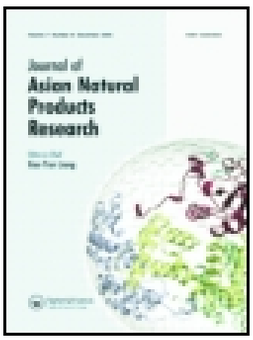

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Design, synthesis and antibacterial activities of pleuromutilin derivatives

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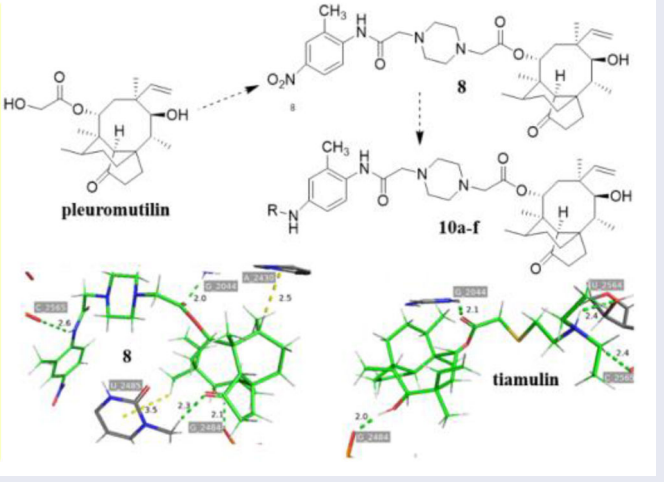
ABSTRACT

We described the design, synthesis and antimicrobial activities of novel pleuromutilin derivatives with substituted piperazine substrate. Minimum inhibitory concentration (MIC) was used to evaluate the activity of the derivatives against six bacteria *in vitro*, and compound **8** was potent against *Staphylococcus aureus* and *Staphylococcus epidermidis* with the MIC value of 0.0625 μg/ml. **10a** and **10 b** showed similar activity to positive control drugs (tiamulin, erythromycin) against *S. aureus* with the MIC value of 0.125 μg/ml. The binding mode of compound **8** and tiamulin to the ribosome pocket showed the correlation between binding parameters and the antibacterial activity, and more bonds and stronger combination could effectively enhance the activity of compounds.



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1. Introduction

The discovery of antibiotics is of great significance in human history, which not only prolongs the life span of human beings, but also improves the quality of human lives. However, the abuse of antibiotics makes many bacteria have increasingly serious drug resistance, which poses a great threat to the safety of human life [1, 2]. It is estimated that as a result of the increasing number of multidrug-resistant bacteria, the global death toll from bacterial infections will climb to about 7 million to 10 million by 2050 [3]. Therefore, the Food and Drug Administration (FDA) launched an incentive program in 2012 to promote antibiotic research and development (generating antibiotics now, GAIN), which aims to cope with the decrease of antibiotics and the increasing number of drug-resistant bacteria [4]. According to related studies, the vast majority of the 42 antibiotics currently in clinical trials are modified from previously marketed antibiotic drugs [5]. Researchers have concentrated their attention on antibiotic analogues that are already on the market and compounds that have previously been ignored. One of them is pleuromutilin.

Pleuromutilin **1** (Figure 1) is a kind of natural product with antibacterial activity discovered in the 1950s, which is a diterpene compound with parallel tricyclic skeleton produced by deep culture of *Pleurotsmutilus* and *Pleurots passeckerianus* from basidiomycetes of higher fungi [6]. Studies have shown that pleuromutilin binds to the 23 s rRNA of bacterial ribosomal 50 s subunit, locates in the peptidyl transferase center of ribosomal 50 s subunit through its tricyclic mother nucleus, and forms a tight pocket at site A. Meanwhile, the side chain partially covers the P site of tRNA binding, which directly inhibits the formation of peptide bond, thus preventing the synthesis of bacterial protein [7]. It is precisely because of this ingenious mode of action that the compound and some of its derivatives have obvious antibacterial activity and excellent pharmacokinetic properties against drug-resistant Gram-positive bacteria and mycoplasma, and have less cross-drug resistance with other antibiotics [8]. In the 1970s, a number of pleuromutilin derivatives were synthesized and studied the structure–activity relationship, implying that introduction of thioether and alkaline group in the C14 side chain would improve antibacterial activity [9, 10].

Tiamulin **2** (Figure 1) is a derivative discovered by the Sandoz Institute in 1974, which is 10 to 100 times higher than pleuromutilin in antibacterial activity [11]. It is

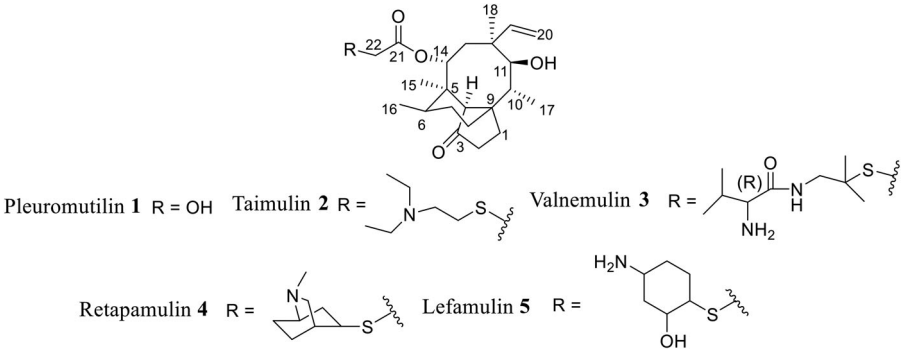

Figure 1. The structures of pleuromutilin and related drugs.

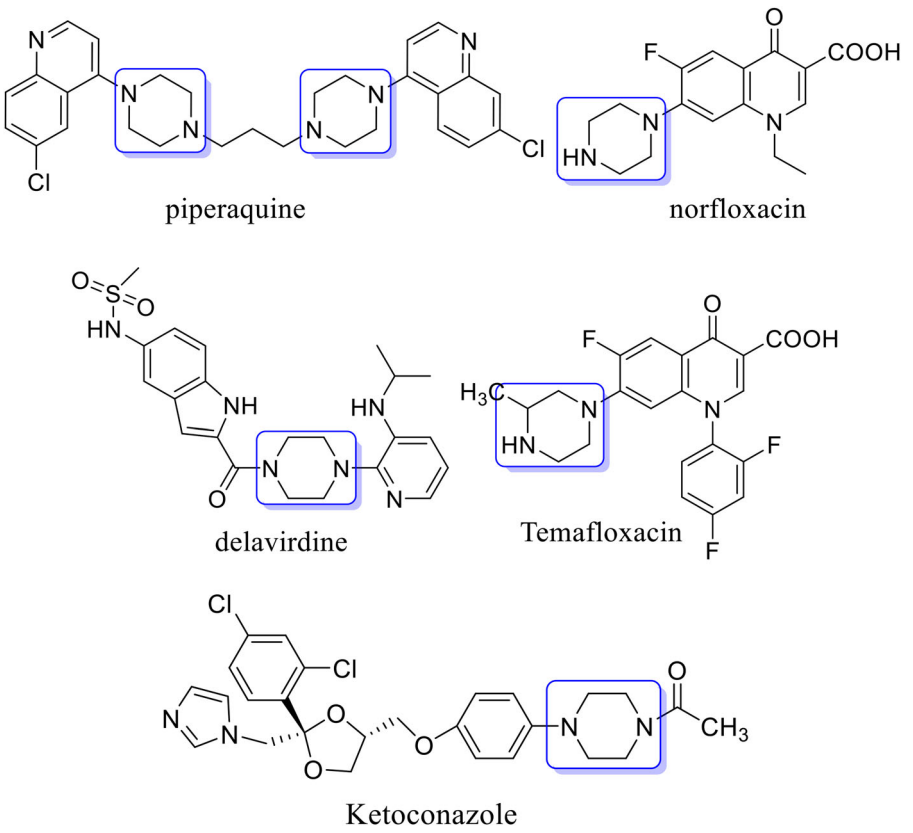

Figure 2. Antimicrobial drugs with piperazine rings.

one of the commonly used veterinary antibiotics in the market and mainly used in the form of feed additives to treat and prevent gastrointestinal and respiratory tract infections in swine and poultry [11, 12]. In 1980, valnemulin **3** (Figure 1) was first synthesized by Sandoz Institute, which is superior to tiamulin in antibacterial activity [13]. In addition, recent studies have shown that valnemulin is effective in the treatment of antibiotic-resistant mycoplasma infections in immunocompromised patients [14]. Retapamulin **4** (Figure 1) is another pleuromutilin antibiotic developed by Glaxo Smith Kline of the United Kingdom. It is the first topical antibiotic for human use and was approved by US FDA in April 2007 [15, 16]. Lefamulin **5** (formally BC-3781) (Figure 1) is a novel pleuromutilin antibiotic for community-acquired bacterial pneumonia (CABP) as intravenous (IV) and oral (PO) formulations, and it is the first to be used for systemic treatment of bacterial infections in humans [17].

Most of pleuromutilin derivatives usually contain sulfide linkage in C14 side chain, either drugs that are on the market or have been reported, either for human or veterinary use, however, it is reported that thioether group suffers from being rapidly and extensively metabolized *in vivo* [18]. Piperazine substrate was widely used in many antimicrobial drugs, such as piperaquine, norfloxacin, temafloxacin, delavirdine and ketoconazole, etc. (Figure 2). We were inspired by those structures and tried to introduce piperazine to pleuromutilin as the linkage of C14 side chain. It accorded with the SAR

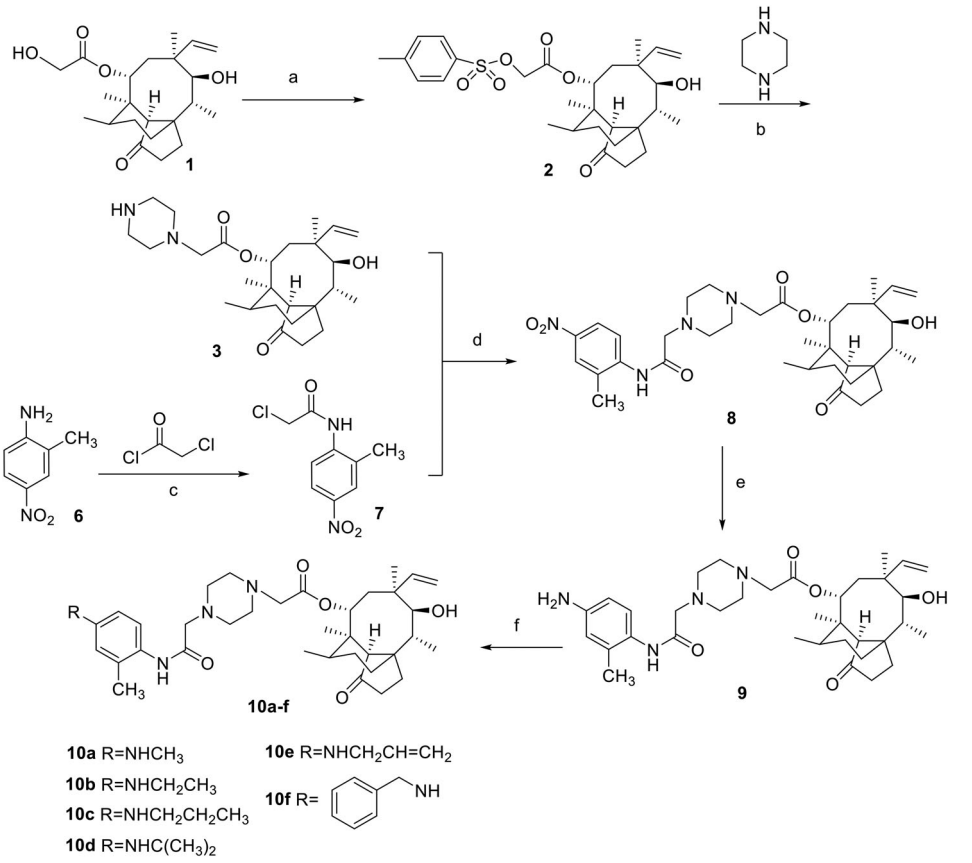

Figure 3. Preparation of the target compounds. a: p-TsCl, Et_3N, DCM; b: K_2CO_3, acetonitrile; c: Et_3N, DCM; d: Et_3N, DMF; e: Fe, NH_4Cl, CH_3COOH, H_2O; f: Et_3N, DMF.

that alkaline group in C14 side chain is beneficial to activity improvement. It can be found by observing pleuromutilin derivatives on the market that all of them owe amide terminal group. Based on these points, we designed and synthesized a series of pleuromutilin derivatives, and the antimicrobial activities were further measured.

2. Results and discussion

2.1. Chemistry

As depicted in Figure 2, pleuromutilin (**1**) was reacted with 1.5 equiv. of *p*-toluene sulfonyl chloride in the presence of 1.5 equiv. of triethylamine to afford compound **2**. Compound **2** was reacted with piperazine and 2-methyl-4-nitroaniline to obtain compound **8**, and then it was reduced to afford compound **9**. At last, we replaced compound **9** with a series of halogenated hydrocarbons to obtain compounds **10a–f**.

2.2. In vitro antibacterial activity

The evaluation of the *in vitro* antibacterial activities of the pleuromutilin derivatives **8**, **9** and **10a–f** was performed against five drug-susceptible Gram-positive pathogens

Table 1. The MIC values (μg/ml) of compounds 8, 9 and 10a–f.

Compounds	*Staphylococcus aureus* ATCC 25923	MRSA NY3	*Staphylococcus epidermidis* ATCC 12228	*Staphylococcus suis* ATCC 43765	*Enterococcus faecalis* ATCC 19433	*Escherichia coli* CVCC 231
10a	0.25	>64	0.125	>64	>64	>64
10b	0.25	>64	0.5	>64	>64	>64
10c	0.5	>64	0.5	>64	>64	>64
10d	0.5	>64	0.5	>64	>64	>64
10e	0.5	>64	0.5	>64	>64	>64
10f	0.5	>64	0.5	>64	>64	>64
8	0.06	>64	0.06	>64	8	>64
9	0.5	>64	0.5	>64	16	>64
Tiamulin fumaric acid	0.125	16	0.125	32	64	16
Erythromycin sulfur	0.125	8	0.125	0.5	4	16

and one drug-resistant isolated from clinic as well as against one Gram-negative bacteria strain. Tiamulin fumaric acid and erythromycin sulfur were used as positive control and the MIC values were provided in Table 1. Most of the derivatives showed general *in vitro* antibacterial activity against the tested strains, and compound **8** showed excellent *in vitro* antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*. However, these synthesized compounds and the reference agents have minimal potency against Gram-negative bacterial, like *Escherichia coli*. The results illustrate that compound **8** synthesized as a lead compound showed almost two-fold increase in activity against *S. aureus* and *S. epidermidis* in comparison with that of reference drugs, and eight-fold increase in activity against *Enterococcus faecalis* compared to that of tiamulin. **10a** and **10 b** showed the same activity with two controlled drugs against *S. aureus*.

2.3. Molecular docking study

Conclusions can be drawn from Table 1 that compound **9** and **10c–f** displayed moderate antimicrobial activity and **10a–b** owned better activity than **9** and **10c–f**, while the most active was compound **8**. The results of molecular docking were consistent with the results of activity determination. **10a** and **10 b**, **9** and **10c–f**, displayed the similar interaction mode with the pocket, respectively. We compared the interaction of **10a**, **10d**, **8** and tiamulin with the active pocket. **10a** was found to form one hydrogen bonds (1.82 Å) with U2485 and two H-π interactions (Table 2). **10d** was found to form two H-π interactions (Table 2). **8** was found to form four hydrogen bonds (1.99 Å, 2.11 Å, 2.26 Å, 2.64 Å) with G2044, G2484, U2485, C2565 and two H-π interactions (Figures 4 and 5), its binding strength was the best of all the compounds. Tiamulin was found to form three hydrogen bonds (1.64 Å, 2.04 Å, 2.24 Å) with G2484, G2044, U2564 and two H-π interactions (Figures 6 and 7). The docking results can explain why **8** exhibited better *in vitro* antibacterial activity, and more bonds and stronger combination could effectively enhance the activity of compounds. At the same time, it is proved that the existence of -NH- at C-14 is crucial. Almost all the designed molecules displayed interactions with U2564, and among those compounds tiamulin and **8** displayed interactions with both G2484 and G2044 (Figures 4–7). The compounds **8**, **10a**, **10d** and tiamulin were suggested a detailed analysis of their possible binding modes in the active pocket of 23 s rRNA, and they showed almost comparable binding energy score (Table 2).

Table 2. Docking scores as "S" (kcal mol^{-1}) and bond interactions of 8, 10a–f and tiamulin with active pocket of 23 s rRNA.

Cpd. No.	S (Kcal mol − 1)	No. of bonds	Distance (A°)	Amino acids involved	Interacting groups
10a	- 9.7638	3	1.82	U2485 U2564 A2430	H-bond with OH of pleuromutilin C11 H-π interaction H-π interaction
10b	- 10.2427	3	2.46	G2484 G2044 A2430	H-bond with C = O of pleuromutilin C3 H-π interaction H-π interaction
10c	- 10.5185	2	1.79 2.39	G2044 G2044	H-bond with OH of pleuromutilin C11 H-bond with OH of pleuromutilin C11
10d	- 9.9347	2	–	U2564 C2565	H-π interaction H-π interaction
10e	- 10.3751	1	–	G2044	H-π interaction
10f	- 10.9199	3	–	U2564 A2045	H-π interaction H-π interaction
8	- 9.9035	6	1.99 2.11 2.26 2.64	G2044 G2484 U2485 C2565 A2430 U2485	H-bond with C = O of ester group H-bond with OH of pleuromutilin C11 H-bond with C = O of pleuromutilin C3 H-bond with NH of acylamino H-π interaction H-π interaction
tiamulin	-8.6283	5	1.64 2.04 2.24	G2484 G2044 U2564 U2483 U2564	H-bond with OH of pleuromutilin C11 H-bond with C = O of ester group H-bond with H of C14 side chain H-π interaction H-π interaction

3. Experimental

3.1. General experimental procedures

Routine monitoring of reaction was performed by TLC using pre-coated GF254 TLC plate, which were purchased from Qingdao Ocean Chemical Co., Ltd. (Qingdao, China). ^{1}H-NMR spectrum was recorded on a Bruker AVANCE 600 spectrometer at 600 MHz instrument (Bruker Daltonics, Bremen, Germany) in $CDCl_3$ using tetramethylsilane (TMS) as the internal reference. MS were performed with electron spray ionization (ESI) mode and recorded on a high performance liquid chromatography tandem mass spectrometer by Waters (Waters, Milford, MA, USA).

All reagents were purchased from commercial sources and were used as received. Pleuromutilin (>90% pure) was obtained from Beijing Ouhe Technology Co., Ltd. (Beijing, China). 2-Methyl-4-nitroaniline, chloroacetyl chloride and alkylamine were purchased from Aladdin Reagent Co. (Shanghai, China). Piperazine and *p*-methyl benzene sulfonic chloride were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

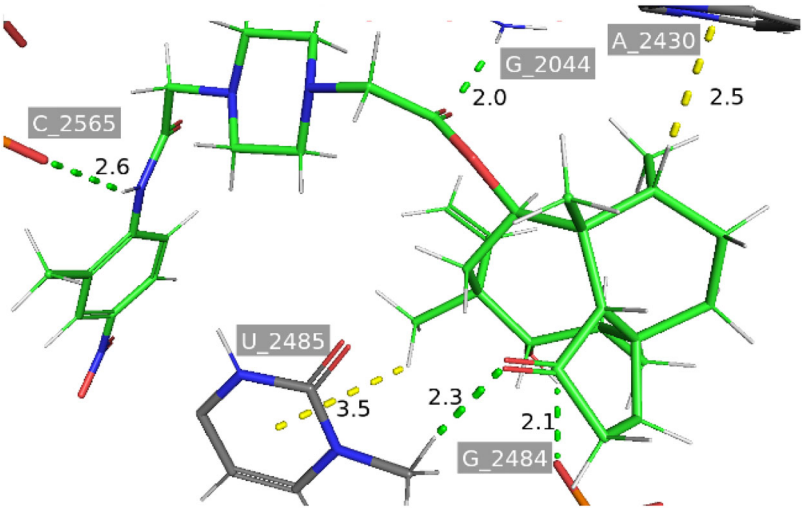

Figure 4. 3 D diagram of compound **8** showing its interactions with the 23 s rRNA active site (distances in Å).

3.2. Synthesis

3.2.1. Synthesis of 14-O-(p-toluene-sulfonyloxyl) mulin (2)

As shown in Figure 1, pleuromutilin **1** (7.0 g, 18.5 mmol) was reacted with *p*-toluene sulfonyl chloride (5.3 g, 28.3 mmol) in the presence of triethylamine (2.8 g, 27.5 mmol) in dichloromethane (50 ml). The mixture was stirred overnight at room temperature, then evaporated in vacuum and ethyl acetate (100 ml) was added to the residue. The organic phase was washed with saturated salt solution, dried with anhydrous sodium sulfate about 2 h, filtered, and the organic solvent was evaporated. The crude product was recrystallized with mixed solvent of ethyl acetate and petroleum ether, and filtered to afford the pure product (**2**, 9.0 g) as white solid in the yield of 91.2%. IR (KBr, cm^{-1}): 3448, 2941, 2864, 1732, 1636, 1597, 1457, 1371, 1224, 1117, 1036, 832, 664, 552. ^{1}H NMR (600 MHz; $CDCl_3$): δ (p.p.m.) 7.75 (d, $J = 7.2$ Hz, 2 H, phenyl H-2, 6), 7.29 (d, $J = 8.4$ Hz, 2 H, phenyl H-3, 5), 6.35 (dd, $J = 10.8$, 6.6 Hz, 1 H, H-19), 5.71 (d, $J = 8.4$ Hz, 1 H, C11-OH), 5.27 (d, $J = 10.8$ Hz, 1 H, H-20), 5.14 (d, $J = 17.4$ Hz, 1 H, H-20), 4.41 (s, 2 H, H-22), 3.27 (dd, $J = 6.6$, 4.2 Hz, 1 H), 2.38 (s, 3 H), 2.09–2.22 (m, 3 H), 1.95–2.01 (m, 2 H), 1.70 (dd, $J = 1.8$ Hz, 1 H), 1.55–1.60 (m, 2 H), 1.37–1.45 (m, 3 H), 1.34 (s, 3 H), 1.28 (dd, $J = 3.0$ Hz, 1 H), 1.18 (d, $J = 16.2$ Hz, 1 H), 1.09 (s, 3 H), 1.02–1.05 (m, 1 H), 0.81 (d, $J = 7.2$ Hz, 3 H), 0.56 (d, $J = 7.2$ Hz, 3 H). ^{13}C NMR (150 MHz; $CDCl_3$): 216.8 (C-3, C = O), 164.9 (C-21, C = O), 145.3 (C-19), 138.7 (phenyl C-1), 132.6 (phenyl C-4), 129.9 (phenyl C-3, 5), 128.1 (phenyl C-2, 6), 117.4 (C-20), 74.5, 70.3, 65.0, 58.0, 45.4, 44.5, 43.9, 41.8, 36.5, 36.0, 34.4, 30.3, 26.8, 26.4, 24.8, 21.7, 16.5, 14.8, 11.5. MS (ESI, *m/z*): 555.3 $[M + Na]^+$.

3.2.2. Synthesis of 14-O-(1-piperazinyl) mulin (3)

Anhydrous piperazine (3.0 g, 35 mmol) and K_2CO_3 (4.8 g, 35 mmol) were added into acetonitrile (80 ml) in three-necked round bottom flask, stirred and heated to reflux. Compound **2** (18.5 g, 35 mmol) was dissolved into acetonitrile (50 ml) and then added

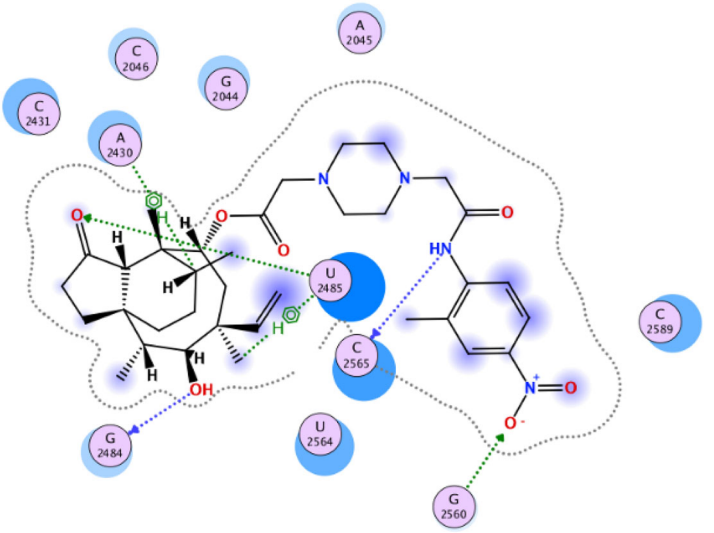

Figure 5. 2 D diagram of compound **8** showing its interactions with the 23 s rRNA active site (distances in Å).

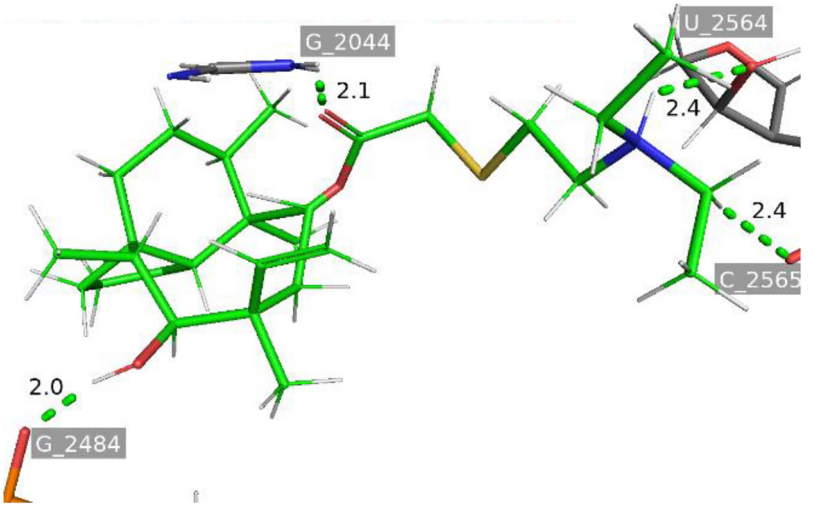

Figure 6. 3 D diagram of tiamulin showing its interaction with the 23 s rRNA active site.

dropwise to the above mixture. The mixture was reacted about 8 h, followed by cooling to room temperature. Double volume of water was added and stirred for 1 h, then filtered and the filter cake was washed to neutral with water. White solid (12.7 g) in 82% yield was obtained after full drying at 80 °C. The next step of reaction could be carried out directly without purification. IR (KBr, cm^{-1}): 3422, 2930, 2860, 1734, 1458, 1287, 1194, 1153, 1117, 1016, 911, 852. ^{1}H NMR (600 MHz; $CDCl_3$): δ (p.p.m.) 6.44 (dd, $J = 10.8$, 6.6 Hz, 1 H, H-19), 5.73 (d, $J = 8.4$ Hz, 1 H, C11-OH), 5.27 (d, $J = 10.8$ Hz, 1 H, H-20), 5.15 (d, $J = 17.4$ Hz, 1 H, H-20), 3.58 (s, 1 H), 3.29 (d, $J = 6.6$ Hz, 1 H), 3.10 (d, $J = 16.8$ Hz, 1 H), 2.98 (d, $J = 16.8$ Hz, 1 H), 2.94–2.95

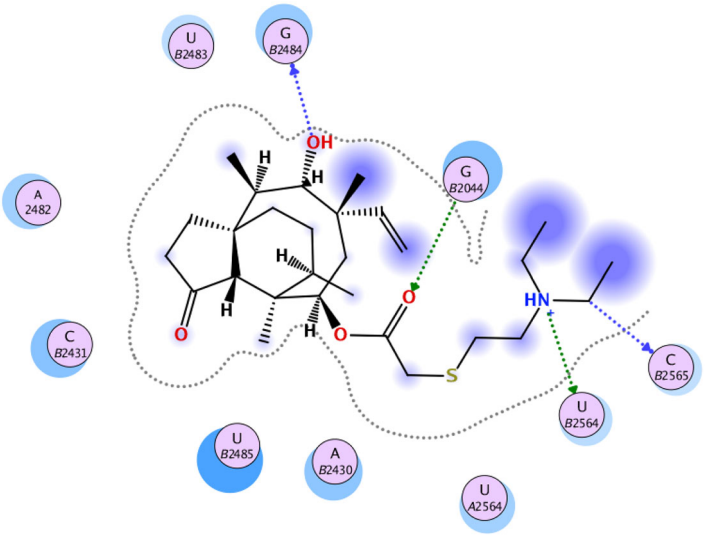

Figure 7. 2 D diagram of tiamulin showing its interaction with the 23 s rRNA active site.

(m, 3 H), 2.49–2.59 (m, 4 H, piperazinyl H-2, 6), 2.24–2.30 (m, 2 H), 2.09–2.22 (m, 4 H, piperazinyl H-3, 5), 1.98–2.03 (m, 2 H), 1.72 (dd, $J = 3.0$ Hz, 1 H), 1.56–1.64 (m, 2 H), 1.45–1.52 (m, 1 H,), 1.38–1.41 (m, 1 H), 1.37 (s, 3 H), 1.26–1.31 (m, 1 H), 1.24 (d, $J = 16.2$ Hz, 1 H), 1.19–1.22 (m, 3 H), 0.81 (d, $J = 6.6$ Hz, 3 H), 0.65 (d, $J = 6.6$ Hz, 3 H). ^{13}C NMR (150 MHz; $CDCl_3$): 217.1 (C-3, C = O), 169.0 (C-21, C = O), 139.0, 117.3, 74.6, 68.3, 60.2, 58.2 (C-22), 53.0 (piperazinyl C-2,6), 45.4 (piperazinyl C-3,5), 45.1, 45.0, 43.9, 41.7, 36.7, 36.0, 34.5, 30.4, 26.8, 26.3, 24.8, 16.7, 14.9, 11.5. MS (ESI, *m/z*): 447.3 $[M + H]^+$.

3.2.3. Synthesis of 2-chloro-N-(2-methyl-4-nitrophenyl) acetamide (7)

2-Methyl-4-nitroaniline (9.8 g, 64.5 mmol) and triethylamine (7.2 g, 70.9 mmol) were dissolved in CH_2Cl_2, and chloroacetyl chloride (7.28 g, 64.5 mmol) was added dropwise to the resultant mixture. The reaction was carried out at room temperature for about 5 h, then most dichloromethane was evaporated in vacuum and ethyl acetate (100 ml) was added to the residue. The extract was washed with saturated sodium chloride to neutral and the organic phase was dried with anhydrous Na_2SO_4 for 2 h. Vacuum concentration afforded the crude product (12.25 g) in 83.2% yield. The next step of reaction could be carried out directly without purification.

3.2.4. Synthesis of 14-O-1-(4-(2-methyl-4-nitrophenyl) acetamide) piperazine mulin (8)

Compound **7** (10 g, 43.8 mmol) and triethylamine (4.87 g, 48.1 mmol) were dissolved in DMF (60 ml), and the mixture was stirred and heated to 80 °C. Compound **3** (19.56 g, 43.8 mmol) was dissolved in DMF (40 ml), and then added dropwise to the above mixture slowly. The majority of DMF was evaporated in vacuum after the end of the reaction and water (100 ml) was added to the residue, then solid was precipitated out by stirring. The crude target product was obtained by filtrating, and the filter cake was rinsed with water (30 ml × 3) and dried at 80 °C. The crude product

was recrystallized using ethyl acetate and petroleum ether to afford pure product (20.41 g), in 73.1% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.85 (d, J = 13.2 Hz, 1 H, phenyl H-5), 8.09–8.13 (m, 2 H, phenyl H-3, 6), 6.51 (dd, J = 16.8, 9.6 Hz, 1 H, H-19), 5.78 (d, J = 12.6 Hz, 1 H, H-19), 5.33 (d, J = 16.2 Hz, 1 H, H-20), 5.19 (dd, J = 12.6 Hz, 1 H, H-20), 3.36–3.38 (m, 1 H), 3.22–3.26 (m, 3 H), 3.09 (d, J = 17.2 Hz, 1 H), 2.54–2.74 (m, 8 H, piperazinyl), 2.39 (s, 1 H), 2.31–2.35 (m, 1 H), 2.19–2.26 (m, 2 H), 2.07–2.10 (m, 2 H), 1.75–1.79 (m, 1 H), 1.62–1.69 (m, 3 H), 1.53–1.56 (m, 1 H), 1.47–1.49 (m, 1 H), 1.43–1.45 (m, 4 H), 1.34–1.38 (m, 1 H), 1.25–1.30 (m, 2 H), 1.16 (s, 3 H), 1.09–1.14 (m, 1 H), 0.88 (d, J = 10.8 Hz, 3 H), 0.72 (d, J = 10.8 Hz, 3 H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 217.0 (C-3, C = O), 168.8 (C-21, C = O), 168.5 (C-24, C = O), 143.0 (C-19), 141.7 (phenyl C-4), 139.1 (phenyl C-1), 126.3 (phenyl C-2), 125.5 (phenyl C-3), 123.1 (phenyl C-5), 119.2 (C-20), 117.2 (phenyl C-6), 74.5, 68.4, 61.9 (C-23), 59.6, 58.1 (C-22), 53.2 (piperazinyl), 53.0, 45.4, 44.9, 43.9, 41.7, 36.6, 36.0, 34.4, 30.3, 26.8, 26.4, 24.8, 17.9, 16.7, 14.8, 11.5. MS (ESI, *m/z*): 661.6 $[M + Na]^+$.

3.2.5. Synthesis of 14-O-1-(4-(2-methyl-4-aminophenyl)acetamide)piperazine mulin (9)

Reduced iron powder (1.53 g, 27.3 mmol), ammonium chloride (2.19 g, 40.9 mmol), acetic acid (10 ml) and water (20 ml) were stirred together and heated to 90 °C. Compound **8** (8.7 g, 13.6 mmol) was dissolved in absolute ethyl alcohol (50 ml) and was added dropwise to the above mixed solution. Most ethyl alcohol was evaporated in vacuum after complete reaction, and saturated sodium bicarbonate was added to the residue to adjust pH to 7, followed by extracting with ethyl acetate and concentrating the organic phase by reduced pressure distillation to give crude dark brown product. It was chromatographed on silica gel and recrystallized with a mixture of ethyl acetate and petroleum ether to give pure product (7.38 g) in 89% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.91 (s, 1 H, NHC = O), 7.66 (d, J = 12.0 Hz, 1 H, phenyl H-6), 6.56 (s, 1 H, phenyl H-3), 6.48–6.53 (m, 1 H, phenyl H-5), 5.79 (d, J = 12.6 Hz, 1 H, H-19), 5.34 (dd, J = 16.8, 1.8 Hz, 1 H, H-20), 5.20 (dd, J = 17.4, 1.8 Hz, 1 H, H-20), 3.56 (s, 2 H, H-22), 3.35 (dd, J = 9.6 Hz, 1 H), 3.22 (d, J = 16.8 Hz, 1 H), 3.15 (s, 2 H), 3.07 (d, J = 16.8 Hz, 1 H), 2.70 (s, 6 H), 2.60 (s, 2 H), 2.31–2.38 (m, 1 H), 2.20–2.28 (m, 2 H), 2.18 (s, 3 H, phenyl C-2, $C\underline{H}_3$), 2.04–2.10 (m, 2 H), 1.77 (dd, J = 4.8 Hz, 1 H), 1.60–1.69 (m, 3 H), 1.51–1.57 (m, 1 H), 1.42–1.49 (m, 5 H), 1.34–1.39 (m, 1 H), 1.24–1.31 (m, 1 H), 1.16 (s, 3 H), 1.09–1.13 (m, 1 H), 0.87 (d, J = 10.8 Hz, 3 H), 0.72 (d, J = 10.8 Hz, 3 H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 217.3 (C-3), 169.1 (C-21), 168.0 (C-24, C = O), 143.7, 139.2 (phenyl C-4), 130.0 (phenyl C-2), 127.1 (phenyl C-1), 123.7 (phenyl C-6), 117.4 (phenyl C-3), 117.0 (phenyl C-5), 113.5 (C-20), 74.7, 68.4, 62.0 (C-23), 59.8, 58.3 (C-22), 53.4 (piperazinyl), 53.2, 45.6, 45.1, 44.0, 41.9, 36.8, 36.1, 34.6, 30.5, 26.9, 26.5, 24.9, 18.1, 16.8, 15.0, 11.6. MS (ESI, *m/z*): 609.4 $[M + H]^+$.

3.2.6. Synthesis of 14-O-1-(4-(2-methyl-4-alkylamino phenyl) acetamide) piperazine mulin (10a–f)

Compound **9** (2.7 g, 4.4 mmol) and equivalent halogenated alkane were dissolved in DMF (15 ml) and triethylamine (0.49 g, 4.8 mmol) was added to the above mixture as

acid-binding agent. Water (20 ml) was added to the residue after complete reaction. And solid was precipitated after stirring. The crude product was obtained by filtrating, rinsing the filter cake with water and drying at 80 °C. It was purified by silica gel chromatography that afforded the pure product.

3.2.6.1. 14-O-1-(4-(2-Methyl-4-methylamino phenyl) acetamide) piperazine mulin (10a). White powdery solid, yield 55.2%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.87 (s, 1 H, NHC = O), 7.65 (d, J = 8.4 Hz, 1 H, phenyl H-6), 6.49–6.53 (m, 1 H, phenyl H-3), 6.43–6.48 (m, 2 H, phenyl H-5 and -NH-), 5.79 (d, J = 8.4 Hz, 1 H), 5.33 (dd, J = 17.4, 1.2 Hz, 1 H, H-20), 5.19 (dd, J = 17.4, 1.2 Hz, 1 H, H-20), 3.35 (s, 1 H), 3.20 (d, J = 16.8 Hz, 1 H), 3.15 (s, 2 H), 3.08 (d, J = 16.8 Hz, 1 H), 2.78 (s, 3 H, NH-C$\underline{H_3}$), 2.60–2.71 (m, 8 H, piperazinyl), 2.34 (t, J = 6.6 Hz, 1 H), 2.22–2.28 (m, 1 H), 2.17–2.20 (m, 3 H), 2.05–2.09 (m, 2 H), 1.77 (dd, J = 14.4, 2.4 Hz, 1 H), 1.62–1.68 (m, 2 H), 1.54–1.57 (m, 1 H), 1.45–1.50 (m, 2 H), 1.44 (s, 3 H), 1.36 (dd, J = 13.8, 3.0 Hz, 1 H), 1.28 (d, J = 16.2 Hz, 1 H), 1.16 (s, 4 H), 0.87 (d, J = 7.2 Hz, 3 H), 0.72 (d, J = 7.2 Hz, 3 H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 217.3 (C-3 C = O), 169.1 (C-21 C = O), 168.0 (C-24 C = O), 147.0, 139.2 (phenyl C-4), 130.2 (phenyl C-2), 126.0 (phenyl C-1), 124.0 (phenyl C-6), 117.4 (phenyl C-5), 114.2 (phenyl C-3), 110.8, 74.7, 68.5, 62.0, 59.9, 58.3, 53.4 (piperazinyl), 53.2, 45.6, 45.2, 44.1, 41.9, 36.9, 36.2, 34.6, 31.1, 30.6, 27.0, 26.5 (-NHC$\underline{H_3}$), 25.0, 18.3, 16.9, 15.0, 11.6. MS (ESI, *m/z*): 623.9 $[M + H]^+$.

3.2.6.2. 14-O-1-(4-(2-Methyl-4-ethylaminophenyl) acetamide) piperazine mulin (10 b). White powdery solid, yield 58.5%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.86 (s, 1 H, NHC = O), 7.64 (d, J = 8.6 Hz, 1 H, phenyl H-6), 6.43–6.53 (m, 3 H, phenyl H-3, 5 and -NH-), 5.78 (d, J = 8.5 Hz, 1 H), 5.32 (dd, J = 17.4, 1.2 Hz, 1 H, H-20), 5.19 (dd, J = 17.4, 1.2 Hz, 1 H, H-20), 3.33–3.36 (m, 1 H), 3.21 (d, J = 16.8 Hz, 1 H), 3.14 (s, 2 H), 3.10–3.14 (m, 2 H, -NHC$\underline{H_2}$CH$_3$), 3.07 (d, J = 16.8 Hz, 1 H) , 2.59–2.70 (m 8 H), 2.35 (t, J = 6.6 Hz, 1 H), 2.20–2.28 (m, 2 H), 2.18 (s, 3 H), 2.04–2.09 (m, 2 H), 1.76 (dd, J = 14.4, 2.4 Hz, 1 H), 1.62–1.67 (m, 2 H), 1.50–1.58 (m, 2 H), 1.45–1.50 (m, 2 H), 1.44 (s, 3 H), 1.36 (dd, J = 13.8, 3.0 Hz, 1 H), 1.28 (d, J = 16.2 Hz, 1 H), 1.23 (t, J = 7.2 Hz, 3 H, -NHCH$_2$C$\underline{H_3}$), 1.14 (s, 4 H), 1.10–1.14 (m, 2 H), 0.87 (d, J = 7.2 Hz, 3 H), 0.72 (d, J = 7.2 Hz, 3 H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 217.3 (C-3 C = O), 169.1 (C-21 C = O), 168.0 (C-24 C = O), 146.0, 139.2 (phenyl C-4), 130.2 (phenyl C-2), 126.0 (phenyl C-1), 123.9 (phenyl C-6), 117.4 (phenyl C-5), 114.6 (phenyl C-3), 111.1, 74.7, 68.4, 62.0, 59.8, 58.3, 53.4, 53.2 (piperazinyl), 45.6, 45.1, 44.1, 41.9 (-NH$\underline{C}$H$_2$CH$_3$), 38.8, 36.8, 36.2, 34.6, 30.5, 26.9, 26.5, 25.0, 18.3, 16.8 (-NHCH$_2\underline{C}$H$_3$), 15.0, 11.6. MS (ESI, *m/z*): 637.8 $[M + H]^+$.

3.2.6.3. 14-O-1-(4-(2-Methyl-4-propylamino phenyl) acetamide) piperazine mulin (10c). White powdery solid, yield 62.3%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.85 (s, 1 H, NHC = O), 7.63 (d, J = 8.6 Hz, 1 H, phenyl H-6), 6.43–6.53 (m, 3 H, phenyl H-3, 5 and -NH-), 5.79 (d, J = 8.5 Hz, 1 H), 5.33 (dd, J = 17.4, 1.2 Hz, 1 H, H-20), 5.19 (dd, J = 17.4, 1.2 Hz, 1 H, H-20), 3.35 (s, 1 H), 3.21 (d, J = 16.8 Hz, 1 H), 3.12–3.16 (m, 2 H, -NHC$\underline{H_2}$CH$_2$CH$_3$), 3.03–3.09 (m, 3 H), 2.60–2.70 (m, 8 H, piperazinyl), 2.34 (t, J = 6.6 Hz, 1 H), 2.22–2.28 (m, 1 H), 2.20–2.26 (m, 2 H), 2.18 (s, 3 H), 2.04–2.09 (m,

2 H), 1.77 (dd, $J = 14.4$, 2.4 Hz, 1 H), 1.64–1.67 (m, 2 H, -NHCH$_2$CH$_2$CH$_3$), 1.60–1.62 (m, 2 H), 1.54–1.57 (m, 1 H), 1.49–1.51 (m, 1 H), 1.45–1.47 (m, 1 H), 1.44 (s, 3 H), 1.36 (dd, $J = 13.8$, 3.0 Hz, 1 H), 1.28 (d, $J = 16.2$ Hz, 1 H), 1.16 (s, 3 H), 1.10–1.15 (m, 1 H), 0.98 (t, $J = 7.2$ Hz, 3 H, -NHCH$_2$CH$_2$CH$_3$), 0.87 (d, $J = 7.2$ Hz, 3 H), 0.72 (d, $J = 7.2$ Hz, 3 H). ^{13}C NMR (150 MHz, CDCl$_3$) δ 217.3 (C-3 C = O), 169.1 (C-21 C = O), 168.0 (C-24 C = O), 146.1, 139.2 (phenyl C-4), 130.2 (phenyl C-2), 125.8 (phenyl C-1), 124.0 (phenyl C-6), 117.4 (phenyl C-5), 114.6 (phenyl C-3), 111.0, 74.7, 68.4, 62.0, 59.9, 58.3, 53.4, 53.2 (piperazinyl), 46.2 (-NHCH$_2$CH$_2$CH$_3$), 45.6, 45.1, 44.1, 41.9, 36.8, 36.2, 34.6, 30.6, 26.9, 26.5, 25.0 (-NHCH$_2$CH$_2$CH$_3$), 22.8, 18.3, 16.9, 15.0, 11.7 (-NHCH$_2$CH$_2$CH$_3$). MS (ESI, *m/z*): 673.7 $[M + Na]^+$.

3.2.6.4. 14-O-1-(4-(2-Methyl-4-isopropylamino phenyl) acetamide) piperazine mulin (10d). White powdery solid, yield 56.3%.^{1}H NMR (600 MHz, CDCl$_3$) δ 8.86 (s, 1 H, NHC = O), 7.64 (d, $J = 8.6$ Hz, 1 H, phenyl H-6), 6.52 (dd, $J = 11.4$, 6.0 Hz, 1 H, phenyl H-3), 6.42–6.46 (m, 2 H, phenyl H-5 and -NH-), 5.79 (d, $J = 8.5$ Hz, 1 H), 5.34 (dd, $J = 17.4$, 1.2 Hz, 1 H, H-20), 5.20 (dd, $J = 17.4$, 1.2 Hz, 1 H, H-20), 3.57–3.61 (m, 1 H, -NHCH(CH$_3$)$_2$), 3.35 (dd, $J = 6.6$, 4.2 Hz, 1 H), 3.21 (d, $J = 16.8$ Hz, 1 H), 3.15 (s, 2 H), 3.08 (d, $J = 16.8$ Hz, 1 H), 2.61–2.67 (m, 8 H, piperazinyl), 2.35 (t, $J = 7.2$ Hz, 1 H), 2.21–2.29 (m, 2 H), 2.18–2.20 (m, 3 H), 2.05–2.10 (m, 2 H), 1.77 (dd, $J = 14.4$, 2.4 Hz, 1 H), 1.63–1.68 (m, 2 H), 1.55–1.61 (m, 3 H), 1.45 (s, 3 H), 1.46–1.48 (m, 2 H), 1.35–1.38 (m, 1 H), 1.28 (d, $J = 16.2$ Hz, 1 H), 1.19 (d, $J = 6.6$ Hz, 6 H, -NHCH(CH$_3$)$_2$), 1.15 (s, 3 H), 0.88 (d, $J = 6.6$ Hz, 3 H), 0.73 (d, $J = 6.6$ Hz, 3 H). ^{13}C NMR (150 MHz, CDCl$_3$) δ 217.3 (C-3 C = O), 169.2 (C-21 C = O), 168.0 (C-24 C = O), 145.1, 139.2 (phenyl C-4), 130.2 (phenyl C-2), 125.8 (phenyl C-1), 124.0 (phenyl C-6), 117.5 (phenyl C-5), 115.3 (phenyl C-3), 111.6, 74.7, 68.5, 62.1, 59.9, 58.3, 53.5, 53.3 (piperazinyl), 45.6 (-NHCH(CH$_3$)$_2$), 45.2, 44.6, 44.1, 41.9, 36.9, 36.2, 34.6, 30.6, 27.0, 26.5, 25.0 (-NHCH(CH$_3$)$_2$), 23.2, 18.3, 16.9, 15.1, 11.7. MS (ESI, *m/z*): 673.7 $[M + Na]^+$.

3.2.6.5. 14-O-1-(4-(2-Methyl-4-allylamino phenyl) acetamide) piperazine mulin (10e). White powdery solid, yield 79.3%. ^{1}H NMR (600 MHz, CDCl$_3$) δ 8.87 (s, 1 H, NHC = O), 7.65 (d, $J = 8.4$ Hz, 1 H, phenyl H-6), 6.49–6.56 (m, 3 H, phenyl H-3, 5 and -NH-), 5.78–5.86 (m, 2 H, -NHCH$_2$CH=CH$_2$ and H-19), 5.30–5.35 (m, 1 H, -NHCH$_2$CH = CH$_2$), 5.14–5.21 (m, 4 H, -NHCH$_2$CH = CH$_2$ and C-11-OH and H-20), 3.88 (d, $J = 4.8$ Hz, 4 H), 3.34–3.37 (m, 1 H), 3.21 (d, $J = 16.8$ Hz, 1 H), 3.15 (s, 2 H), 3.07 (d, $J = 16.8$ Hz, 1 H), 2.47–2.70 (m, 8 H, piperazinyl), 2.32–2.37 (m, 1 H), 2.22–2.25 (m, 1 H), 2.20 (s, 3 H), 2.05–2.09 (m, 2 H), 1.78 (dd, $J = 14.4$, 3.0 Hz, 1 H), 1.63–1.68 (m, 2 H), 1.52–1.59 (m, 1 H), 1.45–1.49 (m, 1 H), 1.44 (s, 3 H), 1.37 (dd, $J = 13.8$, 3.0 Hz, 1 H), 1.28 (d, $J = 16.2$ Hz, 1 H), 1.10–1.18 (m, 4 H), 0.89 (d, $J = 7.2$ Hz, 3 H), 0.73 (d, $J = 7.2$ Hz, 3 H). ^{13}C NMR (150 MHz, CDCl$_3$) δ 217.3 (C-3 C = O), 169.1 (C-21 C = O), 168.0 (C-24 C = O), 146.4, 139.2 (phenyl C-4), 134.1 (-NHCH$_2$CH = CH$_2$), 129.9 (phenyl C-2), 125.2 (phenyl C-1), 123.8 (phenyl C-6), 117.4 (phenyl C-5), 116.1 (phenyl C-3), 114.3 (-NHCH$_2$CH=CH$_2$), 110.9, 74.7, 68.5, 62.0, 59.9, 58.3, 53.4, 53.2, 53.0 (piperazinyl), 45.6 (-NHCH$_2$CH = CH$_2$), 45.2, 44.1, 41.9, 36.9, 36.2, 34.6, 30.6, 27.0, 26.5, 25.0, 18.6, 16.9, 15.0, 11.7. MS (ESI, *m/z*): 649.5 $[M + H]^+$.

3.2.6.6. 14-O-1-(4-(2-Methyl-4-benzylamino phenyl) acetamide) piperazine mulin (10f). White powdery solid, yield 68.3%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.87 (s, 1 H, NHC = O), 7.65 (d, J = 8.5 Hz, 1 H, phenyl H-6), 7.26–7.39 (m, 5 H, phenyl H-3, 5 and -NH- and phenyl H-3', 5') , 6.48–6.54 (m, 3 H, phenyl H-2', 4', 6'), 5.80 (d, J = 8.5 Hz, 1 H, H-19), 5.34 (dd, J = 17.4, 1.2 Hz, 1 H), 5.18–5.21 (m, 1 H), 4.31 (s, 2 H), 3.36 (dd, J = 6.6, 4.2, Hz, 1 H), 3.21 (d, J = 16.8, 1 H), 3.15 (s, 2 H), 3.07 (d, J = 16.8, 1 H), 2.61–2.70 (m, 8 H, piperazinyl), 2.34–2.36 (m, 1 H), 2.18–2.24 (m, 4 H), 2.05–2.10 (m, 2 H), 1.78 (dd, J = 14.4, 2.4 Hz, 1 H), 1.60–1.69 (m, 4 H), 1.53–1.58 (m, 1 H), 1.46–1.48 (m, 2 H), 1.45 (s, 3 H), 1.37 (dd, J = 13.8, 2.4 Hz, 1 H), 1.29 (d, J = 16.2 Hz, 1 H), 1.11–1.17 (m, 4 H), 0.88 (d, J = 7.2 Hz, 3 H), 0.73 (d, J = 7.2 Hz, 3 H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 217.3 (C-3 C = O), 169.1 (C-21 C = O), 168.0 (C-24 C = O), 145.7, 139.6 (phenyl C-1'), 139.2 (phenyl C-4), 130.2 (phenyl C-2), 128.8 (phenyl C-3', 5'), 127.6 (phenyl C-2', 6'), 127.5 (phenyl C-1), 124.0 (phenyl C-6), 117.5 (phenyl C-4'), 114.7 (phenyl C-3), 111.2 (phenyl C-5), 74.7, 68.5, 62.0, 59.9, 58.3, 53.4, 53.2, 48.6, 45.6, 45.2, 44.1, 41.9, 36.9, 36.2, 34.6, 30.6, 27.0, 26.5, 25.0, 18.3, 16.9, 15.1, 11.7. MS (ESI, *m/z*): 699.7 $[M + H]^+$.

3.3. Minimum inhibitory concentrations (MIC) testing

MICs of compounds **8**, **9**, **10a-f** against Gram-positive bacteria (MSSA, MRSA, MSSE, *Staphylococcus suis* and *E. faecalis*) and Gram-negative bacteria (*E. coli*) were investigated using tiamulin fumaric acid and erythromycin sulfur as the reference agents based on the Clinical and Laboratory Standards Institute (CLSI). The tested compounds were dissolved in DMSO (dimethyl sulfoxide), and then diluted with sterile water to make the concentration of stock solutions be 2560 μg/ml. Then, 1 ml of the stock solution was added 9 ml sterile water and diluted to 256 μg/ml as the initial test concentration. The above six bacteria were cultured in Mueller–Hinton Broth (MHB) overnight and cell concentrations of those bacteria were diluted to 10^6–10^7 CFU/ml as the test bacteria solution, respectively. Each well of the 96-well plates was added 100 μl of MHB and 100 μl initial solution was added to the first column to make the concentration of the test compounds be 128 μg/ml. Then 100 μl of the solution in the first column was pipetted into the second column and homogeneously mixed to make the concentration of the test compounds in the second column be 64 μg/ml. And the same two-fold dilution method was employed to adjust the concentrations of the test compounds in columns 3–10 to 32, 16, 8, 4, 2, 1, 0.5 and 0.25 μg/ml, and then each well of the columns 1–10 was added 100 μl of test bacteria solution. Column 11 was added only 200 μl MHB and column 12 was added only 200 μl test bacteria solution. The 96-well plates were cultured at 37 °C for 24 h. The MIC values were defined by the OD values of the lowest concentration which were compared to that of MHB (blank control). All data were tested in parallel triplicate.

3.4. Molecular modeling

Molecular docking study was performed by Molecular Operating Environment (MOE) 2018. 0101 release of Chemical Computing Group Inc., Montreal, Quebec,

Canada to fully understand the interaction between those new derivatives with receptor targets and further study the structure–activity relationship of this series of compounds [19]. The triangle matcher placement method and London δG scoring function were used for evaluation of the binding pattern and binding affinity of the ligands and refinement of the obtained results. The X-ray crystal structure of *Deinococcus radiodurans* with tiamulin (PDB 1 D: 1XBP) was obtained from the Protein Data Bank and was used to construct the initial model [20]. The co-crystallized protein was prepared for docking study by removal of DNA chains, water molecules and ligands, and then the enzyme was prepared using protonate 3 D protocol in MOE with default options. Validation of the docking setup was done by redocking the co-crystallized ligand (tiamulin) in the active site to study the scoring energy(s), root mean standard deviation (RMSD) and amino acid interactions. The validated process was used to predict the binding interactions and affinity of the designed compounds at the active site. Binding score, number and lengths of the hydrogen bonds and amino acid interactions were determined and shown in Table 2.

Disclosure statement

No potential conflict of interest was reported by the authors.

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