Accepted Manuscript

Antibacterial activity evaluation of synthetic novel pleuromutilin derivatives in vitro and in experimental infection mice

Yu Deng, Xiao-Zhong Wang, Shu-Heng Huang, Cheng-Hong Li

PII: S0223-5234(18)30960-7

DOI: https://doi.org/10.1016/j.ejmech.2018.11.006

Reference: EJMECH 10863

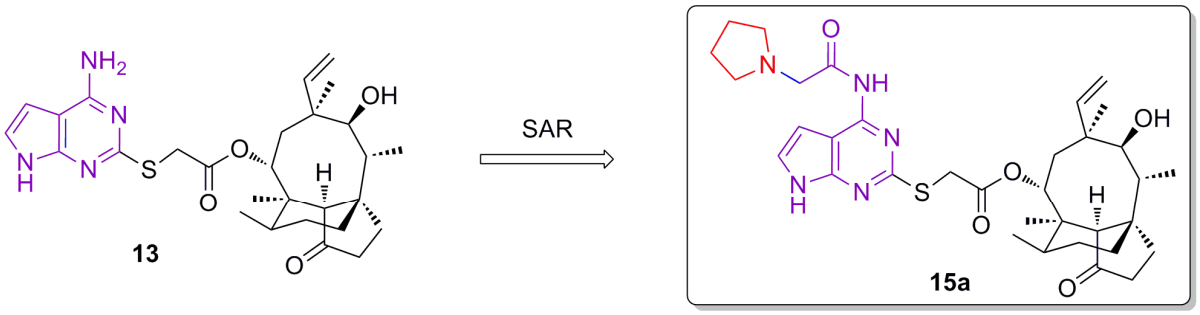
To appear in: *European Journal of Medicinal Chemistry*

Received Date: 20 July 2018

Revised Date: 9 October 2018

Accepted Date: 4 November 2018

Please cite this article as: Y. Deng, X.-Z. Wang, S.-H. Huang, C.-H. Li, Antibacterial activity evaluation of synthetic novel pleuromutilin derivatives in vitro and in experimental infection mice, *European Journal of Medicinal Chemistry* (2018), doi: https://doi.org/10.1016/j.ejmech.2018.11.006.



MIC (MRSA) = 0.0625 μ g/ mL
ED_{50} (MRSA) = 16.0 mg/kg

Antibacterial activity evaluation of synthetic novel pleuromutilin derivatives in vitro and in experimental infection mice

Yu Deng [a], Xiao-Zhong Wang [a], Shu-Heng Huang [b], Cheng-Hong Li [a*]

a Institute of Veterinary Sciences & Pharmaceuticals, Chongqing Academy of Animal Sciences, Rongchang, 402460, China

b College of Bioengineering, Chongqing University, Chongqing 400044, China

Abstract: A series of novel pleuromutilin derivatives embracing 7*H*-pyrrolo[2,3-*d*]pyrimidine moiety were synthesized and evaluated for their in vitro antibacterial activity against Gram-positive and Gram-negative pathogens as well as in vivo efficacy in lethal systemic infected mice. Most compounds displayed good in vitro potency against MSSA, MRSA, MSSE, MRSE and *E. faecium* (MIC = 0.0625 ~ 4 μg/mL), especially **15a**, **15b** and **15o** showed excellent activity that even more active than the comparator valnemulin. The in vivo efficacy investigation exhibited compound **15a** (ED_{50} = 16.0 mg/kg) had comparable activity to valnemulin (ED_{50} =13.5 mg/kg). The results provided by the dose-response study demonstrated **15a** can supply infected mice with 70% survival rate at dose of 40 mg/kg via intragastric (i.g.) administration.

Key words: antibacterial activity; in vivo efficacy; pleuromutilin derivatives; pyrrolopyrimidine; Gram-positive bacteria

1. Introduction

The widespread long-term abuse of antibiotics in the clinic has led to the growing problem of antibiotic resistance, especially the emergence of “super bacteria”, which poses a great threat to human life safety.[1-2] In 2016, the World Health Organization (WHO) figured in the Global Review of Antimicrobial Resistance that about 700,000 people died of superbugs infections every year in the whole world. It is estimated that by 2050, 10 million people’s lives would be threatened by bacterial infections. On February 27, 2017, WHO released the first list of 12 deadliest pathogens including *Staphylococcus aureus* and *Streptococcus pneumoniae* which have serious resistance to traditional antibiotics. Therefore, in order to effectively prevent and treat serious bacterial infections and to solve the current acute shortage of antibiotics, the discovery and development of novel antibacterial drugs are urgent. Natural products are an important source of lead compounds for use in small-molecule discovery. In fact, all new small-molecule-based pharmaceuticals approved during 1981 and 2014, approximately 51% came from natural product whereas 49% were based on chemical syntheses. In terms of the natural product-based drugs, most were natural product-derived or synthetic drugs with natural product backbones.[3]

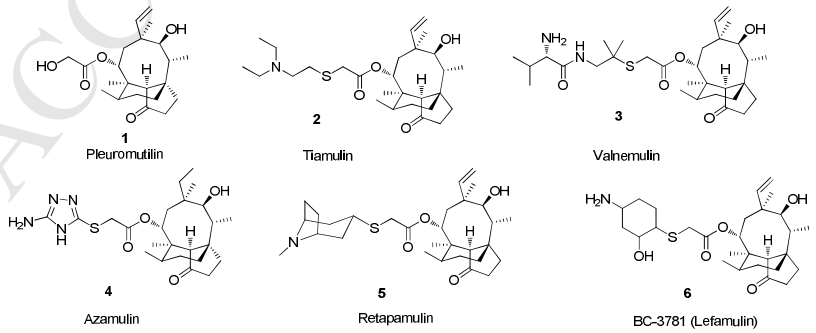


Figure 1. Pleuromutilin and related compounds and drugs

Pleuromutilin (**1**) (Figure 1) is a natural product with an unusual tricyclic diterpenoid structure first isolated from the fungi *Clitopilusscyphoides* in 1950s and has been demonstrated to show modest antibacterial activity.[4] The mechanism research has proved it inhibits bacterial protein synthesis via

* Corresponding author. E-mail: lchong_cqaa@hotmail.com

interaction with prokaryotic 50s ribosome subunit but neither affected protein synthesis of eukaryotes nor bind to mammalian ribosomes.[5] The special structure and action mode of pleuromutilin encourages people to modify it for development of new class of antibiotics to reduce cross-resistance in clinical use. Between 1970s and early 1980s, a number of semisynthetic pleuromutilin derivatives were prepared and in-depth study of structure-activity relationship implied that introduction of thiother and alkaline group in the C14 side chain would improve antibacterial activity.[6-8] Such efforts successfully prompted the approval of tiamulin (**2**) and valnemulin (**3**) as veterinary antibiotics in 1979 and 1999, respectively.[9] Another pleuromutilin analogue azamulin (**4**) was produced in 1980s to aim at human use, which was impeded in the phase I clinical study for its poor water solubility resulting in insufficient bioavailability. [10-11] In 2007, retapmulin (**5**) developed by GlaxoSmithKine was approved as the first pleuromutilin derivative for treatment of human topic skin infection. [12] In late 2006, Nabriva Therapeutics developed compound BC-3781 (now called lefamulin) (**6**), which has obtained important progress in community-acquired bacterial pneumonia (CABP) phase III clinical and acute bacterial skin and skin structure infections (ABSSSI) phase III clinical trials. [13]

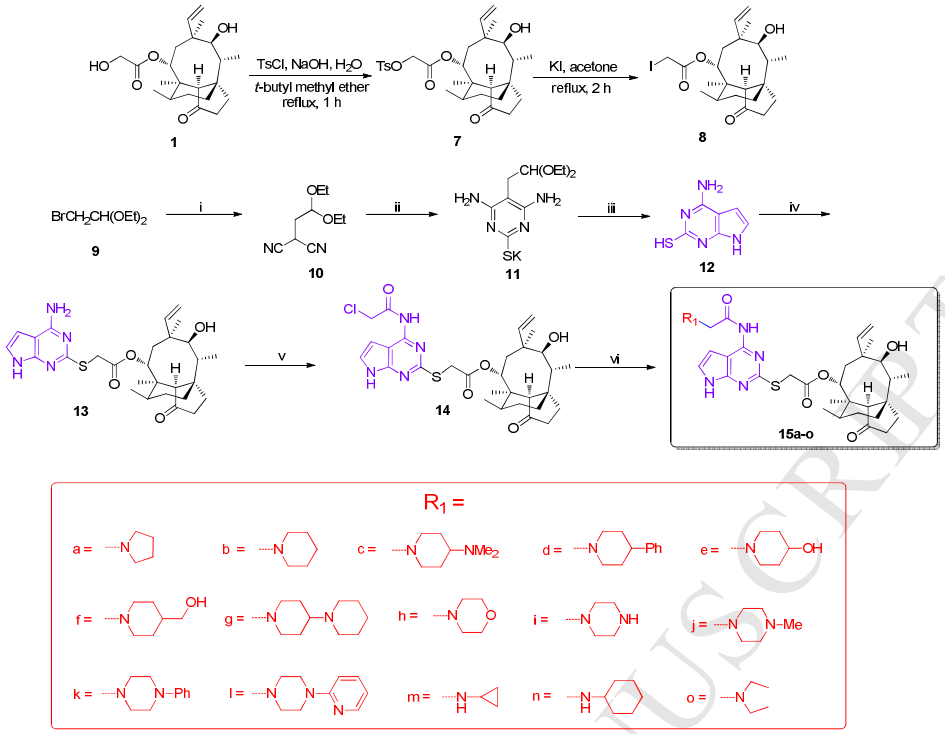
Based on former structure-activity relationship explorations as well as the inherent metabolic instability of pleuromutilin, [14] we exerted our effort to combined hetetoaromatic substitutions into the C14 side chain of pleuromutilin to develop a new class of pleuromutilin derivatives to improve antibacterial activity and water solubility. The design, synthesis, SAR, detailed antibacterial activity and in vivo efficacy of novel semisynthetic pleuromutilin analogues embracing 7*H*-pyrrolo[2,3-*d*]pyrimidine backbone in the C14 side chain will be reported and discussed in the article.

2. Results and discussion

2.1 Chemistry

As shown in scheme 1, pleuromutilin (**1**) was reacted with tosyl chloride in the presence of NaOH in H_2O and *t*-butyl methy ether (TBME) to provide mutilin 14-tosyloxyacetate (**7**) in 90% yield. [15] Compound **8** was prepared by nucleophilic substitution of **7** with KI under a basic condition in 86% yield. [16]

2-bromo-1, 1-diethoxyethane (**9**) was coupled with malononitrile in the presence of K_2CO_3 in DMF overnight to give 2-(2, 2-diethoxyethyl) malononitrile (**10**) in 75% yield. Cyclization reaction of compound **10** with thiourea using *t*-BuOK as the base in refluxing EtOH to lead to potassium 4,6-diamino-5-(2,2-diethoxyethyl)pyrimidine-2-thiolate (**11**) in 62% yield. Subsequent cyclization of compound **11** with 4 N HCl at room temperature for 1 h followed treatment of 4 M NaOH for 10 min to yield 4-amino-7*H*-pyrrolo[2,3-d]pyrimidine-2-thiol (**12**) in 91%. Compound **13** was prepared smoothly from **8** with **12** in the presence of 15% NaOH in 84% yield. Next, the amidation reaction of compound **13** with chloroacetyl chloride employing DCM and DMF as a mixed solvent and Et_3N as a base to provide compound **14** in 45% yield. Finally, a wide range of amines including primary and secondary amine compounds were reacted with compound **14** catalyzed by 2 mol% NaI in refluxing THF to give target compounds **15a-o**.



Scheme 1. Reagents and conditions: (i) malononitrile, K_2CO_3, DMF, 50 °C, overnight; (ii) thiourea, *t*-BuOK, EtOH, 80 °C, overnight; (iii) 4 N HCl for 1 h and 4 M NaOH for 10 min; (iv) compound **8**, 15% NaOH, THF, 0 °C, 2 h; (v) chloroacetyl chloride, Et_3N, DCM/DMF = 5/1, rt, 2 h; (vi) R_1H, 2 mol% NaI, K_2CO_3, THF, 60 °C.

2.2 In vitro antibacterial activity

The evaluation of the in vitro antibacterial activities of the synthesized pleuromutilin derivatives containing 7*H*-pyrrolo[2,3-*d*]pyrimidine were performed against a panel of well characterized drug-susceptible and -resistant Gram-positive pathogens isolated from clinic as well as against two Gram-negative bacteria strain. Valnemulin hydrochloride was used as the positive control and the minimum inhibitory concentration (MIC) values were provided in Table 1. In general, all the newly prepared pleuromutilin derivatives showed good to excellent in vitro antibacterial activity against Gram-positive bacteria except *E. faecalis*. However, none of all of these synthesized compounds and the reference agent have potency against Gram-negative pathogens.

Initially, compound **13** was synthesized as a lead compound to evaluate its in vitro antibacterial activity against Gram-positive bacteria. The results showed that **13** displayed 4 to 8 fold decrease in activity against MSSA, MRSA, MSSE, MRSE and *E. faecium* in comparison with that of valnemulin, with MICs ranging from 1 to 2 μg/mL. Next, several amines compounds including primary and secondary amines were introduced to **13** through chloroacetyl chloride coupling with 4-NH_2 in the pyrrolopyrimidine to optimize its potency. It was found that introduction of pyrrole (**15a**), piperidine (**15b**) and diethylamine (**15o**) exhibited apparent improved activity than valnemulin, especially MICs of **15a** against all strains were lower than that of valnemulin. The substitution of the 4-positon of piperidine of **15b** with aminomethyl, aryl, hydroxyl, hydroxymethyl and piperidinyl (yielding **15c**, **15d**, **15e**, **15f** and **15g**, respectively) led to decreased activity in comparison with that of the parent compound **15b**. The activity of **15c** and **15d** displayed significant decline, with 4 to 8 fold and 8 to 16

fold drop compared to **15b**, respectively. Replacement of **14** with morpholine (**15h**) showed enhanced activity than **13** and gave comparable activity as valnemulin. Compound **15i** bearing piperazine resulted in lower activity against MSSA and MRSA than that of **13** while the 4-substituted piperazine compounds introduced to **14** showed more active than **15i**. This result indicates the substitution effect in the 4-position on the activity of piperazine presented an opposite trend compared to that of piperidine. The data of efficacy of primary amines incorporated to **14** demonstrated that cyclopropylamine (**15m**) showed a better promotion for antibacterial activity in comparison with that of cyclohexylamine (**15n**), with MICs ranging from 0.125 to 0.25 μg/mL and 0.5 to 1 μg/mL, respectively.

In addition, the in vitro activity of those synthesized compounds against Gram-negative bacteria such as *E.coil* and *P. multocida* were investigated. The result showed that this series compounds were not effective to inhibit the growth of Gram-negative pathogens. As the data in Table 1, compound **15o** gave the relative good result against *E.coil*, exhibiting moderate efficacy with a MIC of 16 μg/mL, which had a 2 fold increase in activity against *E.coil* compared to that of valnemulin. The activity against *P. multocida* was also examined, and the most efficient compound **15a** proffered identical MIC (16 μg/mL) as valnemulin.

Table 1. In vitro antibacterial activity of the synthetic compounds and reference agent against Gram-positive and Gram-negative bacteria

compound	MIC [a](μg/mL)							
	MSSA[b]	MRSA[b]	MSSE[b]	MRSE[b]	*E. faecium*[b]	*E. faecalis*[b]	*E.coil*[c]	*P. multocida*[d]
13	1	1	1	2	1	64	>64	64
15a	0.0625	0.0625	0.0625	0.0625	0.0625	64	32	16
15b	0.0625	0.125	0.0625	0.125	0.125	32	64	32
15c	0.5	1	0.5	0.5	1	64	>64	64
15d	1	2	1	1	1	64	>64	>64
15e	0.25	0.25	0.25	0.25	0.5	64	>64	32
15f	0.25	0.25	0.125	0.125	0.25	64	>64	64
15g	0.5	0.5	0.25	0.5	0.0625	64	64	64
15h	0.125	0.25	0.25	0.25	0.25	64	64	32
15i	2	4	1	1	1	64	>64	32
15j	0.125	1	0.25	0.5	0.5	64	>64	32
15k	1	1	0.5	1	1	64	>64	64
15l	1	0.5	0.5	0.5	0.25	64	>64	>64
15m	0.125	0.25	0.125	0.25	0.25	64	>64	32
15n	0.5	1	0.5	0.5	0.5	64	>64	64
15o	0.0625	0.0625	0.0625	0.125	0.125	32	16	64
Valnemulin	0.25	0.25	0.125	0.25	0.125	>64	32	16

a Abbreviations are as follows: MIC, minimum inhibitory concentration; MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSE, methicillin-sensitive *Staphylococcus epidermidis*; MRSE, methicillin-resistant *Staphylococcus epidermidis*; *E. faecium*, *Enterococcus faecium*; *E. faecalis*, *Enterococcus faecalis*; *E.coil*, *Escherichia coli*; *P. multocida*, *Pasteurella multocida.*

b All of the strains were isolated from the clinical bacteria in Chongqing Daping Hospital and reserved in Institute of Veterinary Sciences & Pharmaceuticals, Chongqing Academy of Animal Sciences.

c *E.coil* ATCC11775.

d *P. multocida* was isolated from the clinical bacteria in Institute of Veterinary Sciences & Pharmaceuticals, Chongqing Academy of Animal Sciences.

We also conducted the time-kill kinetics of three concentrations of **15a** and valnemulin during the initial 4 h through inhibiting the viability of exponential-phase of *S.aureus* (MRSA) isolated from clinic to probe their antibacterial activity furtherly. The outcomes revealed that both **15a** and valnemulin displayed concentration-dependent effect (Fig. 2). The pathogen suspension was treated with 8 fold MIC of **15a** and valnemulin to lead the concentration of the bacteria slumped to the undetectable level in 2 h and the result also suggested that **15a** had faster kinetics bactericidal against *S.aureus* than valnemulin. When the concentration of compounds decreased to 4 fold MIC, the bacteria was killed in 4 h. Compared to other two teams, 2 fold MIC treatments provided a more slower speed in inhibiting the growth of *S.aureus.*

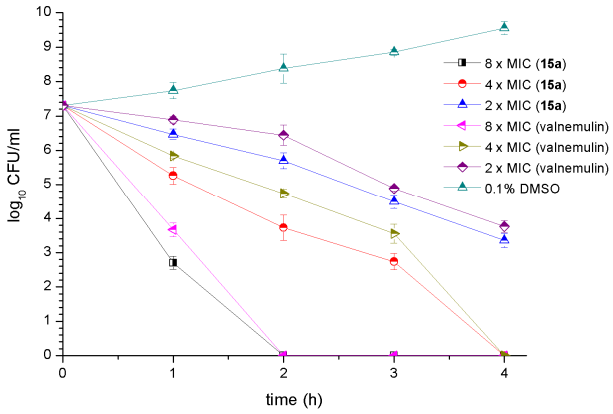


Figure 2. Time-kill kinetics of **15a** and valnemulin against stationary-phase of *S.aureus.* 3 biologically independent samples were performed to give the mean±s.d. of CFU/ml.

*2.3 In vivo efficacy of compounds **15a**, **15b** and **15o***

Since their excellent in vitro antibacterial activity, compounds **15a**, **15b** and **15o** were assessed for their in vivo efficacy against lethal *S.aureus* (MRSA) systemic infection model in mice (Table 2). Compound **15a** showed potent protective effect against MRSA in vivo, with ED_{50} of 16.0 mg/kg that was slightly higher than that of valnemulin. Compounds **15b** exhibited moderate in vivo efficacy and **15o** presented the largest ED_{50} value though **15o** showed equivalent activity in vitro to that of **15a**. The data of **15a** *vs*. valnemulin and **15a** *vs*. **15o** implied that pharmacokinetics and ADME properties have considerable impact on efficacy.

Table 2. In vivo efficacy (ED_{50}) of compounds **15a**, **15b** and **15o** in Mouse Systemic Infection Model

Compound	MRSA [a]	
	MIC(μg/mL) [b]	ED_{50} (mg/kg) [c]
15a	0.0625	16.0 (12.6~19.9)
15b	0.125	19.7 (16.8~24.5)
15o	0.0625	27.7 (22.6~34.5)

valnemulin	0.25	13.5 (10.7~16.8)

[a] Clinical isolate. [b] MICs of compounds against specific strains used for in vivo efficacy estimation. [c] ED_{50} was selected as the efficacy criterion and calculated as the dose at which mice survival rate was 50%. Mice were inoculated intraperitoneally and medication was given intraperitoneal administration 1 h after infection. Values in parentheses indicates 95% confidence ranges.

Next, further investigations of the in vivo efficacy of compound **15a** and valnemulin were conducted in another panel mice infected by MRSA. As shown in Fig. 3, once daily intragastric (i.g.) administration with 40mg/kg of **15a** and valnemulin for consecutive 3 days proffered notable protection against lethal challenge caused by clinical isolate *S.aureus* resistant strain. During the treatment period, the survival percentage of both **15a** and valnemulin kept higher than 90% and the figure experienced a slight decrease between the fifth and sixth days, standing at 70% and 80% at last, respectively. However, when the treatment dose reduced to 20 mg/kg, the survival percent of both **15a** and valnemulin slumped to 30%. The rapid decrease in the survival rate intrigued by the half reduction of dose may attribute to the apparent first-pass effect of **15a** and valnemulin, which lowered the effective plasma concentration. The outcome indicates that the survival percent of infected mice is significantly dose-dependent with the dose of both valnemulin and **15a** via i.g. administration in this experiment model.

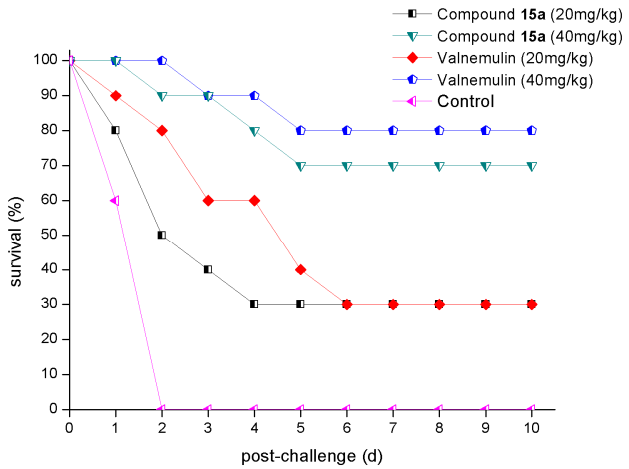


Figure 3. Dose-response study of compound **15a** and valnemulin in lethal systemic infection mice given once daily intragastric administration

*2.4 Molecular docking study of compound **15a***

As its excellent in vitro and in vivo efficacious, we conducted the molecular docking research of **15a** selecting valnemulin as the control. To validate the protocol of Surflex-dock, the co-crystallized tiamulin was re-docked into the binding site of the 50S ribosomal subunit from *Deinococcus radiodurans* (PDB ID: 1XBP). The total score of the optimal conformer was 10.06, and the root mean squared deviation (RMSD) of heavy atoms was 0.47Å (Figure 3). It can be inferred that Surflex-dock can reproduce the native conformation of tiamulin very well.

Crystal structure studies have proposed that pleuromutilin derivatives blocks peptide-bond formation directly by interfering with substrate binding at the ribosome's peptidyl-transferase center

(PTC) domain. [17-18] Taimulin, valnemulin and compound **15a** exhibited the same binding modes in PTC domain (Fig.4). It can be observed that all the three compounds involved hydrophobic interactions with residues G2061, A2062, C2063, A2451, C2452, A2503, U2504, G2505, U2506, U2585, C2586 and formed three hydrogen bonds with G2505 and G2061, which were consistent with previous result reported (nucleotides were numbered according to the *E.coli* 23S rRNA sequence throughout the text) [17]. Furthermore, the total score (-log(KD)) of the optimal conformation of **15a** was calculated to be 12.53, which indicated that the affinity activity of **15a** with 23S rRNA was significantly higher than tiamulin (10.06) and valnemulin (10.46). In terms of **15a**, it should be notable that two extra hydrogen bonds were formed between the-NH of pyrrole ring and residues U2506, G2505, and two π-π stacking interactions were formed between the pyrrolopyrimidine scaffold and U2506. The total score and strong hydrogen bonds as well as the π-π stacking interaction of **15a** may explain why it showed better in vitro antibacterial activity than valnemulin.

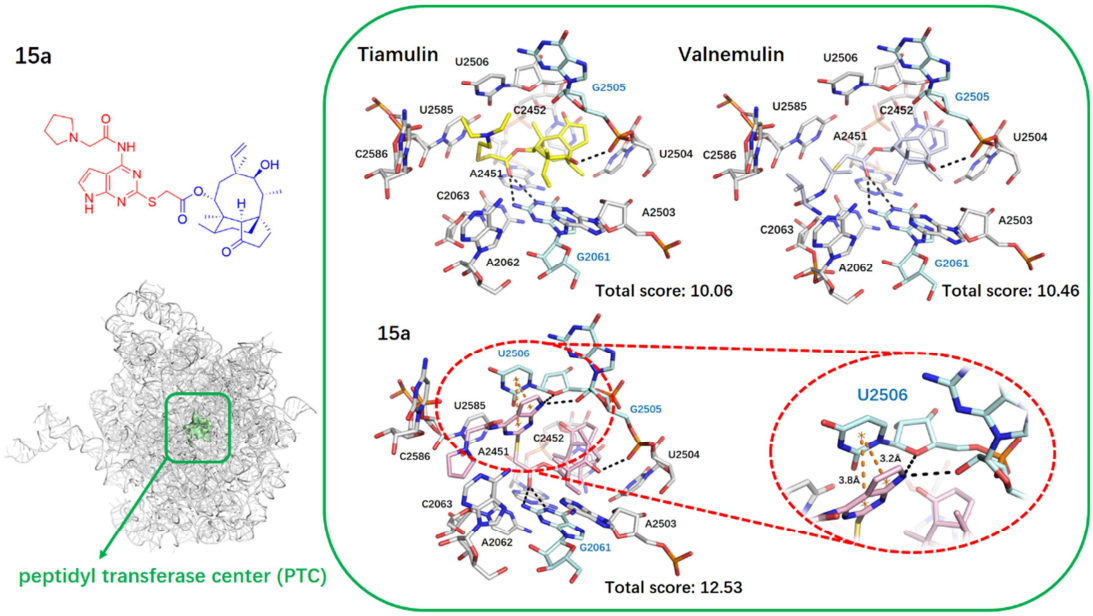


Figure 4. The binding modes of tiamulin, valnemulin and **15a** within the PTC domain. The inhibitors tiamulin, valnemulin and **15a** are represented by yellow, purple and pink. Nucleotides involved in hydrogen bonds and hydrophobic interactions are colored in cyan and white, respectively. Hydrogen bonds and π-π interactions are shown as dotted black and orange lines, respectively.

3. Conclusion

In summary, a series of new semisynthetic thioether pleuromutilin compounds incorporating pyrrolopyrimidine backbone have been reported. Rudimentary SAR study has shown that incorporation of intermediate **13** into pleuromutilin provides moderate antibacterial activity against regardless of sensitive or resistant Gram-positive pathogens. Further optimization using chloroacetyl chloride as a linker to introduce several amines compounds resulted in compounds **15a**, **15b** and **15o** possessing outstanding in vitro antibacterial activity, which were equivalent or even superior to marketed drug valnemulin. The time-kill kinetics assay demonstrated that **15a** was a kind of concentration-dependent antibacterial agent and showed a more rapid kinetics bactericidal against *S.aureus* than valnemulin. The

ED_{50} values evaluation and dose-response study in lethal systemic infection mice of those three compounds have revealed **15a** can be a lead of potential antibacterial agent and indicates hydrophilicity, pharmacokinetics and ADME properties may have significant impact on the in vivo efficacy, which would provide insight into our following research of pleuromutilin derivatives for the treatment of infectious in either animal or human.

4. Experiment section

4.1 Material and methods

Pleuromutilin (~ 92% purity) was purchased from Beijing Couple Technology Co., Ltd. Other chemical were from J&K scientific Co., Ltd. All solvents are analytical grade and without further purification unless otherwise noted. All reactions went under inert atmosphere nitrogen and were monitored by TLC using pre-coated silica gel GF254 glass plates (Qingdao Haiyang Chemical Co., Ltd., Shandong, China) or monitored by LCMS employing the following method: Agilent LCMS 1260-6400; Column: Waters X-Bridge C18-OBD column (4.6 mm × 50 mm, 5 μm); Temperature: 40 °C; Flow rate: 2.0 mL/min; Mobile phase: from 90% [water + 5 mM $HCOONH_4$] and 10% [CH_3CN] to 10% [water + 5 mM $HCOONH_4$] and 90% [CH_3CN] in 0.9 min, then under this condition for 0.9 min, finally changed to 90% [water + 5 mM $HCOONH_4$] and 10% [CH_3CN] in 0.01 min and under this condition for 0.7 min. The target compounds were purified by flash chromatography column or by Prep-HPLC. ^1H NMR and ^{13}C NMR were measured on Agilent 600 DD2 (600 MHz) or Bruker Ultrashield 400 (400 MHz) spectrometer using trimethylsilane as an internal standard. Chemical shifts (δ values) were reported in parts per million (ppm) downfield from tetramethylsilane, and coupling constants (*J*). ESI-HRMS was performed on Bruker solariX XR (FT-ICRMS).

4.2 Synthesis

2-(2,2-diethoxyethyl)malononitrile (**10**). To a solution of malononitrile (20.0 g, 0.30 mol) in DMF (350 mL) in a 500-mL round-bottom flask equipped with magnetic stirrer was added potassium carbonate (41.8 g, 0.30 mol). The resulting purple mixture was then added with bromoacetaldehyde diethyl acetal (29.6 g, 0.15 mol) was added. The suspension was heated to 50 °C and aged overnight. TLC test showed materials were consumed completely. The slurry was cooled to room temperature and treated with water (150 mL) and extracted with toluene (200 mL×3). The combined organic layers were washed with brine (200 mL×3), dried over anhydrous Na_2SO_4 and evaporated in vacuum. The residue was flushed with toluene (100 mL×2) followed anhydrous ethanol (100 mL×2). The concentrate (20.5 g, 75.0 % yield) was used for the next step directly without further purification.

potassium 4,6-diamino-5-(2,2-diethoxyethyl)pyrimidine-2-thiolate (**11**). To a stirred solution of compound **10** (20.5 g, 112.5 mmol) in anhydrous ethanol (200 mL) was added thiourea (10.3 g, 135.0 mmol) under ice bath. The solution was stirred for 15 min and then added potassium *tert*-butoxide solid (15.1 g, 135.0 mmol). The mixture was warmed to 78 °C and keep at this temperature overnight. The slurry was cooled to ambient temperature after the reaction completed. The resulting precipitate was filtered and washed with anhydrous ethanol (100 mL×3). The filter cake was dried in vacuum at 40 °C

overnight to give an off-white solid (20.6 g, 62 % yield). The solid was subjected to the next step without further purification.

4-amino-7*H*-pyrrolo[2,3-d]pyrimidine-2-thiol (**12**). To a stirred solution of compound **11** (20.6 g, 69.5 mmol) in H_2O (150 mL) was added 4N HCl (34.8 mL, 139.0 mmol) at 0 °C. Then the mixture was warmed to 50 °C and aged 1 h. The formed slurry was treated with H_2O (150 mL) and 4 M NaOH (18 mL, 70 mmol) to adjust pH 7 and stirred at room temperature for 10 min. The precipitate was filtered and the filter cake was dried in vacuum at 40 °C to give an off-white solid (10.5 g, 91 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.51 (s, 2H), 7.60 (brs, 2H), 6.85 (s, 1H), 6.46 (d, J = 3.2 Hz, 1H); HRMS (ES) calcd $[M + H]^+$ for $C_6H_6N_4S$ 167.03859, found 167.03900.

14-*O*-(((4-amino-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin (**13**). To a stirred solution of compound **8** (20.0 g, 41.0 mmol) in THF (150 mL) was added compound **12** (6.8 g, 41.0 mmol) and 15% NaOH (aq.) (16.5 mL, 61.5 mmol) at 0 °C and remained 2 h. After the material was consumed completely, the THF was evaporated in vacuum. Then 4 N HCl was employed to adjust the pH of the mixture to 7. The resulting solution was extracted with EtOAc (50 mL×3). The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated in vacuum. The crude product was purified by flash chromatography with DMC/MeOH (V/V = 50/1 to 25/1) as eluent to give **13** as an off-white solid (18.0 g, 84 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.25 (s, 1H), 6.98 (s, 2H), 6.93 – 6.90 (m, 1H), 6.43 (dd, J = 3.3, 1.9 Hz, 1H), 5.52 (d, J = 8.3 Hz, 1H), 5.02 (d, J = 16.0 Hz, 1H), 4.95 (d, J = 12.0 Hz, 1H), 4.49 (d, J = 4.0 Hz, 1H), 3.89 (dd, J = 20.0, 16.0 Hz, 2H), 3.39 (t, J = 6.0 Hz, 1H), 2.37 (s, 1H), 2.23 – 1.95 (m, 4H), 1.66 – 1.58 (m, 2H), 1.46 – 1.36 (m, 2H), 1.34 (s, 3H), 1.29 – 1.20 (m, 3H), 1.03 – 0.92 (m, 4H), 0.80 (d, J = 7.0 Hz, 3H), 0.60 (d, J = 6.8 Hz, 3H); HRMS (ES) calcd $[M + H]^+$ for $C_{28}H_{38}N_4O_4S$ 527.26865, found 527.26778.

14-*O*-(((4-(2-chloroacetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin (**14**). A three-neck round bottom flask equipped with a magnetic stirrer was added compound **13** (10.0 g, 19.0 mmol) and 100 mL of DCM/DMF (V/V = 9/1). The stirred solution was then added Et_3N (2.9 g, 28.5 mmol) and 2-chloroacetyl chloride (3.2 g, 28.5 mmol) at 0 °C under nitrogen atmosphere. The resulting mixture was stirred at rt for 2 h. After completion, the mixture was treated with H_2O (20 mL) and extracted with DCM (30 mL×3). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuum. The residue was purified by flash chromatography with DMC/MeOH (V/V = 40/1 to 20/1) as eluent to give **14** as white solid (5.2 g, 45 % yield). ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.95 (brs, 2H), 6.88 (d, J = 4.0 Hz, 1H), 6.39 (d, J = 4.0 Hz, 1H), 5.47 (d, J = 4.0 Hz, 1H), 5.13 (d, J = 12.0 Hz, 1H), 5.04 (d, J = 8.0 Hz, 1H), 4.90 (d, J = 6.6 Hz, 1H), 4.44 (dd, J = 24.0, 8.0 Hz, 2H), 3.87 (q, J = 16.1 Hz, 2H), 2.30 – 2.25 (m, 1H), 2.20 – 2.14 (m, 2H), 2.11 – 2.04 (m, 2H), 1.78 (q, J = 8.0 Hz, 1H), 1.62 (d, J = 8.0 Hz, 1H), 1.46 – 1.42 (m, 1H), 1.38 (d, J = 12.0 Hz, 1H), 1.33 (s, 3H), 1.24 – 1.22 (m, 3H), 1.01 (t, J = 12.0 Hz, 1H), 0.85 (s, 3H), 0.71 (d, J = 7.0 Hz, 3H), 0.59 (d, J = 7.0 Hz, 3H); LCMS (ES) calcd $[M + H]^+$ for $C_{30}H_{39}ClN_4O_5S$ 602.23, found 603.3.

General procedure for the synthesis of compounds **15a-o**. To a stirred solution of compound **14** (1 mmol) in THF (10 mL) was added K_2CO_3 (207.0 mg, 1 mmol), R_1H (1 mmol) and NaI (3 mg, 0.02 mmol) at rt. The mixture was warmed to 60 °C and remained for 4 h. Then the mixture was added H_2O (5 mL) and extracted with EtOAc (10 mL×3). The combined organic layers were dried over anhydrous

Na_2SO_4 and concentrated in vacuum. The crude product was purified by Prep-TLC or Prep-HPLC to give the target.

14-*O*-(((4-(2-(pyrrolidin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15a)**: slight yellow solid, 65% yield, m. p. : 110.3 – 114.2; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.83 (s, 1H), 10.15 (s, 1H), 7.24 (d, *J* = 2.4 Hz, 1H), 6.76 (d, *J* = 3.1 Hz, 1H), 5.52 (d, *J* = 8.2 Hz, 1H), 4.99 (d, *J* = 16.5 Hz, 1H), 4.93 (d, *J* = 11.2 Hz, 1H), 4.50 (d, *J* = 5.8 Hz, 1H), 4.00 (dd, *J* = 20.0, 16.0 Hz, 2H), 3.42 (s, 2H), 3.18 (s, 1H), 2.64 (s, 4H), 2.37 (s, 1H), 2.18 (dd, *J* = 16.0, 8.0 Hz, 1H), 2.08 (ddd, *J* = 28.0, 16.0, 8.0 Hz, 3H), 1.76 (s, 4H), 1.67 – 1.66 (m, 2H), 1.44 (d, *J* = 8.2 Hz, 1H), 1.33 (d, *J* = 16.0 Hz, 1H), 1.31 (s, 3H), 1.23 (t, *J* = 8.0 Hz, 3H), 1.02 – 0.98 (m, 1H), 0.97 (s, 3H), 0.80 (d, *J* = 6.8 Hz, 3H), 0.58 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.61, 169.49, 168.06, 160.28, 154.38, 150.08, 141.16, 123.46, 115.61, 104.81, 103.49, 73.03, 70.25, 59.32, 58.55, 57.68, 54.05, 45.40, 44.51, 41.97, 36.81, 34.44, 33.84, 30.56, 29.04, 27.03, 26.17, 24.91, 24.17, 23.97, 23.74, 16.51, 14.93, 11.98; HR-MS (ESI) m/z calcd for $C_{34}H_{48}N_5O_5S$ $(M+H)^+$: 638.33707, found: 638.33526.

14-*O*-(((4-(2-(piperidin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15b)**: slight yellow solid, 70% yield, m. p. : 108.4 – 110.5; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.83 (s, 1H), 10.11 (s, 1H), 7.24 (dd, *J* = 3.3, 2.4 Hz, 1H), 6.77 (dd, *J* = 3.4, 1.9 Hz, 1H), 5.52 (d, *J* = 8.2 Hz, 1H), 5.03 – 4.99 (d, *J* = 16.0 Hz, 1H), 4.92 (d, *J* = 8.0 Hz, 1H), 4.49 (d, *J* = 6.0 Hz, 1H), 3.99 (dd, *J* = 20.0, 16.0 Hz, 2H), 3.48 – 3.37 (m, 2H), 3.22 (s, 2H), 2.36 (d, *J* = 14.4 Hz, 2H), 2.18 (q, *J* = 8.0 Hz, 1H), 2.02 (ddd, *J* = 24.2, 17.5, 8.8 Hz, 3H), 1.67 – 1.54 (m, 6H), 1.50 – 1.35 (m, 6H), 1.31 (s, 3H), 1.29 – 1.21 (m, 3H), 1.03 – 0.97 (m, 4H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.57 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.61, 169.23, 168.04, 160.30, 154.45, 149.90, 141.17, 123.52, 115.58, 104.78, 104.47, 103.50, 73.03, 70.25, 62.50, 57.68, 54.42, 54.22, 45.40, 44.50, 44.00, 41.97, 36.84, 36.79, 34.44, 33.90, 30.55, 29.02, 27.02, 26.13, 24.91, 23.90, 16.51, 14.93, 11.97; HR-MS (ESI) m/z calcd for $C_{40}H_{52}N_6O_5S$ $(M+H)^+$: 729.37927, found: 729.37640.

14-*O*-(((4-(2-(4-(dimethylamino)piperidin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15c)**: brown solid, 61% yield, m. p. : 95.6 – 97.6; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.85 (s, 1H), 10.18 (s, 1H), 8.28 (s, 1H), 7.24 (dd, *J* = 3.3, 2.0 Hz, 1H), 6.75 (dd, *J* = 3.4, 1.3 Hz, 1H), 5.52 (d, *J* = 8.2 Hz, 1H), 5.03 – 4.87 (m, 4H), 3.99 (dd, *J* = 20.0, 16.0 Hz, 2H), 3.38 (d, *J* = 5.7 Hz, 1H), 2.97 (d, *J* = 11.1 Hz, 2H), 2.55 (s, 1H), 2.48 – 2.38 (m, 8H), 2.20 (dt, *J* = 32.0, 8.0 Hz, 4H), 2.02 (ddd, *J* = 36.0, 16.0, 8.0 Hz, 3H), 1.82 (s, 2H), 1.65 – 1.55 (m, 4H), 1.47 – 1.42 (m, 1H), 1.37 (d = 16.0 Hz, 1H), 1.31 (s, 3H), 1.29 – 1.20 (m, 3H), 1.18 – 0.98 (m, 1H), 0.96 (s, 3H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.57 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.61, 169.15, 168.04, 164.91, 160.29, 154.40, 150.02, 141.16, 123.52, 115.60, 104.86, 103.43, 73.03, 70.25, 61.72, 61.44, 57.69, 52.52, 45.40, 44.50, 43.99, 41.98, 40.87, 36.84, 36.79, 34.44, 33.90, 30.56, 29.04, 27.60, 27.03, 24.90, 16.50, 14.95, 11.97; HR-MS (ESI) m/z calcd for $C_{37}H_{55}N_6O_5S$ $(M+H)^+$: 695.39492, found: 695.39235.

14-*O*-(((4-(2-(4-hydroxypiperidin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15d)**: slight yellow solid, 64% yield, m. p. : 133.2 – 135.7; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.84 (s, 1H), 10.18 (s, 1H), 7.31 (s, 1H), 7.32 – 7.25 (m, 4H), 7.22 – 7.17 (m, 1H), 6.78 (dd, *J* = 3.5, 1.9 Hz, 1H), 5.53 (d, *J* = 8.2 Hz, 1H), 4.99 (dd, *J* = 17.8, 1.7 Hz, 1H), 4.93 (dd, *J* = 11.2, 1.6 Hz, 1H), 4.49 (d, *J* = 6.1 Hz, 1H), 4.00 (dd, *J* = 24.0, 16.0 Hz, 2H), 3.38 (t, *J* = 6.2 Hz, 1H), 3.35 (s, 2H), 3.02 (d, *J* = 11.0

Hz, 2H), 2.56 – 2.54 (m, 1H), 2.36 – 2.32 (m, 3H), 2.17 (dd, *J* = 19.2, 10.2 Hz, 1H), 2.02 (ddd, *J* = 32.8, 17.1, 8.8 Hz, 3H), 1.79 – 1.74 (m, 4H), 1.60 (dd, *J* = 24.0, 12.0 Hz, 2H), 1.49 – 1.36 (m, 2H), 1.28 – 1.19 (m, 3H), 1.24 (d, *J* = 15.7 Hz, 3H), 1.01 – 0.94 (m, 1H), 0.97 (s, 3H), 0.79 (d, *J* = 7.0 Hz, 3H), 0.58 (d, *J* = 6.7 Hz, 3H)； ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.60, 169.26, 168.05, 160.31, 154.45, 150.01, 146.54, 141.15, 128.80, 127.18, 126.50, 123.54, 115.61, 104.90, 103.51, 73.03, 70.23, 62.09, 57.69, 54.20, 45.39, 44.50, 44.01, 41.98, 41.77, 36.84, 36.80, 34.44, 33.93, 33.62, 30.55, 29.05, 27.03, 24.91, 16.52, 14.96, 11.98; HR-MS (ESI) m/z calcd for $C_{41}H_{54}N_5O_5S$ $(M+H)^+$: 728.38402, found: 728.38151.

14-*O*-(((4-(2-(4-phenylpiperidin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15e)**: white solid, 60% yield, m. p. : 133.2 – 135.3; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.83 (s, 1H), 10.13 (s, 1H), 7.24 (d, *J* = 3.6 Hz, 1H), 6.76 (d, *J* = 3.5 Hz, 1H), 5.52 (d, *J* = 8.2 Hz, 1H), 4.98 (d, *J* = 17.8 Hz, 1H), 4.92 (d, *J* = 11.2 Hz, 1H), 4.62 (s, 1H), 4.49 (d, *J* = 5.9 Hz, 1H), 3.99 (dd, *J* = 20.0, 16.0 Hz, 2H), 3.51 (s, 1H), 3.24 (s, 2H), 2.80 – 2.77 (m, 2H), 2.36 – 2.29 (m, 3H), 2.17 (dd, *J* = 19.0, 10.7 Hz, 1H), 2.01 (ddd, *J* = 24.6, 17.4, 8.8 Hz, 3H), 1.78 – 1.75 (m, 2H), 1.65 – 1.55 (m, 2H), 1.55 – 1.51 (m, 3H), 1.36 (d, *J* = 13.8 Hz, 1H), 1.31 (s, 3H), 1.24 – 1.20 (m, 3H), 1.02 – 0.98 (m, 1H), 0.96 (s, 3H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.58 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.62, 169.24, 168.04, 160.29, 154.41, 149.96, 141.15, 123.50, 115.60, 104.80, 103.48, 73.04, 70.24, 66.02, 61.79, 57.69, 51.36, 45.39, 44.50, 43.98, 41.97, 36.84, 36.80, 34.83, 34.44, 33.86, 30.55, 29.04, 27.03, 24.91, 16.52, 14.95, 11.99; HR-MS (ESI) m/z calcd for $C_{35}H_{50}N_5O_6S$ $(M+H)^+$: 668.34763, found: 668.34543.

14-*O*-(((4-(2-(4-(hydroxymethyl)piperidin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15f)**: white solid, 56% yield, m. p. : 119.5 – 121.0; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.83 (s, 1H), 10.09 (s, 1H), 7.24 (d, *J* = 3.3Hz, 1H), 6.77 (d, *J* = 3.3Hz, 1H), 5.52 (d, *J* = 8.2 Hz, 1H), 4.98 (dd, *J* = 17.8, 1.6 Hz, 1H), 4.92 (dd, *J* = 11.2, 1.6 Hz, 1H), 4.49 (d, *J* = 5.7 Hz, 1H), 4.45 (brs, 1H), 3.99 (q, *J* = 16.2 Hz, 2H), 3.28 (d, *J* = 6.2 Hz, 2H), 3.24 (s, 2H), 2.90 (d, *J* = 10.9 Hz, 2H), 2.37 (s, 1H), 2.20 – 2.15 (m, 3H), 2.02 (ddd, *J* = 36.0, 16.0, 8.0 Hz, 3H), 1.70 – 1.58 (m, 4H), 1.48 –1.34 (m, 3H), 1.31 (s, 3H), 1.28 – 1.18 (m, 5H), 1.02 – 0.98 (m, 1H), 0.96 (s, 3H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.58 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.61, 169.27, 168.03, 160.29, 154.43, 149.93, 141.14, 123.51, 115.60, 104.81, 103.51, 99.99, 73.03, 70.23, 66.34, 62.20, 57.69, 53.66, 45.40, 44.50, 44.01, 41.97, 38.32, 36.79, 34.44, 33.89, 30.55, 29.32, 29.04, 27.03, 24.91, 16.51, 14.96, 11.99; HR-MS (ESI) m/z calcd for $C_{36}H_{52}N_5O_6S$ $(M+H)^+$: 682.36328, found: 682.36091.

14-*O*-(((4-(2-([1,4'-bipiperidin]-1'-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15g)**: yellow solid, 68% yield, m. p. : 123.9 – 125.7; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.85 (s, 1H), 10.17 (s, 1H), 8.26 (s, 1H), 7.24 (dd, *J* = 3.3, 2.3 Hz, 1H), 6.75 (dd, *J* = 3.5, 1.7 Hz, 1H), 5.52 (d, *J* = 8.3 Hz, 1H), 4.98 (dd, *J* = 17.8, 1.6 Hz, 1H), 4.92 (dd, *J* = 11.2, 1.6 Hz, 1H), 4.00 (dd, *J* = 20.0, 16.2 Hz, 2H), 3.39 (d, *J* = 5.7 Hz, 1H), 2.98 (d, *J* = 10.9 Hz, 2H), 2.73 (m, 4H), 2.57 (t, *J* = 11.5 Hz, 1H), 2.37 (s, 1H), 2.24 (t, *J* = 11.8 Hz, 2H), 2.16 (t, *J* = 11.8 Hz, 1H), 2.02 (ddd, *J* = 32.0, 17.5, 8.8 Hz, 3H), 1.82 (d, *J* = 11.1 Hz, 2H), 1.66 – 1.60 (m, 8H), 1.43 (d, *J* = 5.0 Hz, 3H), 1.37 (d, *J* = 13.8 Hz, 1H), 1.31 (s, 3H), 1.25 – 1.21 (m, 3H), 1.01 (d, *J* = 4.3 Hz, 1H), 0.96 (s, 3H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.57 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.61, 169.14, 168.04, 164.63, 160.28, 154.40, 150.01, 141.14, 123.52, 115.60, 104.86, 103.44, 73.02, 70.24, 62.15, 61.45, 57.70, 52.85, 49.79, 45.40,

44.51, 44.00, 41.98, 40.88, 36.85, 36.79, 34.44, 33.89, 30.56, 29.04, 27.33, 27.03, 25.19, 24.91, 23.87, 16.50, 14.94, 11.98; HR-MS (ESI) m/z calcd for $C_{40}H_{59}N_6O_5S$ $(M+H)^+$: 735.42622, found: 735.42309.

14-*O*-(((4-(2-morpholinoacetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15h)**: slight yellow solid, 72% yield, m. p. : 132.4 – 134.0; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.82 (s, 1H), 10.25 (s, 1H), 7.24 (dd, *J* = 3.3, 2.1 Hz, 1H), 6.75 (d, *J* = 3.3 Hz, 1H), 5.52 (d, *J* = 8.2 Hz, 1H), 4.99 (dd, *J* = 17.8, 1.6 Hz, 1H), 4.92 (dd, *J* = 11.2, 1.5 Hz, 1H), 4.49 (d, *J* = 6.0 Hz, 1H), 4.00 (q, *J* = 16.2 Hz, 2H), 3.65 – 3.63 (m, 4H), 3.40 – 3.35 (m, 4H), 2.57 – 2.54 (m, 4H), 2.37 (s, 1H), 2.18 (dd, *J* = 19.1, 10.8 Hz, 1H), 2.02 (ddd, *J* = 24.4, 17.5, 8.8 Hz, 3H), 1.65 – 1.56 (m, 2H), 1.47 – 1.41 (m, 1H), 1.38 – 1.34 (m, 1H), 1.31 (s, 3H), 1.27 – 1.17 (m, 3H), 1.02 – 0.98 (m, 1H), 0.96 (s, 3H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.58 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.62, 168.89, 168.04, 160.28, 154.37, 150.08, 141.15, 123.49, 115.62, 104.89, 103.41, 73.03, 70.25, 66.69, 61.89, 57.68, 53.47, 45.39, 44.51, 43.96, 41.98, 36.84, 36.79, 34.44, 33.87, 30.54, 29.04, 27.03, 24.91, 16.52, 14.95, 11.98; HR-MS (ESI) m/z calcd for $C_{34}H_{48}N_5O_6S$ $(M+H)^+$: 654.33198, found: 654.32979.

14-*O*-(((4-(2-(piperazin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15i)**: slight yellow solid, 52% yield, m. p.: 84.9 – 86.5; ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.79 (s, 1H), 10.10 (s, 1H), 7.21 (s, 1H), 6.71 (d, *J* = 15.1 Hz, 1H), 5.48 (d, *J* = 7.7 Hz, 1H), 4.95 (d, *J* = 17.7 Hz, 1H), 4.89 (d, *J* = 11.1 Hz, 1H), 4.45 (d, *J* = 5.1 Hz, 1H), 3.96 (q, *J* = 16.2 Hz, 2H), 3.35 – 3.29 (m, 4H), 3.19 (s, 2H), 2.5 – 2.44 (m, 4H), 2.33 (s, 1H), 2.13 (dd, *J* = 18.6, 11.1 Hz, 1H), 2.03 (dd, *J* = 18.8, 9.0 Hz, 2H), 1.93 (dd, *J* = 15.5, 8.1 Hz, 1H), 1.62 – 1.54 (m, 2H), 1.41 (s, 1H), 1.33 (d, *J* = 8.0 Hz, 1H), 1.27 (s, 3H), 1.22 – 1.17 (m, 3H), 0.97 – 0.95 (m, 1H), 0.93 (s, 3H), 0.76 (d, *J* = 6.3 Hz, 3H), 0.55 (d, *J* = 6.1 Hz, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 222.32, 173.77, 172.76, 164.99, 159.14, 154.67, 145.88, 128.22, 120.32, 109.53, 108.18, 77.76, 74.98, 67.15, 66.06, 62.41, 59.18, 57.28, 56.34, 50.83, 50.12, 49.23, 48.71, 46.69, 41.57, 41.51, 39.16, 38.59, 35.28, 33.76, 31.75, 29.63, 21.24, 19.67, 16.70; HR-MS (ESI) m/z calcd for $C_{34}H_{49}N_6O_5S$ $(M+H)^+$: 653.34797, found: 653.34576.

14-*O*-(((4-(2-(4-methylpiperazin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15j)**: slight yellow solid, 75% yield, m. p. : 70.2 – 71.9; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.84 (s, 1H), 10.14 (s, 1H), 7.24 (s, 1H), 6.76 (d, *J* = 2.9 Hz, 1H), 5.52 (d, *J* = 8.1 Hz, 1H), 4.98 (d, *J* = 16.0 Hz, 1H), 4.92 (d, *J* = 12.0 Hz, 1H), 4.49 (d, *J* = 4.0 Hz, 1H), 4.00 (q, *J* = 16.0 Hz, 2H), 3.28 (s, 7H), 2.57 – 2.37 (s, 8H), 2.19 (s, 3H), 2.15 (d, *J* = 9.7 Hz, 2H), 2.02 (ddd, *J* = 25.0, 17.5, 8.8 Hz, 3H), 1.65 – 1.56 (m, 2H), 1.47 – 1.42 (m, 1H), 1.39 – 1.34 (m, 1H), 1.32 (s, 3H), 1.25 – 1.17 (m, 3H), 1.00 (m, 1H), 0.97 (s, 3H), 0.80 (d, *J* = 6.8 Hz, 3H), 0.57 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.61, 168.99, 168.03, 160.28, 154.43, 149.95, 141.15, 123.52, 115.61, 104.82, 103.48, 73.03, 70.23, 61.60, 57.69, 55.16, 53.03, 46.17, 45.40, 44.50, 44.02, 41.97, 36.84, 36.79, 33.90, 30.55, 29.03, 27.03, 24.91, 16.53, 14.99, 11.98; HR-MS (ESI) m/z calcd for $C_{35}H_{51}N_6O_5S$ $(M+H)^+$: 667.36362, found: 667.36139.

14-*O*-(((4-(2-(4-phenylpiperazin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15k)**: white solid, 73% yield, m. p. : 137.4 – 138.6; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.84 (s, 1H), 10.26 (s, 1H), 7.26 – 7.19 (m, 3H), 6.95 (d, *J* = 8.3 Hz, 2H), 6.81 – 6.77 (m, 2H), 5.52 (d, *J* = 8.2 Hz, 1H), 4.99 (d, *J* = 20.0 Hz, 1H), 4.92 (d, *J* = 11.2 Hz, 1H), 4.49 (d, *J* = 6.0 Hz, 1H), 4.00 (dd, *J* = 20.0, 16.0 Hz, 2H), 3.38 (s, 3H), 3.21 – 3.19 (m, 4H), 2.74 – 2.72 (m, 4H), 2.36 (s, 1H), 2.17 (dd, *J* = 18.8, 10.7 Hz, 1H), 2.02 (ddd, *J* = 32.0, 17.5, 8.8 Hz, 3H), 1.60 (dd, *J* = 23.1, 12.2 Hz, 2H), 1.44 – 1.35 (m,

2H), 1.31 (s, 3H), 1.25 – 1.19 (m, 3H), 1.02 – 0.92 (m, 4H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.57 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.60, 168.96, 168.03, 160.30, 154.40, 151.42, 150.07, 141.14, 129.37, 123.52, 119.36, 115.96, 115.63, 104.89, 103.43, 73.03, 70.24, 61.56, 57.69, 53.04, 48.79, 45.39, 44.50, 43.99, 41.97, 36.78, 34.44, 33.87, 30.55, 29.05, 27.03, 24.91, 16.54, 14.96, 11.99; HR-MS (ESI) m/z calcd for $C_{40}H_{53}N_6O_5S$ $(M+H)^+$: 729.37927, found: 729.37640.

14-*O*-(((4-(2-((4-(pyridin-2-yl)piperazin-1-yl)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl) mutilin **(15l)** : off-white solid, 66% yield, m. p. : 136.4 – 138.6; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.83 (s, 1H), 10.29 (s, 1H), 8.12 (dd, *J* = 4.8, 1.5 Hz, 1H), 7.53 (t, *J* = 6.9 Hz, 1H), 7.27 – 7.22 (m, 1H), 6.83 (d, *J* = 8.6 Hz, 1H), 6.76 (dd, *J* = 3.5, 1.9 Hz, 1H), 6.64 (dd, *J* = 6.9, 5.0 Hz, 1H), 5.52 (d, *J* = 8.2 Hz, 1H), 4.98 (dd, *J* = 17.8, 1.6 Hz, 1H), 4.91 (dd, *J* = 11.2, 1.5 Hz, 1H), 4.49 (d, *J* = 6.0 Hz, 1H), 4.00 (dd, *J* = 20.0, 16.0 Hz, 2H), 3.56 – 3.54 (m, 4H), 3.35 (s, 2H), 2.71 – 2.64 (m, 4H), 2.36 (s, 1H), 2.17 (dd, *J* = 18.9, 10.5 Hz, 1H), 2.02 (ddd, *J* = 24.8, 17.3, 8.8 Hz, 3H), 1.65 – 1.56 (m, 2H), 1.43 (d, *J* = 9.3 Hz, 1H), 1.36 (d, *J* = 10.9 Hz, 1H), 1.30 (s, 3H), 1.24 – 1.19 (m, 3H), 1.01 – 0.98 (m, 1H), 0.98 (s, 3H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.57 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.60, 168.98, 168.04, 160.30, 159.43, 154.39, 150.09, 148.01, 141.14, 137.96, 123.51, 115.62, 113.49, 107.55, 104.90, 103.42, 73.04, 70.24, 61.61, 57.69, 52.83, 45.39, 45.16, 44.51, 43.99, 41.97, 36.84, 36.78, 34.45, 33.88, 30.54, 29.04, 27.02, 24.91, 16.53, 14.95, 11.99; HR-MS (ESI) m/z calcd for $C_{39}H_{52}N_7O_5S$ $(M+H)^+$: 730.37451, found: 730.37125.

14-*O*-(((4-(2-(cyclopropylamino)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15m)**: slight yellow solid, 76% yield, m. p. : 116.2 – 120.6; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.82 (s, 1H), 10.39 (s, 1H), 7.23 (d, *J* = 3.6 Hz, 1H), 6.77 (d, *J* = 3.6 Hz, 1H), 5.52 (d, *J* = 8.2 Hz, 1H), 4.98 (d, *J* = 17.8 Hz, 1H), 4.93 (d, *J* = 11.2 Hz, 1H), 4.49 (d, *J* = 6.0 Hz, 1H), 4.00 (dd, *J* = 20.0, 16.0 Hz, 2H), 3.51 (s, 2H), 3.35 (s, 2H), 2.96 (brs, 1H), 2.36 (s, 1H), 2.23 – 2.13 (m, 2H), 2.02 (ddd, *J* = 28.0, 16.0, 8.0 Hz, 3H), 1.65 – 1.56 (m, 2H), 1.45 – 1.34 (m, 2H), 1.31 (s, 3H), 1.24 – 1.20 (m, 3H), 1.02 – 0.98 (m, 1H), 0.96 (s, 3H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.58 (d, *J* = 6.7 Hz, 3H), 0.38 (dt, *J* = 16.0, 3.3 Hz, 2H), 0.31 (dt, *J* = 8.0, 3.3 Hz, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.61, 171.56, 168.03, 160.29, 154.30, 150.27, 141.14, 123.36, 115.63, 104.71, 103.36, 73.04, 70.25, 57.69, 53.19, 45.39, 44.50, 43.95, 41.97, 36.84, 36.80, 34.44, 33.81, 30.53, 29.02, 27.03, 24.91, 16.53, 14.93, 11.98, 6.61; HR-MS (ESI) m/z calcd for $C_{33}H_{46}N_5O_5S$ $(M+H)^+$: 624.32142, found: 624.31970.

14-*O*-(((4-(2-(cyclohexylamino)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15n)**: off-white solid, 74% yield, m. p. : 123.0 – 125.8; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.83 (s, 1H), 7.24 (d, *J* = 3.0 Hz, 1H), 6.81 (d, *J* = 3.2 Hz, 1H), 5.52 (d, *J* = 3.2 Hz, 1H), 4.98 (dd, *J* = 17.8, 1.4 Hz, 1H), 4.93 (d, *J* = 12.0 Hz,1H), 4.49 (d, *J* = 6.0 Hz, 1H), 3.99 (dd, *J* = 24.0, 16.0 Hz, 2H), 3.44 (s, 2H), 3.38 (t, *J* = 4.0 Hz, 1H), 2.43 – 2.37 (m, 2H), 2.16 (dd, *J* = 24.0, 16.0 Hz, 1H), 2.02 (ddd, *J* = 24.4, 17.5, 8.7 Hz, 3H), 1.83 (d, *J* = 10.8 Hz, 2H), 1.70 – 1.54(m, 5H), 1.47 – 1.35 (m, 2H), 1.32 (s, 3H), 1.25 – 1.18 (m, 5H), 1.10 (d, *J* = 10.6 Hz, 2H), 1.02 – 0.98 (m, 5H), 0.96 (s, 3H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.57 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 217.59, 171.67, 168.02, 160.32, 154.40, 149.94, 141.16, 123.42, 115.60, 104.57, 103.47, 73.04, 70.25, 57.69, 56.76, 50.72, 45.39, 44.49, 43.98, 41.97, 36.79, 34.44, 33.86, 33.39, 30.55, 29.02, 27.04, 26.15, 24.84, 16.51, 14.93, 11.98; HR-MS (ESI) m/z calcd for $C_{36}H_{52}N_5O_5S$ $(M+H)^+$: 666.36837, found: 666.36627.

14-*O*-(((4-(2-(diethylamino)acetamido)-1*H*-pyrrolo[2,3-d]pyrimidin-6-yl)thio)acetyl)mutilin **(15o)** slight yellow solid, 62% yield, m. p. : 111.3 – 114.7; ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.80 (s, 1H), 10.04 (s, 1H), 7.21 (s, 1H), 6.76 (s, 1H), 5.48 (d, J = 8.2 Hz, 1H), 4.95 (d, J = 17.8 Hz, 1H), 4.89 (d, J = 11.2 Hz, 1H), 4.45 (d, J = 5.9 Hz, 1H), 3.96 (q, J = 16.2 Hz, 2H), 3.35 (t, J = 5.7 Hz, 1H), 3.30 (s, 3H), 2.61 (q, J = 6.9 Hz, 4H), 2.47 (s, 1H), 2.33 (s, 1H), 2.14 (d, J = 12.0, 8.0 Hz, 1H), 2.05 – 1.99 (m, 3H), 1.94 (dd, J = 12.0, 8.0 Hz, 1H), 1.61 – 1.54 (m, 2H), 1.41 – 1.32 (m, 2H), 1.27 (s, 3H), 1.24 – 1.17 (m, 3H), 1.01 (t, J = 7.1 Hz, 6H), 0.97 – 0.95 (m, 1H), 0.93 (s, 4H), 0.76 (d, J = 6.8 Hz, 3H), 0.54 (d, J = 6.9 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 222.30, 175.09, 172.78, 165.04, 159.20, 154.45, 145.88, 128.27, 120.31, 109.48, 108.29, 77.76, 74.99, 62.41, 53.02, 50.12, 49.23, 48.72, 46.69, 41.56, 41.51, 39.16, 38.56, 35.27, 33.75, 31.74, 29.63, 21.23, 19.64, 17.30, 16.69; HR-MS (ESI) m/z calcd for $C_{35}H_{52}N_5O_5S$ $(M+H)^+$: 640.35272, found: 640.35034.

4.3 Minimum Inhibitory Concentrations Testing.

MICs of compounds **15a-15o** against Gram-positive bacteria (MSSA, MRSA, MSSE, MRSE, *E. faecalis* and *E.faecium*) and Gram-negative bacteria (*E.coil and P. multocida*) were investigated using valnemulin hydrochloride as the reference agent based on the National Committee for Clinical Laboratory Standards (NCCLS). The test compounds were dissolved in aqueous solution containing 10 – 20% DMSO to make the concentration of stock solutions be 1280 μg/mL. Then 1 mL of the stock solution was added 9 mL sterile water and diluted to 128 μg/mL as the initial test concentration. Each well of the 96-well plates was added 100 μL of Mueller-Hinton Broth (MHB) and the initial solution was added to the first column to make the concentration of the test compounds be 64 μg/mL. Then pipetting 100 μL of the solution in the first column into the second column and employing the same 2-fold dilution method to adjust the concentrations of the test compounds in column 2 to 10 to 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 μg/mL. Column 11 was just added only MHB solution while column 12 was only filled with bacterial suspension. The test bacteria were cultured in MHB overnight and cell concentrations of those bacteria were diluted to 10^6~10^7 CFU/mL. Bacterial suspension with a mount of 100 μL was added to each well of the 96-well plates containing 100 μL of a serial of diluted compounds or drug and the 96-well plates were incubated at 37°C for 24 h. The MIC values of the test compounds and drug were determined by the OD values which were compared to that of MHB (blank control). Each concentration of the test compounds inhibiting each bacteria contained three parallels.

4.4 Killing kinetics assay

To get exponential phase cells of *S. aureus*, the bacterial suspension was incubated at 37°C, with shaking at 120 rpm for 4 h. The culture were diluted to ~ 2 ×10^7 CFU/mL with MHB. 1 ml of the exponential phase cell culture was added to the wells of a 96-well assay block (Corning Costar 3960, Corning, NY, USA) containing 1 ml of MHB with twice the desired concentrations of **15a** and valnemulin. The assay block was incubated at 37°C shaking at 120 rpm. At specific times (1, 2, 3 and 4 h), 400 μl samples were drawed and washed once with PBS to remove the antibiotics. The samples were serially diluted 10^5 fold with PBS and spot-plated onto MHB plates. After incubating the plates overnight (~18h) at 37°C, the colonies were counted to enumerate the number of cells which was represented as $\log_{10}$ (CFU/mL). These experiments were conducted in triplicate.

4.5 ED_{50} testing

Clinical Isolate of MRSA. Kunming mice (half male and half female, Chongqing Tengxin Bill Experimental Animal Sales Co. Ltd. Limited) weighing between 19 and 23 g were used in the study, with 10 mice in each group. The mice were infected by a lethal systemic MRSA via intraperitoneal injection with 0.5 mL of bacterial suspension (~10^7 CFU/mL). Compound **15a**, **15b** and **15o** dissolved in 0.5 mL vehicle (DMSO: Tween-80: sterile water = 1:1:8) and valnemulin dissolved in 0.5 mL sterile water were given intraperitoneal administration after 1 h infection at doses of 8.0, 12.0, 18.0, 27.0, 40.0 mg/kg, 9.8, 14.3, 21.0, 30.5, 45.0 mg/kg, 10.0, 15.0, 23.0, 34.5, 50.0mg/kg and 7.0, 10.8, 16.7, 25.7 and 40.0 mg/kg, respectively. ED_{50} values were calculated after 7 days treatment using the Probit analysis method. The protocol for this study was reviewed and approved by Chongqing Academy of Animal Sciences

4.6 MRSA Infection Model

Clinical Isolate of MRSA. Kunming mice (female, Chongqing Tengxin Bill Experimental Animal Sales Co. Ltd. Limited) weighting 22±2 g, were subjected to neutropenic for intraperitoneal treatment with 150 mg/kg cyclophosphamide 4 days before infection and with 100 mg/kg 1 day before infection. The neutropenic mice were treated with 0.5 mL bacteria suspension via intraperitoneal injection. At 1 h after infection, the infected mice were conducted intragastric administration with the test compounds **15a** and valnemulin at doses of 20 mg/kg/d and 40 mg/kg/d for uninterrupted 3 days. The protocol for this study was reviewed and approved by Chongqing Academy of Animal Sciences

4.7 Molecular docking

Surflex-dock (Sybyl 8.2, Tripos Inc.) as a flexible molecular docking method was employed in this paper [19]. The crystal structures of the 50s ribosomal subunit from *Deinococcus radiodurans* in complex with tiamulin (PDB ID: 1XBP) was used for molecular docking. Surflex-dock adopted protomol, an idealized ensemble of CH_4, NH and CO probes, to provide the generation of docking conformers of the ligands.

The protomol was generated based on the residues within 5Å distance from tiamulin. The search grid and the number of additional starting conformations were set as 2Å and 3 Å, respectively. The self-scoring, molecule fragmentation, soft grid, pre- and post-dock minimizations were performed in the docking processes. Total score indicating -log(KD) was used for ligand ranking. Before docking, all ligands were optimized by Tripos force field with conjugate gradient minimizer (Sybyl 8.2). The maximum iteration steps and energy gradient were set to 5000 times and 0.05kcal/mol·Å, respectively.

Acknowledgement

This work was supported by Chongqing Agriculture Development Found (No. 17412, Chongqing Municipal Finance Bureau) and Chongqing Basic Scientific Research Project (No. 16438, Chongqing Science and Technology Commission).

Reference

[1] S. Donadio, S. Maffioli, P. Monciardini, *et al*. J. Antibiot. 63 (2010) 423-430.

[2] K. K. Kumarasamy, M. A.Toleman, T. R.Walsh, *et al*. Lancet Infect. Dis. 10 (2010) 597-602.
[3] D.J. Newman, G. M. Cragg. J. Nat. Prod. 79 (2016) 629-661.
[4] F. Kavanagh, A. Hervey, W. J. Robbins. Proc. Natl. Acad. Sci. U.S.A. 37 (1951) 570.
[5] G. Hogenauer. Eur. J. Biochem. (52) 1975 93–98.
[6] K. Riedl. J. Antibiot. 29 (1976) 132-139.
[7] H. Egger, H. Reinshagen. J. Antibiot. 29 (1976) 915-922.
[8] H. Egger, H. Reinshagen. J. Antibiot. 29 (1976) 923-927.
[9] E. van Duijkeren, C. Greko, M. Pringle, *et al*. J. Antimicrob. Chemother. 69 (2014) 2022-2031.
[10] H. Berner, G. Fischer, J. F. Hildebrandt, G. Laber, H. Vyplel. Curr. Chemother. Immunother. 1 (1982) 345.
[11] J. F. Hildebrandt, H. Berner, G. Laber, F. Turnowsky, E. Schütze. Curr. Chemother. Immunother. 1 (1982) 346-347.
[12] S. Rittenhouse, C. Singley, J. Hoover, R. Page, D. Payne. Antimicrob. Agents Chemother. 50 (2006) 3886-3888.
[13] S. Paukner, R. Riedl. Cold Spring Harb. Perspect. Med. 7 (2017) a027110.
[14] R. Novak. Ann. N.Y. Acad. Sci. 1241 (2011) 71-81.
[15] R. Shang, X. Pu, X. Xu, Z. Xin, C. Zhang, W. Guo, Y. Liu, J. Liang. J. Med. Chem. 57 (2014) 5664-5678.
[16] X. Wang , Y. Ling , H. Wang , J. Yu , J. Tang , H. Zheng , X. Zhao, D. Wang , G. Chen , W. Qiu , J. Tao. Bioorg. & Med. Chem. Letters. 22 (2012) 6166-6172.
[17] F. Schlünzen, E. Pyetan, P. Fucini, A. Yonath and Jörg M. Harms. Mol. Microbiol. 54 (2004) 1287-1294.
[18] C. Davidovich, A. Bashan, T. Auerbach-Nevo, R. D. Yaggie, R. R. Gontarek, A. Yonath. Proc. Natl. Acad. Sci. USA 104 (2007) 4291-4296.
[19] A.N. Jain. J. Med. Chem. 46 (2003) 499-511.

Research Highlights

- Fifteen novel peluromutilin derivatives linking 7*H*-pyrrolo[2,3-*d*]pyrimidine were synthesized
- Compounds **15a** presented the best in vitro antibacterial activity
- The in vivo efficacy of compounds **15a** was equal to valnemulin
- **15a** might be a promising lead compound for new antibacterial agent