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Synthesis and antibacterial activities of novel pleuromutilin derivatives

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Abstract

Pleuromutilin derivatives **4a–h**, **5a–g**, and **6a–d** were synthesized and characterized by IR, ^{1}H NMR, and ^{13}C NMR. All synthetic compounds were screened for their *in vitro* antibacterial activity against *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *S. aureus* (MRSA, ATCC 43300), *Pasteurella multocida* (CVCC 408), *Escherichia coli* (ATCC 25922), and *Salmonella typhimurium* (ATCC 14028). Most compounds with quaternary amine showed higher antibacterial activities against both Gram-positive and Gram-negative bacteria strains. Among the screened compounds, compound **5a** bearing an *N*,*N*,*N*-trimethyl group at the C-14 side chain of pleuromutilin was found to be the most active agent. Furthermore, preliminary molecular docking was performed to predict the binding interaction of the compounds in the binding pocket.

KEYWORDS
antibacterial activity, molecular docking, pleuromutilin derivatives, synthesis

1 | INTRODUCTION

Multidrug resistance bacteria are one of major threats to human health, especially *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, and *Streptococcus pneumonia*.[1] Without ascending the discovery and approval of new antibiotics and introducing new scaffolds with the ability to combat the rapid rise in bacterial resistance to traditional classes of antibiotics, we risk moving back toward the pre-antibiotic era.[2]

Pleuromutilin was isolated from *Pleurotus mutilus* and *P. passeckerianus* in 1951.[3] Its structure includes two parts: the rather rigid 5-6-8 tricyclic carbon skeleton and the C-14 side chain.[4,5] Pleuromutilin derivatives selectively inhibited bacterial protein synthesis through interaction with prokaryotic ribosomes and preventing the peptidyl transferase reaction.[6–8] The tricyclic skeletons of pleuromutilin derivatives orient at the A-site of the peptidyl transferase center (APT) and alter the conformation of U-2506 to tightly close to APT pocket.[9,10] Preliminary studies showed that the modification of pleuromutilin side chain improved its biological activity.[4] Therefore, more researchers focused on the studies of pleuromutilin derivatives and thus resulted in three drugs, tiamulin, valnemulin, and retapamulin (Figure 1).[11] Nabriva's lefamulin received FDA fast-track status to treat community-acquired bacterial pneumonia (CABP) and acute bacterial skin and skin structure (ABSSS).[12,13]

It is well known that thioether and tertiary amine were electron isosteres.[14] In view of this situation and as an extension of our studies

Yunpeng Yi and Yunxing Fu contributed equally to this work.

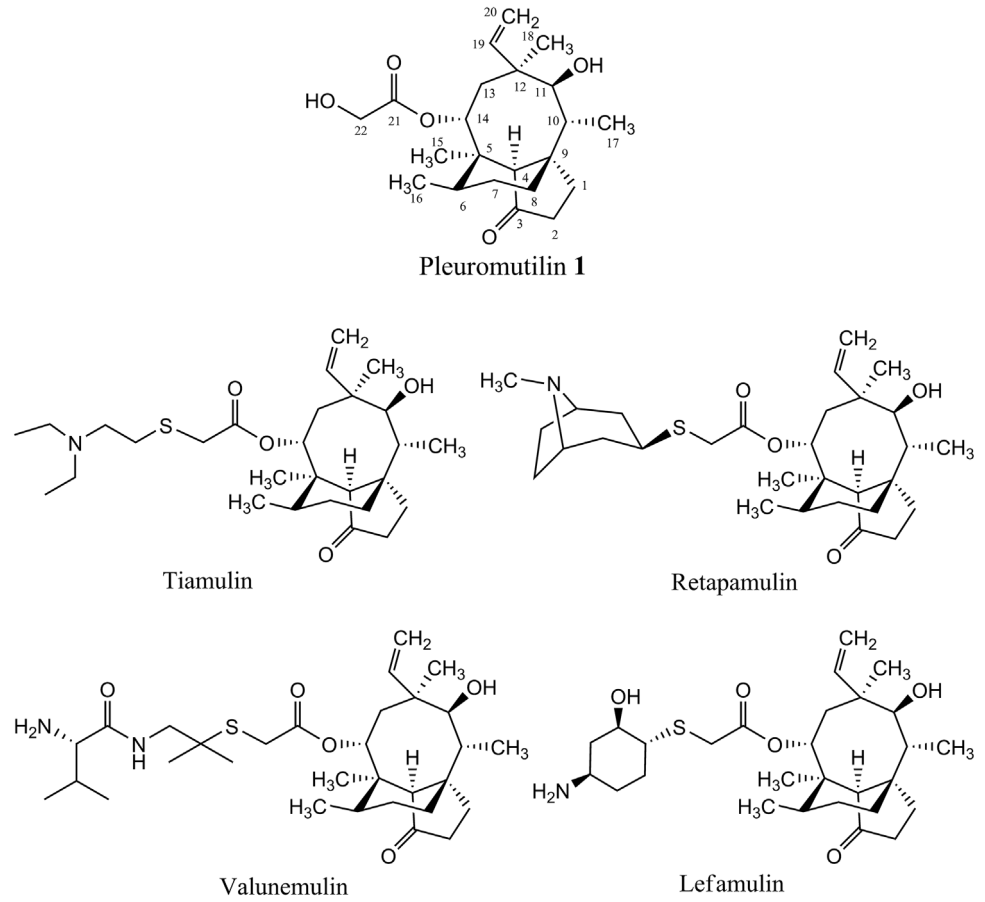


FIGURE 1 Structural formulas of pleuromutilin, tiamulin, valnemulin, retapamulin, and lefamulin

on the development of antimicrobial agents, it is of great interest for us to combine the amine fragment and pleuromutilin to generate a new structural type of potentially antimicrobial agents. In the current study, a series of novel pleuromutilin analogues was designed and synthesized to introduce different amine groups, tertiary amine groups, and N-containing heterocycles in the C-14 side chain. Their antibacterial activities *in vitro* were evaluated, as well as docking studies were also performed to clarify the antibacterial mechanism.

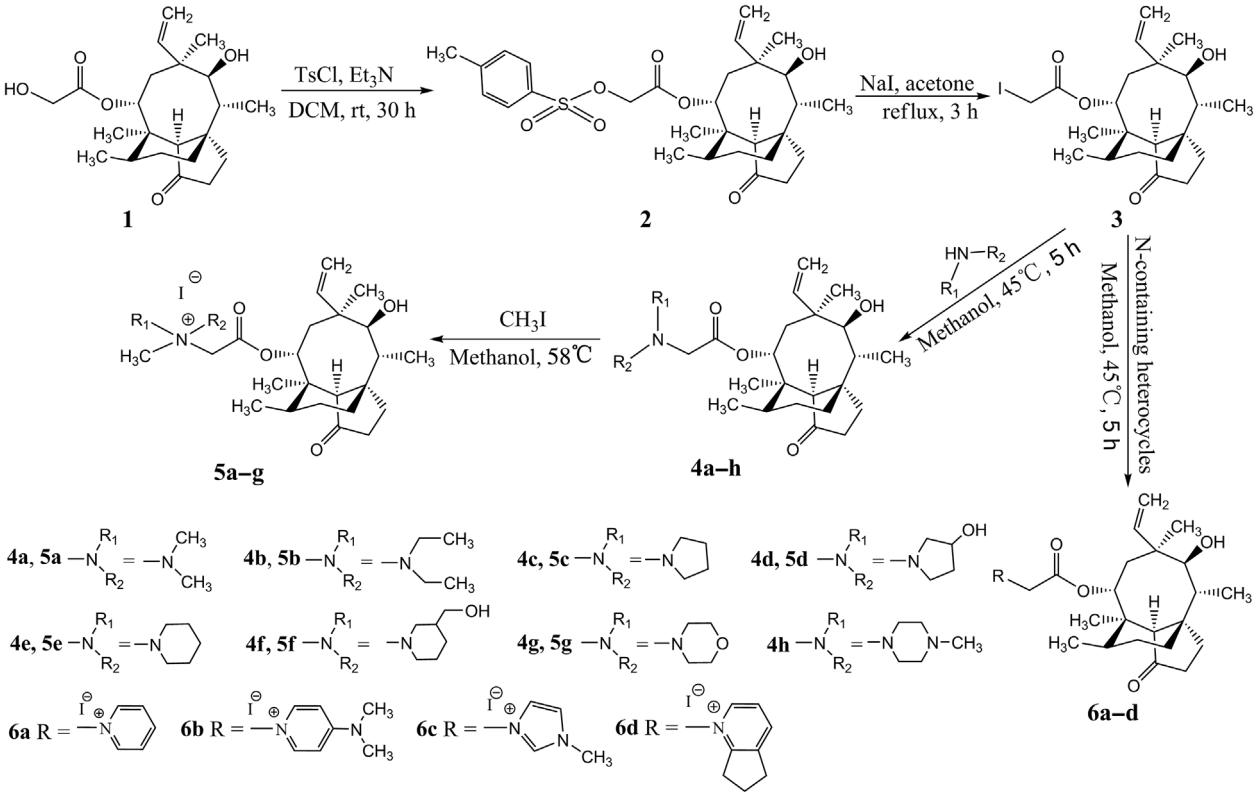
2 | RESULTS AND DISCUSSION

2.1 | Chemistry

The semisynthesis of pleuromutilin derivatives was performed as outlined in Scheme 1. In general, the target compounds were obtained via 22-*O*-tosylpleuromutilin **2**, a key intermediate for synthesizing almost all pleuromutilin derivatives. Compound **3** was prepared by nucleophilic substitution of **2** with sodium iodide in acetone reaction. Analogues **4a–h** were obtained from the reaction of iodide group of compound **3** and the secondary amines. Compound **4h** has been reported by Luo and coworkers.[15] Here we synthesized these compounds again for the supplements to compounds **4a–g** and compared their antibacterial activities. Further treatment of **4a–g** with iodomethane gave the target compounds **5a–g** with yield of 47–82%. Compounds **6a–d** were directly obtained from **3** and various heterocyclic compounds containing nitrogen atom in dry methanol. The structures of the synthesized derivatives were characterized by IR, ^{1}H NMR, ^{13}C NMR, and HRMS spectra (Supporting Information). For further confirming the structure and conformation, a crystal of compound **5a** was obtained as a yellow, block-like from a solution of methanol and acetone by slow evaporation method at room temperature (Figure 2). Data were deposited in data bank as CCDC 1827079.

2.2 | Bioactivity

The *in vitro* antibacterial activities of **4a–h**, **5a–g**, **6a–d**, tiamulin fumarate, and 5% DMSO (control group) against *S. aureus* (ATCC 25923), methicillin-resistant *S. aureus* (MRSA, ATCC 43300), *Pasteurella multocida* (*P. multocida*, CVCC 408), *Escherichia coli* (*E. coli*, ATCC 25922), and *Salmonella typhimurium* (*S. typhimurium*, ATCC 14028) were determined and the results are presented in Table 1. Most of the compounds exhibited middle antibacterial efficiency against *S. aureus*, MRSA, with minimum inhibitory concentrations (MIC) ranging from 0.125 to 8 μg/mL, except **5e** and **5g**. Three compounds **5a**, **5c**, and **5d** showed higher antibacterial activity than



SCHEME 1 Synthesis of pleuromutilin derivatives **4a–h**, **5a–g**, and **6a–d**

their corresponding analogues **4a**, **4c**, and **4d**. Compound **5a** displayed lower MIC values for MRSA, *P. multocida* and *E. coli* than tiamulin fumarate. However, these compounds showed comparatively lower antibacterial activities for *S. typhimurium* and *E. coli*, with MIC values ranging from 8 to >64 μg/mL, respectively. Because DMSO was demonstrated to inhibit growth of some bacteria,[16] we also determined the MIC of 0.5% DMSO which was used as solvent to dissolve all the synthesized compounds. However, the 0.5% DMSO showed no antibacterial activities against the five tested strains (MIC values >5000 μg/mL).

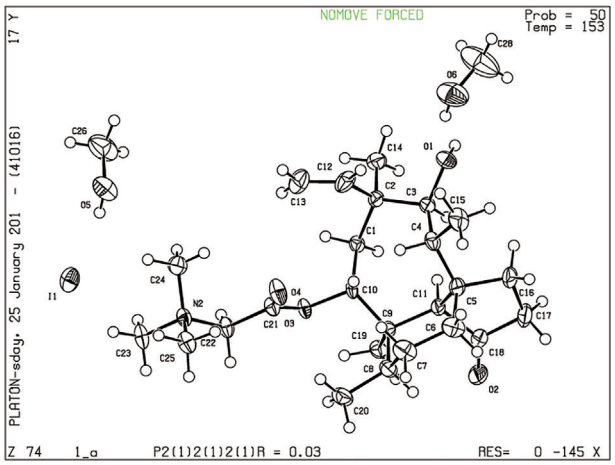


FIGURE 2 Crystal structure of compound **5a**

Compound **5a** displayed promising antibacterial activity and therefore was further evaluated for *in vitro* time-kill assay.[17–19] The bactericidal properties of **5a** were compared at 1 × MIC and 6 × MIC against *S. aureus* and *P. multocida*. Compound **5a** displayed a concentration-dependent effect with faster killing kinetics at higher concentrations (Figure 3). Although 1 × MIC of compound **5a** and tiamulin fumarate slowed bacterial propagation, the 6 × MIC of compound **5a** achieved 3-$\log_{10}$ reduction in 2–4 h. At 1 × MIC, **5a** showed more rapid bactericidal kinetics against MRSA and *P. multocida* than tiamulin fumarate.

The cytotoxic potencies of quaternary ammonium salts **5a–g** and tiamulin fumarate were evaluated on BRL-3A cells. The IC_{50} values (the concentration at which 50% of the cell are viable) of tested compounds were calculated via viability percentages of cell (Table 1). The IC_{50}s for the tested compounds were 164–276 μg/mL, which was significantly higher than that of tiamulin fumarate (31 μg/mL). These results indicated that the synthesized quaternary ammonium salts **5a–g** showed the lower cell toxicity than the reference drug tiamulin fumarate.

2.3 | Molecular docking studies

The most potent compound **5a** was employed to investigate the binding modes to the peptidyl transferase center (PTC) domain of ribosome by molecular docking experiment. The optimal conformation of the compound obtained from all conformations in the docking study in the active site of PTC (Figure 4A). The red areas showed

TABLE 1 Antibacterial activity and the IC_{50} of the synthesized pleuromutilin derivatives

	MIC (μg/mL)					
Compounds	MRSA	*S. aureus*	*P. multocida*	*E. coli*	*S. typhimurium*	IC_{50} (μg/mL)
4a	0.25	0.5	16	8	32	–
4b	8	8	64	32	64	–
4c	2	2	4	64	16	–
4d	0.25	0.25	4	16	16	–
4e	4	4	64	64	32	–
4f	0.125	0.125	16	8	16	–
4g	0.5	0.5	4	16	32	–
4h	2	1	4	16	16	–
5a	0.25	0.125	0.25	8	16	276
5b	8	4	16	32	32	250
5c	0.125	0.25	4	8	16	249
5d	0. 25	0.125	2	16	32	164
5e	16	16	2	64	>64	174
5f	1	1	1	16	8	229
5g	16	16	4	>64	16	198
6a	1	2	8	16	32	–
6b	0.25	0.25	1	16	16	–
6c	2	2	32	64	64	–
6d	1	0.5	32	32	64	–
Tiamulin	0.25	0.125	2	32	16	31
DMSO	>5000	>5000	>5000	>5000	>5000	–

lipotropy or hydrophobicity and blue areas showed hydrophobicity. A comparison of optimal conformation of compounds **5a**, **5b**, and **5f** displayed that the steric hindrance of side chain end hinders the interaction between compound **5b** and **5f** and PTC (Figure 4B), which may explain the decreases of the antibacterial activities of compounds **5b** and **5f**. The C-14 side chain extended to the pocket and the mutilin ring was wrapped by G-2530, G-2531, G-2532, U-2533, and C-2479 (Figure 4C). The carbonyl in C-14 side chain formed hydrogen bonding with purine ring of G-2088. Furthermore, the C-11 hydroxyl closed to C-2532. The docking results showed that **5a** was generally accommodated in the active pocket of the receptor and entered the bound region of PTC.

3 | CONCLUSIONS

In summary, a series of novel pleuromutilin derivatives with amine groups, tertiary amine groups, and N-containing heterocycles at their

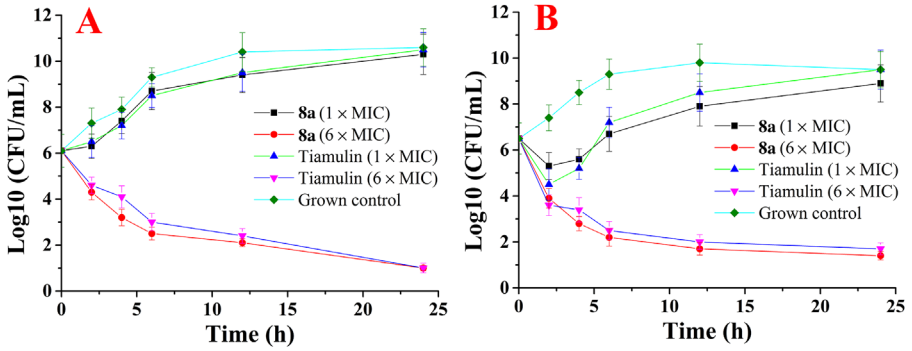


FIGURE 3 Time-kill kinetics of compound **5a** against *S. aureus* (A) and *P. multocida* (B). Mean values of the CFU/mL (colony forming units per milliliter) were obtained from measurements taken in triplicate

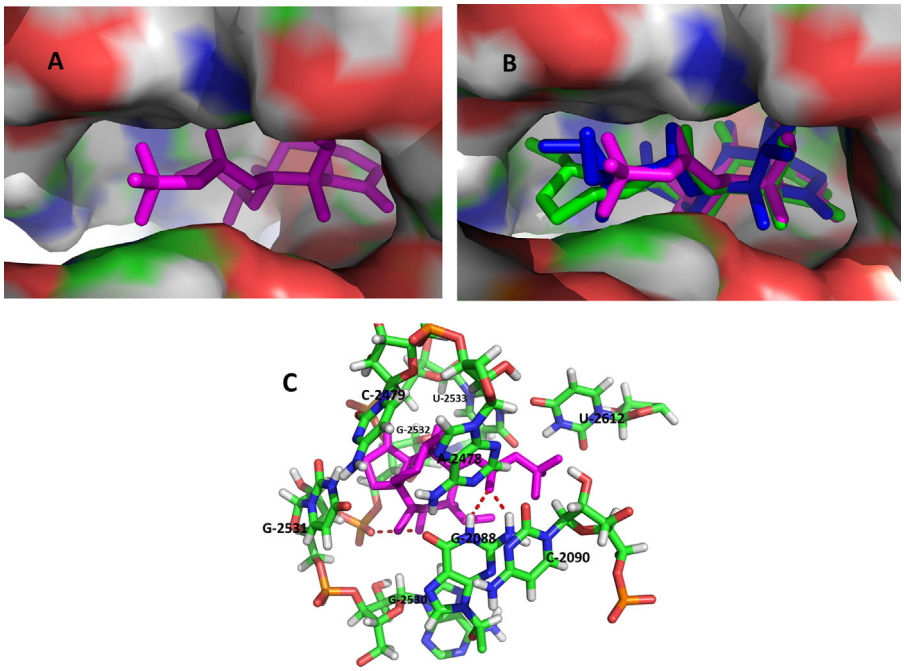


FIGURE 4 (A) The best pose of **5a** (magenta) obtained from the docking study in the active site of the peptidyl transferase center PTC. (B) **5a** (magenta), **5b** (blue), and **5f** (green) in 5HL7. (C) Docking mode of the **5a** into 5HL7. Important residues are depicted as drawn in sticks and in different color. Hydrogen bonds are showed as dashed red lines

C-14 side chain were prepared and evaluated for their antibacterial activities against *S. aureus*, MRSA, *P. multocida*, *E. coli*, and *S. typhimurium*. Most compounds with quaternary amine showed higher antibacterial activities against both Gram-positive and Gram-negative bacteria strains. Compound **5a** displayed excellent inhibition activity against *S. aureus*, MRSA, and *P. multocida*. Furthermore, molecular docking study of **5a** was carried out to understand the molecular interaction of compounds with PTC. These results suggested that compound **5a** may be a potent therapeutic agent for antibacterial treatment.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All the materials and solvents used for the isolation/purification of the compounds were purchased from commercial suppliers and without further purification. 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 bismuth nitrate solution and 0.05% $KMnO_4$ aqueous solution. Chromatographic purification was carried out on silica gel columns (60 Å, 200–300 mesh). 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 by 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.

The original spectra of the investigated compounds are provided as Supporting Information. The InChI codes of the investigated compounds together with some biological activity data are also provided as Supporting Information.

4.1.2 | Synthesis of compound 2

A solution of 4.25 g of pleuromutilin and 2.15 g of 4-toluenesulfonyl chloride in 15 mL of CH_2Cl_2 at 10–15°C was treated with 2.02 g triethylamine, maintaining a temperature <25°C. The resulting off-white suspension was heated to reflux for 20 h and the reaction was followed until completion determined by HPLC. Upon reaction completion the mixture obtained was cooled to 20–30°C, diluted with 52 mL of water, stirred at 15–25°C for 10 min, and the organic layers obtained were separated. The organic phase obtained was washed several times with 52 mL saturated NH_4Cl. The organic layer obtained was concentrated under vacuum. The solid was added 50 mL hexane and stirred overnight. The filter cake obtained was washed with 15 mL of heptane and pulled dry on the filter. The solid was dried under vacuum at <40°C for at least 12 h. It was used in the next step without further purification. Yield: 93%. IR (KBr): 3446, 2924, 2863, 1732, 1633, 1597, 1456, 1371, 1297, 1233, 1117, 1035, 832, 664, 560 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, J = 8.3 Hz, 2H), 7.26

(t, J = 13.3 Hz, 2H), 6.34 (dd, J = 17.4, 11.0 Hz, 1H), 5.70 (d, J = 8.5 Hz, 1H), 5.19 (dd, J = 55.1, 14.2 Hz, 2H), 4.47–4.33 (m, 2H), 3.28 (s, 1H), 2.38 (s, 3H), 2.25–2.09 (m, 3H), 2.01 (s, 1H), 1.99–1.88 (m, 1H), 1.71–1.62 (m, 1H), 1.61–1.51 (m, 2H), 1.46–1.38 (m, 2H), 1.35 (s, J = 7.8 Hz, 3H), 1.27 (d, J = 11.5 Hz, 1H), 1.18 (d, J = 11.6, 4.5 Hz, 2H), 1.12–1.00 (m, 4H), 0.80 (d, J = 7.0 Hz, 3H), 0.55 (d, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 215.71, 163.87, 144.29, 137.70, 131.63, 128.91, 127.09, 116.38, 73.54, 69.29, 64.03, 57.02, 44.39, 43.51, 42.97, 40.84, 35.54, 35.03, 33.40, 29.34, 25.77, 25.39, 23.81, 20.68, 15.53, 13.76, 10.47. HRMS (ESI) calcd. $[M+H]^+$ for $C_{29}H_{40}O_7S$ 533.2501. Found 533.2507.

4.1.3 | Synthesis of compound 3

A solution of 5.25 g of compound **2** and 5 mL of acetone was charged into a vessel and to the obtained mixture 1.49 g of sodium iodide was added. The mixture solution was heated to reflux and stirred for 3 h. The solution was evaporated to 15 mL. The solution was cooled to 20–30°C and filtered. Filter residue was washed with 15 mL water. The solid was dried at 35°C. The obtained crude residue was purified by column chromatography (petroleum ether/ethyl acetate = 1:6–1:1 v/v) to afford the desired compound **3** in 72% yield as a white solid. IR (KBr): 3307, 2979, 2933, 1736, 1712, 1456, 1417, 1279, 1084, 1023, 985, 967 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (s, 1H), 6.41 (dd, J = 17.4, 11.0 Hz, 1H), 5.70 (d, J = 8.5 Hz, 1H), 5.34 (d, J = 11.0 Hz, 1H), 5.20 (dd, J = 17.4, 1.1 Hz, 1H), 3.65 (d, J = 10.4 Hz, 1H), 3.57 (d, J = 10.5 Hz, 1H), 3.35 (s, 1H), 2.36–2.28 (m, 1H), 2.26–2.14 (m, 2H), 2.07 (dd, J = 16.0, 8.5 Hz, 2H), 1.79–1.60 (m, 4H), 1.57–1.47 (m, 2H), 1.47–1.42 (m, 4H), 1.35 (dd, J = 26.4, 9.7 Hz, 2H), 1.17 (s,3H), 1.12 (d, J = 4.3 Hz, 1H), 0.87 (d, J = 7.0 Hz, 3H), 0.73 (d, J = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.76, 166.88, 138.57, 117.28, 74.71, 70.49, 58.20, 45.48, 44.20, 43.93, 41.98, 36.71, 35.86, 34.31, 30.10, 26.78, 26.42, 24.80, 16.75, 14.78, 11.46. HRMS (ESI): $[M+NH_4]^+$ calcd. for $C_{22}H_{33}IO_4$, 506.1762. Found 506.1758.

4.1.4 | General procedure for the synthesis of compounds 4a–h

To a solution of compound **3** (0.488 g, 1 mmol) in 10 mL methanol, amine (3 mmol) was added and stirred for 5–8 h at room temperature and evaporated under reduced pressure to dryness. The crude product was extracted with a solution of ethyl acetate (30 mL) and water (10 mL) and treated with saturated $NaHCO_3$. The target compound **4a–h** was then precipitated and purified by flash silica column chromatography (petroleum ether/ethyl acetate = 1:10–1:3 v/v).

Compound 4a

Compound **4a** was prepared according to the general procedure from compound **3** and dimethylamine. The crude product was purified over silica gel column chromatography to give 0.315 g (78%). IR (KBr): 3254, 2986, 2928, 2880, 2859, 2827, 1736, 1471, 1448, 1283, 1205, 1165, 1151, 1120, 1062, 1041 cm^{-1}. ^{1}H NMR (400 MHz, d-DMSO) δ 6.18 (dd, J = 18.2, 10.7 Hz, 1H), 5.60 (d, J = 8.1 Hz, 1H), 5.07 (d, J = 3.8 Hz, 1H), 5.04 (s, 1H), 4.51 (d, J = 6.1 Hz, 1H), 3.43 (t, J = 6.0 Hz, 1H), 3.04 (d, J = 59.9 Hz, 2H), 2.41 (s, 1H), 2.21 (s, 6H), 2.11 (d, J = 7.6 Hz, 4H), 1.77–1.57 (m, 2H), 1.48 (t, J = 9.4 Hz, 1H), 1.44 (d, J = 13.3 Hz, 1H), 1.35 (s, 3H), 1.30–1.21 (m, 3H), 1.06 (s, 4H), 0.83 (d, J = 6.9 Hz, 3H), 0.62 (d, J = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, d-DMSO) δ 217.71, 169.36, 141.48, 115.54, 73.11, 68.59, 60.68, 57.80, 45.46, 45.02, 44.60, 44.52, 41.89, 36.92, 36.82, 34.47, 30.63, 28.99, 27.04, 24.99, 16.38, 15.08, 12.03. HRMS (ESI) calcd. $[M+H]^+$ for $C_{24}H_{39}NO_4$ 406.2951. Found 406.2959.

Compound 4b

Compound **4b** was prepared according to the general procedure from compound **3** and diethylamine. The crude product was purified over silica gel column chromatography to give 0.398 g (91%). IR (KBr): 3448, 2980, 2955, 2881, 2863, 1734, 1459, 1215, 1195, 1121 cm^{-1}. ^{1}H NMR (400 MHz, d-DMSO) δ 6.18 (dd, J = 17.4, 11.5 Hz, 1H), 5.59 (d, J = 8.1 Hz, 1H), 5.07 (d, J = 6.3 Hz, 1H), 5.03 (s, 1H), 4.51 (d, J = 5.7 Hz, 1H), 3.43 (t, J = 5.9 Hz, 1H), 3.18 (d, J = 28.3 Hz, 2H), 2.56 (dd, J = 7.1, 4.0 Hz, 4H), 2.40 (s, 1H), 2.11 (d, J = 8.8 Hz, 4H), 1.65 (dd, J = 20.7, 11.8 Hz, 2H), 1.55–1.44 (m, 2H), 1.44–1.38 (m, 1H), 1.36 (s, 3H), 1.27 (d, J = 15.4 Hz, 3H), 1.06 (s, 3H), 0.99 (s, 1H), 0.93 (t, J = 7.1 Hz, 5H), 0.83 (d, J = 6.9 Hz, 3H), 0.63 (d, J = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, d-DMSO) δ 217.67, 170.14, 141.52, 115.47, 73.13, 68.57, 57.84, 54.49, 47.31, 45.46, 44.65, 44.50, 41.90, 36.94, 36.85, 34.47, 30.64, 29.04, 27.02, 25.00, 16.37, 15.09, 12.88, 12.03. HRMS (ESI) calcd. $[M+H]^+$ for $C_{26}H_{43}NO_4$ 434.3264. Found 434.3268.

Compound 4c

Compound **4c** was prepared according to the general procedure from compound **3** and pyrrolidine. The crude product was purified over silica gel column chromatography to give 0.354 g (82%). IR (KBr): 3240, 2982, 2954, 2927, 2881, 2859, 1737, 1466, 1452, 1427, 1237, 1211, 1188, 1152, 1117, 1040, 977, 951, 939, 925, 913, 880, 867 cm^{-1}. ^{1}H NMR (400 MHz, d-DMSO) δ 6.26–6.09 (m, 1H), 5.60 (d, J = 8.1 Hz, 1H), 5.11–5.06 (m, 1H), 5.03 (s, 1H), 4.51 (d, J = 6.1 Hz, 1H), 3.43 (s, 1H), 3.18 (dd, J = 93.4, 16.9 Hz, 2H), 2.49 (dd, J = 7.1, 5.0 Hz, 3H), 2.40 (s, 1H), 2.11 (d, J = 8.2 Hz, 4H), 1.67 (s, 6H), 1.49 (d, J = 4.2 Hz, 3H), 1.35 (s, 3H), 1.30–1.20 (m, 3H), 1.06 (s, 4H), 0.83 (d, J = 6.9 Hz, 3H), 0.62 (d, J = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, d-DMSO) δ 217.64, 169.36, 141.43, 115.53, 73.12, 68.62, 57.81, 57.23, 53.48, 45.46, 44.51, 41.89, 40.63, 36.93, 36.82, 34.47, 30.64, 28.99, 27.03, 24.99, 23.91, 16.37, 15.05, 12.02. HRMS (ESI) calcd. $[M+H]^+$ for $C_{26}H_{41}NO_4$ 432.3108. Found 432.3115.

Compound 4d

Compound **4d** was prepared according to the general procedure from compound **3** and 3-pyrrolidinol. The crude product was purified over silica gel column chromatography to give 0.356 g (80%). IR (KBr): 3483, 2987, 2935, 2860, 1735, 1458, 1203, 1156, 1117 cm^{-1}. ^{1}H NMR (400 MHz, d-DMSO) δ 6.18 (dd, J = 17.5, 11.4 Hz, 1H), 5.58 (d, J = 8.0 Hz, 1H), 5.08 (d, J = 7.6 Hz, 1H), 5.05 (s, 1H), 4.59 (d, J = 4.9 Hz, 1H), 4.51 (d, J = 6.0 Hz, 1H), 3.42 (d, J = 5.0 Hz, 2H), 3.19 (dd, J = 17.0, 7.2 Hz, 1H), 3.00 (dd, J = 17.0, 7.6 Hz, 1H), 2.89–2.73 (m, 1H), 2.61 (dd,

J = 19.2, 10.9 Hz, 1H), 2.40 (s, 1H), 2.09 (d, *J* = 8.7 Hz, 5H), 1.93 (d, *J* = 14.6 Hz, 1H), 1.77 (d, *J* = 9.1 Hz, 1H), 1.66 (s, 1H), 1.59 (s, 2H), 1.48 (s, 2H), 1.35 (s, 3H), 1.28 (s, 3H), 1.06 (s, 4H), 0.83 (d, *J* = 6.8 Hz, 3H), 0.63 (d, *J* = 5.9 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.67, 169.29, 141.46, 115.63, 73.12, 68.62, 66.43, 60.97, 59.77, 57.79, 52.56, 45.46, 44.50, 41.89, 36.90, 36.83, 34.48, 33.50, 30.63, 28.98, 27.04, 24.98, 23.71, 16.46, 15.08, 12.02. HRMS (ESI) calcd. $[M+H]^+$ for $C_{26}H_{41}NO_5$ 448.3057. Found 448.3063.

Compound 4e

Compound **4e** was prepared according to the general procedure from compound **3** and piperidine. The crude product was purified over silica gel column chromatography to give 0.369 g (83%). IR (KBr): 3448, 2988, 2955, 2936, 2860, 1736, 1205 cm^{-1}. ^{1}H NMR (400 MHz, *d*-DMSO) δ 6.18 (dd, *J* = 17.4, 11.4 Hz, 1H), 5.58 (d, *J* = 8.1 Hz, 1H), 5.08 (d, *J* = 6.3 Hz, 1H), 5.04 (s, 1H), 4.51 (d, *J* = 6.1 Hz, 1H), 3.43 (t, *J* = 5.9 Hz, 1H), 3.04 (dd, *J* = 75.4, 16.8 Hz, 2H), 2.48–2.33 (m, 5H), 2.11 (d, *J* = 7.8 Hz, 4H), 1.65 (d, *J* = 6.7 Hz, 2H), 1.46 (d, *J* = 5.7 Hz, 5H), 1.39 (d, *J* = 21.1 Hz, 2H), 1.35 (s, 3H), 1.31 (d, *J* = 7.2 Hz, 1H), 1.25 (d, *J* = 15.6 Hz, 3H), 1.06 (s, 4H), 0.83 (d, *J* = 6.9 Hz, 3H), 0.62 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.69, 169.29, 141.49, 115.58, 73.12, 68.58, 60.53, 57.79, 53.74, 45.46, 44.61, 44.48, 41.89, 36.89, 36.83, 34.48, 30.63, 28.96, 27.03, 25.95, 24.97, 24.09, 16.44, 15.06, 12.02. HRMS (ESI) calcd. $[M+H]^+$ for $C_{27}H_{43}NO_4$ 446.3264. Found 446.3268.

Compound 4f

Compound **4f** was prepared according to the general procedure from compound **3** and 3-piperidinemethanol. The crude product was purified over silica gel column chromatography to give 0.323 g (68%). IR (KBr): 3414, 2951, 1731, 1655, 1637, 1459, 1420, 1370, 1239, 1181, 1155, 1119, 1021, 941, 911 cm^{-1}. ^{1}H NMR (400 MHz, *d*-DMSO) δ 6.12 (dd, *J* = 17.7, 11.3 Hz, 1H), 5.56 (dd, *J* = 55.0, 5.3 Hz, 2H), 5.10 (d, *J* = 4.6 Hz, 2H), 4.57 (dd, *J* = 58.5, 16.9 Hz, 3H), 4.01 (s, 1H), 3.69–3.48 (m, 2H), 3.31 (s, 5H), 3.17 (s, 1H), 2.47 (s, 1H), 2.16 (dd, *J* = 24.5, 9.2 Hz, 2H), 2.05 (d, *J* = 7.6 Hz, 2H), 1.83 (d, *J* = 42.3 Hz, 2H), 1.66 (s, 2H), 1.56 (d, *J* = 29.9 Hz, 1H), 1.49 (d, *J* = 15.8 Hz, 3H), 1.38 (s, 3H), 1.29 (d, *J* = 11.4 Hz, 2H), 1.09 (s, 4H), 0.86 (d, *J* = 6.6 Hz, 3H), 0.66 (d, *J* = 6.3 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.44, 164.14, 141.51, 115.78, 72.87, 71.79, 64.62, 61.60, 61.29, 57.58, 51.09, 49.07, 45.39, 44.93, 43.56, 42.02, 37.24, 36.65, 34.44, 30.57, 29.84, 29.55, 26.99, 24.96, 16.92, 16.48, 14.94, 12.13. HRMS (ESI) calcd. $[M+H]^+$ for $C_{28}H_{45}NO_5$ 476.3770. Found 476.3765.

Compound 4g

Compound **4g** was prepared according to the general procedure from compound **3** and morpholine. The crude product was purified over silica gel column chromatography to give 0.379 g (85%). IR (KBr): 3449, 2957, 2931, 2869, 1735, 1452, 1298, 1285, 1215, 1163, 1117, 1034, 1018, 915, 871 cm^{-1}. ^{1}H NMR (400 MHz, *d*-DMSO) δ 5.93 (dd, *J* = 17.1, 11.7 Hz, 1H), 5.35 (d, *J* = 8.1 Hz, 1H), 4.89–4.82 (m, 1H), 4.80 (s, 1H), 4.26 (d, *J* = 6.0 Hz, 1H), 3.31 (t, *J* = 4.5 Hz, 4H), 3.18 (s, 1H), 2.96 (d, *J* = 17.0 Hz, 1H), 2.76 (d, *J* = 17.0 Hz, 1H), 2.27–2.15 (m, 5H), 1.96–1.71 (m, 4H), 1.51–1.32 (m, 2H), 1.24 (dd, *J* = 12.6, 7.5 Hz, 2H), 1.11 (s, 3H), 1.02 (d, *J* = 15.9 Hz, 3H), 0.82 (s, 4H), 0.59 (d, *J* = 6.9 Hz, 3H), 0.38 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.62, 168.92, 141.44, 115.60, 73.11, 68.76, 66.58, 59.81, 57.77, 52.93, 45.45, 44.55, 44.51, 41.89, 36.90, 36.81, 34.47, 30.62, 28.96, 27.04, 24.97, 16.46, 15.04, 12.02. HRMS (ESI) calcd. $[M+H]^+$ for $C_{26}H_{41}NO_5$ 448.3057. Found 448.3051.

Compound 4h

Compound **4h** was prepared according to the general procedure from compound **3** and 4-methyl piperazine. The crude product was purified over silica gel column chromatography to give 0.423 g (92%). IR (KBr): 3374, 2961, 2935, 2864, 2792, 2765, 1731, 1454, 1414, 1376, 1324, 1310, 1287, 1213, 1192, 1172, 1155, 1130, 1115, 1080, 1015, 990, 975, 939, 917 cm^{-1}. ^{1}H NMR (400 MHz, *d*-DMSO) δ 6.18 (dd, *J* = 17.4, 11.4 Hz, 1H), 5.58 (d, *J* = 8.1 Hz, 1H), 5.07 (dd, *J* = 12.7, 6.4 Hz, 2H), 4.51 (d, *J* = 6.1 Hz, 1H), 3.43 (t, *J* = 5.9 Hz, 1H), 3.18 (d, *J* = 16.9 Hz, 1H), 2.98 (d, *J* = 16.9 Hz, 1H), 2.46 (s, 3H), 2.40 (s, 1H), 2.28 (s, 3H), 2.18 (s, 1H), 2.18–2.07 (m, 6H), 2.06 (s, 1H), 1.65 (dd, *J* = 18.6, 12.9 Hz, 2H), 1.49 (s, 1H), 1.46 (d, *J* = 12.7 Hz, 1H), 1.41 (s, 1H), 1.35 (s, 3H), 1.26 (d, *J* = 15.8 Hz, 3H), 1.06 (s, 4H), 0.83 (d, *J* = 6.9 Hz, 3H), 0.62 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.66, 169.10, 141.46, 115.60, 73.11, 68.70, 59.65, 57.78, 55.08, 52.44, 46.24, 45.45, 44.58, 44.49, 41.89, 36.89, 36.81, 34.47, 30.62, 28.96, 27.04, 24.97, 16.47, 15.06, 12.02. HRMS (ESI) calcd. $[M+H]^+$ for $C_{27}H_{44}N_2O_4$ 461.3374. Found 461.3367.

4.1.5 | General procedure for synthesis of compounds 5a–g

A mixture of **4a–g** (1 mmol) and iodomethane (1.2 mmol) in methanol (3 mL) was stirred at 50°C in a closed bottle. The reaction was stirred for 3–5 h. The progress of the reaction was checked by using TLC. Upon completion, the reaction bottle was cooled to 0 to −20°C and filtered. The solid residue was further purified by recrystallization using acetone to afford compounds **5a–h** in 47–82% yield.

Compound 5a

Compound **5a** was prepared according to the general procedure from compound **4a** and iodomethane. The crude product was purified over silica gel column chromatography to give as a white solid (0.256 g, 47%). IR (KBr): 3448, 2991, 2948, 1733, 1460, 1446, 1407, 1387, 1369, 1259, 1224, 1212, 1157, 1140, 1123, 1063, 1002, 973 cm^{-1}. ^{1}H NMR (400 MHz, *d*-DMSO) δ 6.13 (dd, *J* = 17.4, 11.6 Hz, 1H), 5.64 (d, *J* = 8.0 Hz, 1H), 5.11 (s, 1H), 5.07 (d, *J* = 6.8 Hz, 1H), 4.65–4.43 (m, 3H), 3.45 (t, *J* = 5.8 Hz, 1H), 3.24 (s, 9H), 2.48 (s, 1H), 2.24–1.99 (m, 4H), 1.73–1.59 (m, 2H), 1.52 (dd, *J* = 7.1, 3.2 Hz, 1H), 1.49–1.39 (m, 2H), 1.35 (s, 3H), 1.29 (d, *J* = 11.4 Hz, 2H), 1.13–0.96 (m, 4H), 0.85 (d, *J* = 6.8 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.40, 164.26, 141.44, 115.84, 72.86, 71.67, 63.17, 57.55, 53.46, 45.39, 44.90, 43.64, 41.99, 37.20, 36.61, 34.44, 30.57, 29.48, 27.00, 24.94, 16.55, 14.86, 12.11. HRMS (ESI) calcd. $[M]^+$ for $C_{25}H_{42}NO_4$ 420.3108. Found 420.3101.

Compound 5b

Compound **5b** was prepared according to the general procedure from compound **4b** and iodomethane. The crude product was purified over silica gel column chromatography to give as a white solid (0.472 g, 82%). IR (KBr): 3447, 2984, 2945, 1731, 1458, 1409, 1238, 1121, 1023 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 6.20–6.06 (m, 1H), 5.63 (d, *J* = 8.0 Hz, 1H), 5.11 (s, 1H), 5.08 (d, *J* = 2.8 Hz, 1H), 4.63 (d, *J* = 5.8 Hz, 1H), 4.44 (dd, *J* = 48.3, 17.6 Hz, 2H), 3.51 (dd, *J* = 23.9, 14.3 Hz, 5H), 3.10 (s, 3H), 2.48 (s, 1H), 2.20 (d, *J* = 9.3 Hz, 2H), 2.14–1.99 (m, 2H), 1.67 (s, 2H), 1.48 (d, *J* = 16.0 Hz, 2H), 1.39 (s, 4H), 1.29 (d, *J* = 13.0 Hz, 2H), 1.23 (dd, *J* = 11.7, 7.0 Hz, 6H), 1.10 (s, 4H), 0.86 (d, *J* = 6.8 Hz, 3H), 0.66 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.40, 164.25, 141.55, 115.78, 72.89, 71.76, 58.20, 57.54, 57.39, 47.97, 45.38, 44.85, 43.81, 41.99, 37.13, 36.62, 34.44, 30.57, 29.40, 27.02, 24.92, 16.54, 14.92, 12.10, 8.15. HRMS (ESI) calcd. $[M]^+$ for $C_{27}H_{46}NO_4$ 448.3421. Found 448.3426.

Compound 5c

Compound **5c** was prepared according to the general procedure from compound **4c** and iodomethane. The crude product was purified over silica gel column chromatography to give as a white solid (0.368 g, 64%). IR (KBr) 3530, 2949, 1742, 1717, 1458, 1231, 1209, 1126, 1013 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 6.12 (dd, *J* = 18.1, 10.9 Hz, 1H), 5.63 (d, *J* = 8.0 Hz, 1H), 5.20–4.96 (m, 2H), 4.74 (dt, *J* = 21.6, 10.9 Hz, 1H), 4.59 (d, *J* = 17.0 Hz, 2H), 4.08–3.82 (m, 4H), 3.68 (dd, *J* = 18.8, 13.4 Hz, 1H), 3.56 (dd, *J* = 29.4, 15.8 Hz, 3H), 3.45 (d, *J* = 5.3 Hz, 1H), 3.33 (s, 3H), 2.48 (s, 1H), 2.27–2.12 (m, 2H), 2.12–2.06 (m, 1H), 2.06–2.00 (m, 1H), 1.73–1.59 (m, 2H), 1.50 (d, *J* = 15.9 Hz, 2H), 1.48–1.40 (m, 1H), 1.36 (s, 3H), 1.33–1.26 (m, 2H), 1.12–0.98 (m, 4H), 0.85 (d, *J* = 6.8 Hz, 3H), 0.67 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.41, 163.97, 141.48, 115.86, 72.87, 71.93, 61.78, 60.51, 60.10, 57.53, 47.53, 45.39, 44.93, 43.55, 42.02, 37.21, 36.63, 34.44, 30.55, 29.52, 27.01, 24.94, 16.56, 14.91, 12.12. HRMS (ESI) calcd. $[M]^+$ for $C_{27}H_{44}NO_4$ 446.3215. Found 446.3221.

Compound 5d

Compound **5d** was prepared according to the general procedure from compound **4d** and iodomethane. The crude product was purified over silica gel column chromatography to give as a yellow solid (0.326 g, 55%). IR (KBr): 3630, 3316, 2985, 2960, 2920, 2862, 1732, 1458, 1422, 1407, 1259, 1226, 1171, 1155, 1124, 1091, 1053, 1016, 941, 913 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 6.13 (dd, *J* = 17.7, 11.3 Hz, 1H), 5.64 (dd, *J* = 10.3, 5.8 Hz, 2H), 5.16–5.03 (m, 2H), 4.66–4.61 (m, 1H), 4.60–4.46 (m, 3H), 3.90–3.80 (m, 1H), 3.72 (dd, *J* = 12.4, 5.1 Hz, 1H), 3.60 (d, *J* = 12.1 Hz, 2H), 3.49–3.37 (m, 2H), 3.32 (s, 1H), 2.47 (s, 2H), 2.24–2.01 (m, 5H), 1.64 (d, *J* = 13.3 Hz, 2H), 1.49 (s, 2H), 1.45 (s, 1H), 1.37 (s, 3H), 1.30 (t, *J* = 8.9 Hz, 2H), 1.28 (s, 1H), 1.09 (s, 4H), 0.85 (d, *J* = 6.8 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.44, 164.66, 141.43, 115.87, 72.88, 72.58, 71.77, 68.74, 65.65, 63.78, 57.51, 52.36, 45.39, 44.91, 43.53, 42.00, 37.15, 36.61, 34.44, 32.83, 30.53, 29.45, 27.02, 24.93, 16.58, 14.85, 12.08. HRMS (ESI) calcd. $[M]^+$ for $C_{27}H_{44}NO_5$ 462.3214. Found 462.3217.

Compound 5e

Compound **5e** was prepared according to the general procedure from compound **4e** and iodomethane. The crude product was purified over silica gel column chromatography to give yellow solid (0.423 g, 72%). IR (KBr): 3462, 3393, 3023, 3001, 2985, 2974, 2943, 2874, 1734, 1474, 1458, 1227, 1197, 1121, 1021, 986, 938, 911 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 6.22–6.01 (m, 1H), 5.63 (d, *J* = 8.0 Hz, 1H), 5.09 (dd, *J* = 14.5, 1.9 Hz, 2H), 4.64 (d, *J* = 17.1 Hz, 2H), 4.45 (d, *J* = 17.3 Hz, 1H), 3.70–3.61 (m, 1H), 3.54 (d, *J* = 4.1 Hz, 2H), 3.45 (s, 2H), 3.21 (s, 3H), 2.47 (s, 1H), 2.26–2.01 (m, 4H), 1.91–1.76 (m, 4H), 1.66 (s, 2H), 1.53 (s, 3H), 1.48 (d, *J* = 11.6 Hz, 1H), 1.41 (d, *J* = 9.7 Hz, 1H), 1.39 (s, 3H), 1.30 (t, *J* = 10.4 Hz, 2H), 1.09 (s, 4H), 0.86 (d, *J* = 6.8 Hz, 3H), 0.66 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.42, 164.19, 141.49, 115.81, 72.88, 71.71, 61.60, 60.28, 57.59, 48.59, 45.39, 44.93, 43.58, 42.02, 37.23, 36.65, 34.45, 30.59, 29.54, 27.00, 24.95, 21.04, 19.58, 16.50, 14.95, 12.13. HRMS (ESI) calcd. $[M]^+$ for $C_{28}H_{46}NO_4$ 460.3421. Found 460.3428.

Compound 5f

Compound **5f** was prepared according to the general procedure from compound **4f** and iodomethane. The crude product was purified over silica gel column chromatography to give yellow solid (0.361 g, 59%). IR (KBr): 3422, 2926, 1730, 1458, 1240, 1222, 1022, 912 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 6.11 (dd, *J* = 17.3, 11.2 Hz, 1H), 5.62 (d, *J* = 6.3 Hz, 1H), 5.47 (s, 1H), 5.09 (d, *J* = 12.7 Hz, 2H), 4.62 (s, 2H), 4.47 (d, *J* = 17.6 Hz, 1H), 4.10–3.97 (m, 1H), 3.53 (s, 3H), 3.45 (s, 2H), 3.35 (s, 2H), 3.24 (d, *J* = 7.4 Hz, 2H), 3.16 (d, *J* = 4.1 Hz, 1H), 2.47 (s, 1H), 2.28–1.94 (m, 5H), 1.80 (s, 2H), 1.66 (s, 2H), 1.49 (s, 3H), 1.37 (s, 3H), 1.29 (s, 3H), 1.08 (s, 4H), 0.85 (d, *J* = 5.4 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.41, 164.11, 141.48, 115.83, 72.87, 71.77, 64.58, 63.86, 61.59, 60.98, 57.59, 51.04, 49.72, 49.07, 45.38, 44.94, 43.58, 41.98, 37.24, 36.65, 34.45, 30.60, 29.56, 27.00, 24.95, 17.00, 16.52, 14.95, 12.14. HRMS (ESI) calcd. $[M]^+$ for $C_{29}H_{48}NO_5$ 490.3527. Found 490.3529.

Compound 5g

Compound **5g** was prepared according to the general procedure from compound **4g** and iodomethane. The crude product was purified over silica gel column chromatography to give white solid (0.412 g, 70%). IR (KBr): 3537, 2983, 2960, 2949, 2876, 1742, 1717, 1458, 1406, 1231, 1209, 1153, 1126, 1012, 998 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 6.13 (dd, *J* = 17.8, 11.2 Hz, 1H), 5.63 (d, *J* = 8.1 Hz, 1H), 5.22–4.98 (m, 2H), 4.59 (dd, *J* = 46.0, 17.2 Hz, 3H), 3.82–3.61 (m, 3H), 3.48 (dd, *J* = 19.8, 8.2 Hz, 3H), 3.17 (d, *J* = 1.4 Hz, 3H), 2.48 (s, 1H), 2.31–2.05 (m, 7H), 2.04 (s, 1H), 1.66 (s, 2H), 1.50 (d, *J* = 15.9 Hz, 2H), 1.37 (s, 3H), 1.30 (s, 2H), 1.09 (s, 4H), 0.85 (d, *J* = 6.8 Hz, 3H), 0.66 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.41, 164.68, 141.45, 115.87, 72.89, 71.74, 65.62, 65.12, 62.40, 57.55, 49.43, 45.39, 44.92, 42.00, 37.18, 36.62, 34.45, 30.57, 29.48, 27.02, 24.93, 21.70, 16.58, 14.89, 12.10. HRMS (ESI) calcd. $[M]^+$ for $C_{27}H_{44}NO_5$ 434.3265. Found 434.3274.

4.1.6 | General procedure for synthesis of compounds 6a–d

A mixture of **3** (1 mmol) and heteroaromatic compounds with nitrogen atom (1.2 mmol) in methanol (3 mL) was stirred at 60°C. The reaction was stirred for 3 to 4 h. The progress of the reaction was checked by using TLC. Upon completion, the reaction bottle was cooled to 0 to −20°C and filtered. The solid residue was further purified by recrystallization using methanol to afford compound **6a–d** in 60–89% yield.

Compound 6a

Compound **6a** was prepared according to the general procedure from compound **3** and pyridine. The crude product was purified over silica gel column chromatography to give as a white solid (0.502 g, 89%). IR (KBr): 3505, 3012, 2935, 2858, 1747, 1721, 1637, 1485, 1466, 1371, 1237, 1211, 1158, 1123, 1018, 934, 914, 677 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 9.09 (d, *J* = 5.7 Hz, 2H), 8.77 (t, *J* = 7.7 Hz, 1H), 8.31 (t, *J* = 7.0 Hz, 2H), 6.11 (dd, *J* = 17.7, 11.3 Hz, 1H), 5.86–5.67 (m, 2H), 5.57 (d, *J* = 8.0 Hz, 1H), 5.12 (dd, *J* = 59.5, 14.5 Hz, 2H), 4.55 (s, 1H), 3.45 (s, 1H), 2.46 (s, 1H), 2.21 (dd, *J* = 18.7, 11.0 Hz, 1H), 2.07 (dt, *J* = 16.8, 8.6 Hz, 2H), 2.00 (dd, *J* = 12.5, 6.2 Hz, 1H), 1.62 (t, *J* = 10.6 Hz, 2H), 1.56 (d, *J* = 15.9 Hz, 1H), 1.48 (s, 1H), 1.35 (s, 3H), 1.15 (d, *J* = 15.9 Hz, 3H), 1.08 (d, *J* = 25.9 Hz, 3H), 0.99 (t, *J* = 11.1 Hz, 1H), 0.81 (d, *J* = 6.6 Hz, 3H), 0.61 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.34, 164.81, 147.40, 146.55, 141.25, 128.62, 116.10, 72.91, 72.71, 61.30, 57.41, 45.36, 44.81, 43.30, 41.96, 36.90, 36.61, 34.46, 30.50, 29.15, 27.01, 24.83, 17.03, 14.52, 12.03. HRMS (ESI) calcd. $[M]^+$ for $C_{27}H_{38}NO_4$, 440.2795. Found 440.2804.

Compound 6b

Compound **6b** was prepared according to the general procedure from compound **3** and 4-dimethylaminopyridine. The crude product was purified over silica gel column chromatography to give a blue solid (0.439 g, 72%). IR (KBr): 3387, 2923, 2860, 1730, 1655, 1575, 1457, 1403, 1369, 1248, 1211, 1181, 1153, 1120, 1032, 903 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 8.20 (d, *J* = 7.6 Hz, 2H), 7.12 (d, *J* = 7.6 Hz, 2H), 6.11 (dd, *J* = 17.8, 11.2 Hz, 1H), 5.56 (d, *J* = 8.2 Hz, 1H), 5.16 (dd, *J* = 23.5, 5.3 Hz, 3H), 5.04 (dd, *J* = 11.2, 1.2 Hz, 1H), 4.55 (d, *J* = 5.9 Hz, 1H), 3.50–3.37 (m, 1H), 3.28 (d, *J* = 44.5 Hz, 6H), 3.16 (d, *J* = 4.8 Hz, 1H), 2.44 (s, 1H), 2.26–1.92 (m, 4H), 1.62 (dd, *J* = 18.6, 12.7 Hz, 2H), 1.48 (d, *J* = 15.6 Hz, 2H), 1.27 (s, 2H), 1.24 (s, 3H), 1.09 (s, 4H), 0.81 (d, *J* = 6.8 Hz, 3H), 0.61 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.44, 166.28, 156.48, 143.36, 141.25, 115.98, 108.01, 72.93, 71.80, 57.59, 57.52, 49.08, 45.38, 44.77, 43.50, 41.95, 36.93, 36.68, 34.47, 30.53, 29.17, 27.01, 24.87, 16.93, 14.73, 12.05. HRMS (ESI) calcd. $[M]^+$ for $C_{29}H_{43}N_2O_4$ 483.3217. Found 483.3224.

Compound 6c

Compound **6c** was prepared according to the general procedure from compound **3** and iodomethane. The crude product was purified over silica gel column chromatography to give as a white solid (0.461 g, 81%). IR (KBr): 3512, 3094, 3041, 1751, 1723, 1562, 1459, 1374, 1271, 1229, 1202, 1170, 1123, 1093, 1035, 1019, 997, 983, 935, 915, 864, 739, 620 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 9.18 (s, 1H), 7.76 (dd, *J* = 20.2, 1.4 Hz, 2H), 6.12 (dd, *J* = 17.5, 11.3 Hz, 1H), 5.57 (d, *J* = 7.0 Hz, 1H), 5.18 (s, 4H), 4.54 (s, 1H), 3.95 (s, 3H), 3.46 (s, 1H), 3.17 (s, 1H), 2.47 (s, 1H), 2.26–1.96 (m, 4H), 1.64 (d, *J* = 7.3 Hz, 2H), 1.48 (d, *J* = 15.6 Hz, 2H), 1.40–1.31 (m, 1H), 1.29 (s, 3H), 1.13 (s, 1H), 1.10 (s, 4H), 0.82 (d, *J* = 5.8 Hz, 3H), 0.63 (d, *J* = 6.2 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.42, 165.63, 141.23, 138.07, 124.11, 124.02, 116.01, 72.92, 71.89, 57.51, 50.56, 49.07, 45.38, 44.74, 43.52, 41.98, 36.92, 36.65, 34.47, 30.55, 29.13, 27.02, 24.88, 16.87, 14.68, 12.05. HRMS (ESI) calcd. $[M]^+$ for $C_{26}H_{39}N_2O_4$ 443.2904. Found 443.2912.

Compound 6d

Compound **6d** was prepared according to the general procedure from compound **3** and iodomethane. The crude product was purified over silica gel column chromatography to give as a white solid (0.403 g, 66%). IR (KBr): 3424, 2986, 2921, 1724, 1617, 1457, 1431, 1363, 1338, 1280, 1235, 1150, 1119, 1016, 978, 911 cm^{-1}. ^1H NMR (400 MHz, *d*-DMSO) δ 8.86 (d, *J* = 6.1 Hz, 1H), 8.58 (d, *J* = 7.7 Hz, 1H), 8.05 (t, *J* = 6.9 Hz, 1H), 6.10 (dd, *J* = 17.8, 11.2 Hz, 1H), 5.77 (q, *J* = 17.5 Hz, 2H), 5.60 (d, *J* = 8.0 Hz, 1H), 5.11 (dd, *J* = 50.0, 14.5 Hz, 2H), 4.54 (d, *J* = 5.9 Hz, 1H), 3.49 (t, *J* = 5.3 Hz, 1H), 3.21 (d, *J* = 6.9 Hz, 5H), 2.33–2.14 (m, 4H), 2.01 (d, *J* = 6.6 Hz, 2H), 1.73–1.61 (m, 2H), 1.60–1.46 (m, 2H), 1.32 (s, 4H), 1.27 (d, *J* = 11.0 Hz, 2H), 1.13 (s, 4H), 0.82 (d, *J* = 6.7 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, *d*-DMSO) δ 217.27, 164.79, 162.52, 145.56, 143.33, 142.67, 141.39, 126.24, 115.93, 72.89, 59.31, 57.47, 49.09, 45.34, 44.80, 43.74, 42.00, 36.89, 36.58, 34.47, 32.25, 31.21, 30.57, 29.15, 27.02, 24.83, 22.65, 16.80, 14.71, 12.04. HRMS (ESI) calcd. $[M]^+$ for $C_{30}H_{42}NO_4$ 480.3108. Found 480.3116.

4.2 | Biological evaluation

4.2.1 | MIC testing

The antibacterial activity was determined by broth microdilution[13] against *S. aureus*, MRSA, *P. multocida*, *E.coli*, *S. typhimurium*. Stock solutions of compounds were prepared in 0.5% DMSO. Tiamulin fumarate as reference drugs were dissolved in distilled water directly. The compounds as well as 0.5% DMSO used as control were added to the test tube and serially diluted in Mueller–Hinton broth (the final concentration is 0.0625 μg/mL). The bacteria were cultivated and added to the tube. The initial concentration of bacteria cannot be lower than 10^5 CFU/mL. The broth was incubated at 36.7°C for 18–24 h. MICs were read when the change of clarity in the broth was observed in the control test tube.

4.2.2 | Bactericidal time-kill kinetics

The time-kill kinetics were determined for MRSA and *P. multocida*.[17–19] The bacteria were prepared in Mueller–Hinton broth at 37°C for 6 h with shaking, followed by dilution to a final concentration of

1×10^6 cfu/mL in 50 mL fresh MHB. Compound **5a** and tiamulin fumarate were diluted to one- and sixfold MIC concentration, respectively, and added to the bacterial suspension. After specified time intervals (0, 2, 4, 6, 12, and 24 h), 20 mL aliquots were serially diluted in 0.9% saline, plated on sterile Mueller–Hinton agar plates, and incubated at 37°C for 24 h. The same procedure was repeated in triplicate.

4.2.3 | Cytotoxicity assay

The determination of intrinsic toxicity of compounds **5a–g** was conducted as described in the literature with minor modifications.[20,21] Briefly, 100 µL/mL of medium containing BRL-3A cells at a density of 2×10^5 mature cells/mL were seeded in each well of a flat-bottom 96-well plate. Cells were permitted to adhere to the plate for 48 h. Then media were replaced with 100 µL/mL of serial dilutions of the tested compounds and tiamulin fumarate (used as reference drug), and incubated for 16 h. After that, all media in cells were removed and 110 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reagent was added, followed by incubation at 37°C for 4 h. Then the resulting formazan crystals were dissolved by adding MTT solubilizing solution equal to the original culture medium volume. DMSO was used as the positive control and wells were left with no cells for the negative control. The absorbance of each well was determined by a spectrophotometer at dual wavelengths of 490 nm for the background. The viability percentage was calculated by the following formula: cell viability% = $(OD_{sample} - OD_{blank}/OD_{control} - OD_{blank}) \times 100$. The concentration providing 50% inhibition (IC_{50}) was calculated from a graph plotting inhibition percentage against different APTM, retapamulin and tiamulin fumarate concentration. The measurements were repeated at least three times.

4.2.4 | Molecular modeling studies

Molecular docking studies were performed using the Homdock from Chil[2] package (University of Pittsburgh, Pittsburgh, USA, 0.99). The package contains a graph-based molecular alignment (GMA) tool and a Monte-Carlo/Simulated Annealing (MC/SA) algorithm-based docking (GlamDock) (University of Pittsburgh company, Pittsburgh, USA, 0.99) tool. In the current study, we used the inactive conformation co-crystallized of 50S ribosomal subunit with lefamulin as inhibitor (PDB ID: 5HL7[9]) which was downloaded from the Protein Data Bank and prepared for docking. Dock binding affinities of those compounds were evaluated according to many parameters including: the binding free energies (ΔG_b, kcal/mol), hydrogen bonding or other noncovalent molecular interaction, and RMSD values in comparison to the native co-crystallized ligand. The lowest binding free energies and the lowest RMSD values were considered as the best fitted ones. Lefamulin was the template for flexible molecular alignment, and the interaction was optimized by GlamDock according to the ChillScore scoring function based on ChemScore with a smooth, improved potential. All the compounds were prepared with Avogadro software,[22] including a 5000 steps Steepest Descent and 1000 steps Conjugate Gradients geometry optimization based on the MMFF94 force field. Initially, the docking protocol was validated by docking of the co-crystallized ligand lefamulin in the PTC domain of ribosome. All compounds were compared to original conformation of 5HL7, which was kept for binding affinity comparison. The receptor grid (center *x*, *y*, and *z* was 17.0, 77.9, and −2.6) could be set up and generated from the site of lefamulin. As a result of calculations, we obtained the output files of the acceptor-ligand complex with flexible residues. The similarity of docked structures was measured by computing the RMSD between the coordinates of the atoms. All the compounds were superposed to lefamulin by the GMA, and the placement of compounds were optimized by a MC/SA algorithm in Glamdock according to ChillScore. Hydrogen bonds and other interactions were detected by PoseView. ChillScore is the evaluation standard of ligand-receptor binding. Hydrogen bonds and other interactions were detected and generated by PyMol 1.5.03.[23]

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Samples of compounds **2**, **3**, **4a–h**, **5a–g**, and **6a–d** are available as supplementary materials.

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