxy-2-mercaptopyrimidine monohydrate (1.65 g, 10 mmol) in 20 mL methanol, 10 M NaOH (1.1 mL, 11 mmol) was added and stirred for 30 min. A solution of 22-O-tosylpleuromutilin (5.33, 10 mmol) in 20 mL of DCM was added dropwise to the reaction mixture. The mixture was stirred for 36–40 h at room temperature and evaporated under reduced pressure to dryness. The crude product was extracted with a solution of ethyl acetate (60 mL) and water (20 mL) and treated with saturated $NaHCO_3$. The target compound **3** was then precipitated from the solution and purified by flash silica column chromatography (ethyl acetate: ethanol 20:1 v/v) to yield 3.93 g (78%). IR (KBr) 3368, 2930, 1729, 1629, 1575, 1542, 1457, 1286, 1117, 1019, 981, 809 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.47 (dd, J = 17.4, 11.1 Hz, 1H), 5.74 (d, J = 8.3 Hz, 1H), 5.32 (d, J = 11.0 Hz, 1H), 5.24–5.13 (m, 2H), 4.79 (s, 1H), 4.11 (q, J = 7.1 Hz, 1H), 3.85 (d, J = 16.5 Hz, 1H), 3.69 (d, J = 16.5 Hz, 1H), 3.35 (d, J = 6.2 Hz, 1H), 2.29–1.98 (m, 6H), 1.75 (d, J = 14.1 Hz, 1H), 1.63 (dd, J = 21.3, 11.1 Hz, 2H), 1.56–1.18 (m, 9H), 1.10 (d, J = 20.2 Hz, 4H), 0.86 (d, J = 6.8 Hz, 3H), 0.71 (d, J = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.93, 166.90, 165.97, 162.65, 160.33, 139.23, 117.16, 84.04, 74.59, 70.10, 60.39, 58.10, 45.43, 43.94, 41.85, 36.69, 36.00, 33.25, 30.39, 26.88, 26.34, 24.81, 21.03, 16.79, 14.89, 11.45; HRMS (ES) calcd $[M+H]^+$ for $C_{26}H_{37}N_3O_5S$ 504.2526, found 504.2520.

4.2.2. 14-O-[(4-Amino-6-methoxyl-pyrimidine-2-yl) thioacetyl] mutilin (*4*) and 14-O-[4-amino-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl) thioacetyl] mutilin (*5*)

Methyl iodide (0.71 g, 5.0 mmol) was added dropwise into a suspension of compound **3** (2.51 g, 5.0 mmol) in anhydrous DMF (20 mL) and K_2CO_3 (15 mmol). The reaction mixture was stirred at 80–85 °C for 4 h. After the mixture was cooled, the inorganic material was taken off by filtration and the solvent was removed in vacuum. The solid residue, consisting of a mixture of **4** and **5** isomers, was obtained and purified using silica column chromatography (petroleum ether: ethyl acetate 1:15 v/v) to yield 0.75 g (29%) and 1.58 g (61%) of compounds **4** and **5**, respectively. Compound **4**: IR (KBr) 3374, 2928, 1732, 1626, 1585, 1548, 1390, 1307, 1273, 1212, 1152, 1117,1048, 1017, 982, 917 cm^{-1}; ^1H NMR (400 MHz, DMSO) δ 6.67 (s, 2H), 6.11 (dd, J = 17.7, 11.2 Hz, 1H), 5.52 (d, J = 8.2 Hz, 1H), 5.42 (s, 1H), 5.02 (dd, J = 23.6, 14.4 Hz, 2H), 4.49 (d, J = 6.0 Hz, 1H), 3.85 (s, 1H), 3.75 (s, 2H), 3.41 (t, J = 5.7 Hz, 1H), 2.39 (s, 1H), 2.32–1.92 (m, 5H), 1.63 (s, 2H), 1.49–1.15 (m, 9H), 1.02 (s, 5H), 0.80 (d, J = 6.8 Hz, 3H), 0.58 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO) δ 217.59, 169.52, 168.33, 168.09, 165.63, 141.25, 115.71, 82.04, 73.12, 70.20, 60.23, 57.72, 53.71, 45.43, 44.46, 42.01, 36.82, 34.48, 33.69, 30.58, 28.93, 27.10, 24.92, 21.24, 16.56, 14.99, 11.96; HRMS (ES) calcd $[M+H]^+$ for $C_{27}H_{39}N_3O_5S$ 518.2683, found 518.2681.

Compound **5**: IR (KBr) 3422, 2929, 1730, 1630, 1509, 1457, 1414, 1282, 1154, 1117, 1094, 807; ^1H NMR (400 MHz, DMSO) δ 6.28 (s, 2H), 6.10 (dd, J = 17.8, 11.2 Hz, 1H), 5.52 (d, J = 8.1 Hz, 1H), 5.05 (dd, J = 27.5, 14.5 Hz, 2H), 4.92 (s, 1H), 4.52 (d, J = 5.9 Hz, 1H), 4.07–3.96 (m, 2H), 3.42 (d, J = 5.4 Hz, 1H), 3.34 (s, 1H), 2.40 (s, 1H), 2.22–1.93 (m, 5H), 1.62 (dd, J = 24.8, 12.6 Hz, 2H), 1.52–1.13 (m, 9H), 1.05 (s, 4H), 0.82 (d, J = 6.8 Hz, 3H), 0.61 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO) δ 217.55, 170.79, 166.83, 161.88, 161.51, 160.51, 141.22, 115.85, 81.23, 73.13, 70.83, 60.22, 57.65, 45.43, 44.63, 43.90, 42.04, 36.82, 34.56, 30.57, 29.07, 27.09, 24.93, 21.23, 16.61, 14.89, 11.98; HRMS (ES) calcd $[M+H]^+$ for $C_{27}H_{39}N_3O_5S$ 518.2683, found 518.2696.

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

A mixture of the carboxylic acids derivative (2.2 mmol), 0.38 g 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (2.0 mmol), 0.27 g 1-Hydroxybenzotriazole (2.0 mmol) and 0.39 g N,N-diisopropylethylamine (3.0 mmol) were dissolved in DCM (15 ml) and stirred for 15 min. Then, 0.50 g compound **3** (1 mmol) was added in one portion and the reaction was stirred at room temperature for 36–48 h. The mixture was washed with

saturated aqueous $NaHCO_3$ and brine, and then dried with Na_2SO_4 overnight. Before the synthesis of compounds **6g** and **6h**, the amino of valine should be protected by ditertbutyl dicarbonate (BOC_2O). After treatment with $NaHCO_3$ and brine, the residue was treated with a mixture of 10 mL trifluoroacetic acid (TFA) and 10 mL DCM at room temperature for 30 min. The reaction mixture was quenched with 25% aqueous $NaHCO_3$ (15 mL) 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 1:2 v/v) to afford the desired compounds.

4.2.4. 14-O-[(4-Acetamido-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin (**6a**)

White solid, 0.80 g (yield 73%); IR (KBr): 3448, 2931, 1779, 1729, 1627, 1588, 1549, 1459, 1400, 1372, 1299, 1201, 1162, 1117, 1030, 981, 939 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.47 (dd, J = 17.2, 11.0 Hz, 1H), 5.73 (d, J = 7.9 Hz, 1H), 5.32 (d, J = 10.8 Hz, 1H), 5.17 (d, J = 17.5 Hz, 2H), 4.69 (s, 2H), 3.85 (d, J = 16.4 Hz, 1H), 3.78–3.56 (m, 3H), 3.34 (d, J = 4.6 Hz, 1H), 2.16 (ddd, J = 75.4, 48.8, 32.3 Hz, 5H), 1.75 (d, J = 13.9 Hz, 2H), 1.63 (d, J = 9.1 Hz, 3H), 1.28 (dd, J = 45.4, 39.0 Hz, 9H), 1.13 (s, 4H), 0.86 (d, J = 6.2 Hz, 3H), 0.71 (d, J = 6.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.96, 167.08, 166.35, 162.71, 160.59, 139.24, 117.18, 84.18, 74.59, 70.05, 58.41, 58.11, 45.44, 44.32, 43.95, 41.86, 36.70, 36.01, 34.45, 33.29, 30.40, 26.89, 26.34, 24.82, 18.43, 16.81, 14.90, 11.47; HRMS (ES) calcd $[M+H]^+$ for $[C_{29}H_{41}N_3O_5S$ 560.2788, found 560.2784.

4.2.5. 14-O-[(4-(3-Chlorobenzamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin (**6b**)

White solid, 0.53 g (yield 41%); IR (KBr): 3368, 3215, 2931, 1735, 1625, 1586, 1547, 1459, 1283, 1240, 1116, 981, 739 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.20–7.91 (m, 2H), 7.73–7.34 (m, 2H), 6.49 (s, 1H), 6.08 (s, 1H), 5.72 (s, 1H), 5.21 (dd, J = 50.2, 22.6 Hz, 4H), 4.11 (d, J = 6.7 Hz, 1H), 3.97–3.57 (m, 2H), 3.34 (d, J = 6.1 Hz, 1H), 2.40–1.90 (m, 6H), 1.63 (td, J = 47.2, 13.3 Hz, 3H), 1.42 (s, 4H), 1.36–1.18 (m, 3H), 1.10 (s, 4H), 0.85 (d, J = 5.5 Hz, 3H), 0.72 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 217.15, 170.39, 167.95, 165.03, 164.49, 162.37, 139.15, 134.89, 134.19, 130.37, 130.00, 129.70, 128.54, 117.12, 91.07, 74.63, 69.84, 60.42, 58.17, 45.46, 43.98, 41.89, 36.77, 36.00, 34.48, 34.05, 30.43, 26.89, 26.37, 21.05, 16.84, 14.90, 11.45; HRMS (ES) calcd $[M+H]^+$ for $C_{33}H_{40}ClN_3O_6$ 642.2399, found 642.2397.

4.2.6. 14-O-[(4-(4-Methylbenzamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin (**6c**)

White solid, 0.53 g (yield 43%); IR (KBr): 3375, 2930,1735, 1624, 1586, 1458, 1298, 1255, 1166, 1117, 1071, 980, 745 cm^{-1}; 1H NMR (400 MHz, $CDCl_3$) δ8.06–7.97 (m, 2H), 7.29 (s, 1H), 6.59–6.36 (m, 1H), 6.09 (dd, J = 6.6, 3.0 Hz, 1H), 5.72 (d, J = 6.3 Hz, 1H), 5.36–5.23 (m, 1H), 5.21–5.06 (m, 2H), 3.91–3.67 (m, 2H), 3.33 (s, 1H), 2.44 (d, J = 1.1 Hz, 2H), 2.33–2.01 (m, 5H), 1.74 (d, J = 14.5 Hz, 2H), 1.66–1.50 (m, 3H), 1.51–1.18 (m, 9H), 1.16–1.04 (m, 4H), 0.85 (d, J = 6.4 Hz, 3H), 0.73 (dd, J = 4.3, 2.3 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 217.10, 170.22, 167.98, 164.83, 163.60, 145.16, 139.19, 130.52, 129.39, 125.89, 117.11, 91.10, 74.63, 69.72, 60.40, 58.18, 45.46, 43.98, 41.89, 36.79, 36.01, 34.49, 34.08, 30.45, 26.89, 26.36, 24.84, 21.81, 21.04, 16.84, 14.91, 11.45; HRMS (ES) calcd $[M+H]^+$ for $C_{34}H_{43}N_3O_6S$ 622.2945, found 622.2938.

4.2.7. 14-O-[(4-(4-Methoxylbenzamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin (**6d**)

White solid, 0.59g (yield 46%); IR(KBr):3369, 2933, 1734, 1605, 1581, 1549, 1511, 1458, 1401, 1299, 1251, 1160, 1116, 1069, 1024, 846 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.07 (d, J = 8.8 Hz, 2H), 6.94 (d, J = 8.8 Hz, 2H), 6.47 (dd, J = 17.4, 11.0 Hz, 1H), 6.07 (s, 1H), 5.70 (d, J = 8.4 Hz, 1H), 5.33–5.22 (m, 2H), 5.14 (d, J = 17.4 Hz, 1H), 3.86 (d, J = 8.8 Hz, 3H), 3.79–3.61 (m, 2H), 3.32 (d, J = 6.2 Hz, 1H), 2.36–1.93 (m, 5H), 1.74–1.50 (m, 3H), 1.47–1.14 (m, 9H), 1.15–1.04 (m, 4H), 0.84 (d, J = 6.9 Hz, 3H), 0.71 (d, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 217.16, 170.12, 168.02, 164.87, 164.37, 163.23, 139.16, 132.67, 120.85, 117.09, 113.96, 91.10, 74.61, 69.74, 58.17, 55.57, 45.45, 44.49, 43.97, 41.88, 36.77, 35.99, 34.48, 34.06, 30.43, 26.87, 26.36, 24.82, 18.41, 16.82, 14.90, 11.44; HRMS (ES) calcd $[M+H]^+$ for $C_{34}H_{43}N_3O_7S$ 638.2894, found 638.2892.

4.2.8. 14-O-[(4-(Piperidine-2-L(-)carboxamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin) (6e)

White solid, 0.62 g (yield 50%); IR(KBr): 3448, 2918, 2849, 1729, 1654, 1617, 1458, 1438, 1279,1362, 1265, 1151, 1240, 1115, 1042, 1081, 984, 957, 747 cm^{-1}.^1H NMR (400 MHz, CDCl3) δ 6.49 (dd, J = 17.2, 11.3 Hz, 1H), 5.96 (s, 1H), 5.73 (d, J = 8.5 Hz, 1H), 5.32 (d, J = 11.0 Hz, 1H), 5.30–5.01 (m, 3H), 4.29–3.99 (m, 1H), 3.79 (dd, J = 41.0, 16.3 Hz, 2H), 3.35 (s, 1H), 2.27 (s, 6H), 2.06 (d, J = 17.5 Hz, 5H), 1.63 (d, J = 10.3 Hz, 5H), 1.45–1.17 (m, 9H), 1.14 (s, 4H), 0.86 (d, J = 6.8 Hz, 3H), 0.74 (d, J = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.99, 170.41, 167.89, 167.61, 164.80, 139.28, 129.94, 128.13, 117.06, 90.73, 74.66, 69.73, 60.39, 58.20, 45.48, 44.54, 44.01, 41.92, 36.81, 36.04, 34.49, 34.09, 30.47, 26.93, 26.40, 24.86, 21.41, 16.84, 16.55, 14.91, 14.21, 11.43; HRMS (ES) calcd $[M+H]^+$ for $C_{32}H_{46}N_4O_6S$ 615.3210, found 615.3213.

4.2.9. 14-O-[(4-(Piperidine-2-(D)-carboxamide)-6-hydroxypyrimidine-2-yl) thioacetyl] mutilin (**6f**)

White solid, 0.60 g (yield 48%); IR(KBr): 3447, 1733, 1637, 1577, 1560, 1541, 1508, 1457, 1245, 1151, 1116, 969, 547 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.48 (ddd, J = 15.2, 11.0, 4.2 Hz, 1H), 5.96 (s, 1H), 5.74 (t, J = 8.6 Hz, 1H), 5.45–5.27 (m, 1H), 5.23–5.10 (m, 3H), 4.11 (q, J = 7.1 Hz, 1H), 3.91–3.64 (m, 2H), 3.34 (d, J = 6.3 Hz, 1H), 2.41–2.14 (m, 6H), 2.11–1.95 (m, 5H), 1.82–1.44 (m, 5H), 1.47–1.16 (m, 9H), 1.17–1.09 (m, 4H), 0.85 (d, J = 7.0 Hz, 3H), 0.73 (t, J = 6.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.90, 170.32, 167.87, 166.77, 164.80, 139.22, 117.21, 117.08, 90.78, 83.91, 74.62, 69.70, 60.40, 58.17, 45.46, 44.47, 43.97, 41.88, 36.78, 36.67, 34.48, 34.06, 30.43, 26.90, 26.36, 24.83, 21.42, 21.05, 16.83, 14.89, 14.20, 11.45; HRMS (ES) calcd $[M+H]^+$ for $C_{32}H_{46}N_4O_6S$ 615.3210, found 615.3202.

4.2.10. 14-O-[(4-(3-Methyl-2-(L)-amino-butyrylamide) -6-hydroxypyrimidine-2-yl) thioacetyl] mutilin (**6g**)

White solid, 0.72 g (yield 60%); IR(KBr): 3462, 2931, 1733, 1605, 1544, 1432, 1275, 1152, 1117,1043, 785, 547 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.48 (ddd, J = 15.2, 11.0, 4.2 Hz, 1H), 5.96 (s, 1H), 5.73 (d, J = 8.6 Hz, 1H), 5.40–5.24 (m, 1H), 5.17 (d, J = 17.1 Hz, 3H), 4.11 (q, J = 7.1 Hz, 1H), 3.92–3.63 (m, 2H), 3.34 (d, J = 6.3 Hz, 1H), 2.40–2.13 (m, 5H), 2.05 (dd, J = 17.3, 9.5 Hz, 4H), 1.69 (ddd, J = 11.8, 11.2, 3.3 Hz, 6H), 1.55–1.20 (m, 12H), 1.13 (s, 4H), 0.85 (d, J = 7.0 Hz, 3H), 0.74 (d, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.92, 167.05, 163.88, 159.63, 157.52, 139.20, 130.90, 128.84, 117.24, 94.45, 75.38, 74.59, 70.06, 67.78, 58.10, 45.44, 44.37, 43.95, 41.87, 36.72, 36.01, 34.45, 33.24, 30.41, 26.89, 26.27, 25.61, 24.83, 16.86, 14.88, 11.47; HRMS (ES) calcd $[M+H]^+$ for $C_{32}H_{48}N_4O_6S$ 617.3367, found 617.3360.

4.2.11. 14-O-[(4-(3-Methyl-2-(D)-amino-butyrylamide) -6-hydroxypyrimidine-2-yl) thioacetyl] mutilin (**6h**)

White solid, 0.69 g (yield 58%); IR(KBr): 3463, 3363, 2922, 1731, 1604, 1544, 1433, 1278, 1152, 1117, 1042, 785.40 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.48 (dd, J = 17.3, 11.0 Hz, 1H), 5.74 (d, J = 7.9 Hz, 1H), 5.46 (s, 1H), 5.33 (d, J = 10.9 Hz, 1H), 5.23–4.95 (m, 3H), 4.01 (dd, J = 14.5, 7.4 Hz, 1H), 3.93–3.62 (m, 2H), 3.34 (d, J = 6.0 Hz, 1H), 2.42–2.09 (m, 5H), 2.00 (dd, J = 15.7, 9.6 Hz, 4H), 1.69 (ddd, J = 23.1,

21.8, 14.5 Hz, 6H), 1.57–1.19 (m, 12H), 1.14 (s, 4H), 0.86 (d, J = 6.8 Hz, 3H), 0.73 (d, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.93, 167.03, 163.87, 159.62, 157.51, 139.20, 130.91, 128.84, 117.25, 94.41, 75.39, 74.59, 70.05, 67.78, 58.10, 45.44, 44.36, 43.95, 41.86, 36.72, 36.01, 34.45, 33.24, 30.41, 26.89, 26.26, 25.61, 24.83, 16.86, 14.87, 11.47; HRMS (ES) calcd $[M+H]^+$ for $C_{32}H_{48}N_4O_6S$ 617.3367, found 617.3361.

4.3. MIC determination

The MIC studies were performed on MRSA, MRSE, *B. subtilis* and *E. coli* using the agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS). Compounds were dissolved in 15%–30% DMSO in water to prepare a solution that had a concentration of 1.28 mg/mL. Tiamulin fumarate used as reference drug was directly dissolved in 10 mL distilled water. All the solutions were then diluted two-fold with distilled water to provide 11 dilutions (final concentration is 0.625 μg/mL). The dilutions (2 mL) of each test compound/drug were incorporated into 18 mL hot Mueller–Hinton agar medium, which resulted in the final concentration of each dilutions decreasing tenfold.

Inoculums of MRSA, MRSE, *B. subtilis* and *E. coli* were prepared from blood slants and adjusted to approximately 10^5–10^6 CFU/mL with sterile saline (0.90% NaCl). A 10 μL amount of bacterial suspension was spotted onto Mueller–Hinton agar plates containing serial dilutions of the compounds/drugs. The plates were incubated at 36.5 °C for 24–48 h. The MIC was defined as the minimum concentration of the compound needed to completely inhibit bacterial growth. The same procedure was repeated in triplicate.

4.4. In vivo efficacy in mouse model

Male and female 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 (10 per group) received a 0.5 mL MRSA inoculum of 10^9 CFU/mL via intraperitoneal (ip) injection. About 1 h after infection, the mice were then intravenously (iv) administered compound **6a** dissolved in 0.5 mL vehicle (soybean lecithin: sterile water = 1:30) at doses of 2, 3, 4.46, 6.64 and 10 mg/kg body weight. Tiamulin fumarate was used as a control in the same manner at the same doses as **6a**. The survival of the mice at 4 d after infection was used as the end-point, and the ED_{50} was calculated by the method described by Reed Muench [21] using the Hill equation.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2015.06.034. These data include MOL files and InChiKeys of the most important compounds described in this article.

References

[1] H.A. Kirst, Expert Opin. Drug Discov. 8 (2013) 479–493.
[2] F. Kavanagh, A. Hervey, W.J. Robbins, J. Proc. Natl. Acad. Sci. U. S. A. 37 (1951) 570–574.
[3] S.M. Poulsen, M. Karlsson, L.B. Johansson, B. Vester, Mol. Microbiol. 41 (2001) 1091–1099.
[4] F. Schluenzen, E. Pyetan, P. Fucini, A. Yonath, J.M. Harms, Mol. Microbiol. 54 (2004) 1287–1294.
[5] C. Davidovich, A. Bashan, T. Auerbach-Nevo, R.D. Yaggie, R.R. Gontarek, A. Yonath, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 4291–4296.
[6] R.F. Shang, Y. Liu, Z.J. Xin, W.Z. Guo, Z.T. Guo, B.C. Hao, J.P. Liang, Eur. J. Med. Chem. 63 (2013) 231–238.
[7] M.A. Fischbach, C.T. Walsh, Science 325 (2009) 1089–1093.
[8] R. Novak, D.M. Shlaes, Curr. Opin. Investig. Drugs 11 (2010) 182–191.
[9] Y.Z. Tang, Y.H. Liu, J.X. Chen, Mini-Rev. Med. Chem. 12 (2012) 53–61.
[10] R. Novak, Ann. N. Y. Acad. Sci. 1241 (2011) 71–81.
[11] M.N. Moody, L.K. Morrison, S.K. Tyring, Skin Ther. Lett. 15 (2010) 1–4.
[12] R.F. Shang, J.T. Wang, W.Z. Guo, J.P. Liang, Curr. Top. Med. Chem. 13 (2013) 3013–3025.
[13] I. Dreier, L.H. Hansen, P. Nielsen, B. Vester, Bioorg. Med. Chem. Lett. 24 (2014) 1043–1046.
[14] X.Y. Wang, Y. Ling, H. Wang, J.H. Yu, J.M. Tang, H. Zheng, X. Zhao, D.G. Wang, G.T. Chen, W.Q. Qiu, J.H. Tao, Bioorg. Med. Chem. Lett. 22 (2012) 6166–6172.
[15] Y.Z. Tang, Y.H. Liu, J.X. Chen, Mini Rev. Med. Chem. 12 (2012) 53–61.
[16] C.Y. Ling, L.Q. Fu, S. Gao, W.J. Chu, H. Wang, Y.Q. Huang, X.Y. Chen, Y.S. Yang, J. Med. Chem. 57 (2014) 4772–4795.
[17] R.F. Shang, X.Y. Pu, X.M. Xu, Z.J. Xin, C. Zhang, W.Z. Guo, Y. Liu, J.P. Liang, J. Med. Chem. 57 (2014) 5664–5678.
[18] R.F. Shang, G.H. Wang, X.M. Xu, S.J. Liu, C. Zhang, Y.P. Yi, J.P. Liang, Y. Liu, Molecules 19 (2014) 19050–19065.
[19] I.M. Lagoja, Chem. Biodivers. 2 (2005) 1–50.
[20] B. Cosimelli, G. Greco, M. Ehlardo, E. Novellino, F. Da Settimo, S. Taliani, C. La Motta, M. Bellandi, T. Tuccinardi, A. Martinelli, O. Ciampi, M.L. Trincavelli, C. Martini, J. Med. Chem. 51 (2008) 1764–1770.
[21] L.J. Reed, H. Muench, Am. J. Hyg. 27 (1938) 493–497.