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Original article

Synthesis and antibacterial evaluation of novel pleuromutilin derivatives

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ABSTRACT

A series of novel pleuromutilin derivatives possessing thioether moiety has been synthesized *via* acylation reaction under mild conditions. Their *in vitro* antibacterial activity against methicillin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus epidermidis*, *Escherichia coli*, and *Streptococcus agalactiae* were tested by agar dilution method and Oxford cup assay. Among the 17 compounds screened, 14-O-[(4-methoxybenzamide-2- methylpropane-2-yl) thioacetate] mutilin 4i, 14-O-[(2-aminobenzamide-2-methylpropane-2-yl) thioacetate] mutilin 5a and 14-O-[(4-aminobenzamide-2-methylpropane-2-yl) thioacetate] mutilin 5c were resulted as most active antibacterial agents.

1. Introduction

The abuse of antibiotics has made more pathogenic bacteria resistance to drugs, which leads to many available drugs reducing or losing curative effect. Drug-resistance bacteria, especially the *Staphylococcus aureus*, *Staphylococcus pneumoniae*, *Mycobacterium tuberculosis*, etc. kill more than two million people each year and endanger human health seriously [1]. Pleuromutilin and its derivatives (Fig. 1) display high activities against drug-resistant Gram-positive bacteria and mycoplasmas *in vitro* and *in vivo* [2], pharmacodynamic properties [3] and no target-specific cross-resistance to other antibiotics [4].

Pleuromutilin was first isolated in a crystalline form from cultures of two species of basidiomycetes, *Pleurotus mutilus* and *P. passeckerianus* in 1951 [5]. Pleuromutilin is a diterpene, constituted of a rather rigid 5–6–8 tricyclic carbon skeleton with eight stereogenic centers [6,7]. The molecular modifications of pleuromutilin were focused essentially on the C-14 glycolic acid chain. Research reported that modifications of this moiety with a thioether group and a basic group together offered the best bioactivities [8]. Further alterations within this group led to the development of three drugs: tiamulin, valnemulin, and retapamulin (Fig. 1). The first two drugs were used as veterinary medicines. And the retapamulin became the first pleuromutilin approved for human use in 2007. Besides retapamulin, BC-3781, BC-3205 and BC-7013 (Fig. 1) are developing for human use [9,10]. BC-3781 is especially currently under a clinical phase II for complicated skin and skin structure infections and severe respiratory infections by the biopharmaceutical company Nabriva Therapeutics [9,11]. Another primary modification of pleuromutilin was the synthesis of tricyclic carbon core or its analogs in order to improve pharmacokinetic properties [12]. According to structure characteristics of the core, 5–6 fused bicyclic framework was constructed first. Then a condensation reaction was employed to form the pleuromutilin core or its analogs [12–14].

Further studies have shown that tiamulin, valnemulin and others pleuromutilin derivatives interfered with bacterial protein synthesis *via* a specific interaction with the 23S rRNA of the 50S bacterial ribosome subunit [15–17]. The domain V of 23S rRNA at the peptidyl transferase center (PTC) is mutilins derivatives binding site, in which the tricyclic core of the pleuromutilin is positioned in a pocket close to the A-tRNA binding site, whereas the C-14 extension points toward the P-tRNA binding site [11]. Thus these compounds prevent the correct positioning of the tRNAs for peptide transfer, and inhibit the peptidyl transferase [2,18].

Structure activity relationship (SAR) studies show that the presence of thioether group at C-22 position of pleuromutilin

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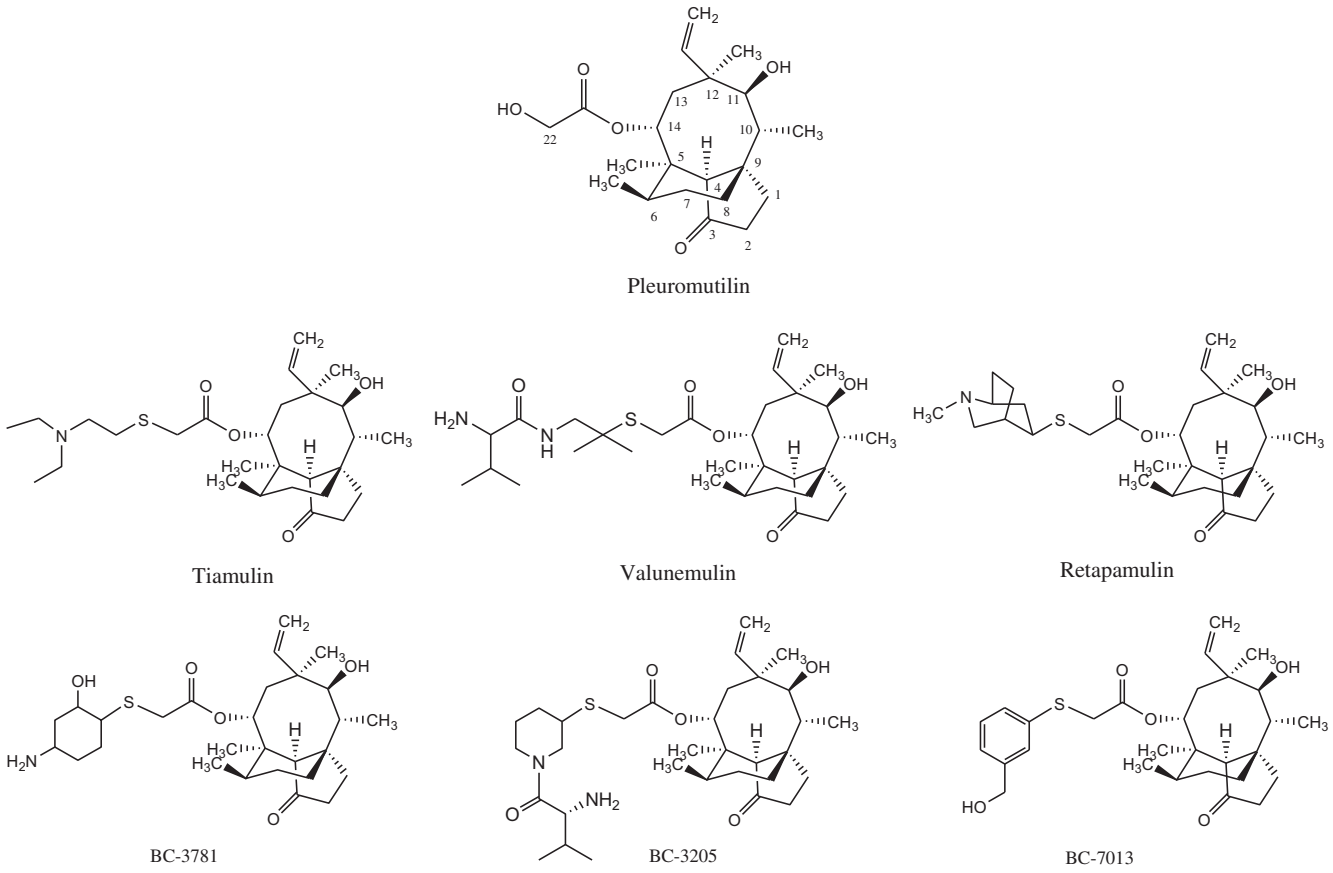


Fig. 1. Structures of pleuromutilin and its derivative drugs.

enhances antibacterial activity. The thioether group moiety is key to their pharmacological properties, especially with side chain [11,19]. For example, antibacterial activity of valnemulin containing dimethyl propane moiety is more effective than that of tiamulin *in vitro* as well *in vivo* [20]. However related analogs of valnemulin are still scarce. A serial of new pleuromutilin derivatives (**4a**–**i**, **5a**–**f**, **6a**–**b**) were synthesized based on the structure of valnemulin. After bacteriostatic test *in vitro*, we demonstrated an attempt to identify novel lead structure for antibiotics against some Gram-positive bacteria, such as methicillin-resistant *S. aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), *Escherichia coli* (*E. coli*), and *Streptococcus agalactiae* (*S. agalactiae*).

2. Results and discussion

2.1. Chemistry

All pleuromutilin derivatives were synthesized by acylation reaction starting from 14-O-[(1-Amino-2-methylpropan-2-yl) thioacetyl] mutilin 3, which was synthesized with 22-O-tosylpleuromutilin 2 and dimethylcysteamine-hydrochloride [21] according to the pathway reported in Scheme 1.

Compound **4a**–**i** was obtained in two steps: First, the correlative carboxylic acids were converted into acyl chlorides with $SOCl_2$ in the refluxing condition and excess $SOCl_2$ was evaporated. Then the acyl chloride was directly reacted with compound **3** to form amide **4a**–**i**. Compound **5a**–**f** was obtained from amino acid derivative which amino-group was protected by tert-butoxycarbonyl (BOC). Condensation reaction between the amino-group of compound **3** and carboxyl group of amino acid derivative was carried out in the presence of N,N′-dicyclohexylcarbodiimide (DCC) and 1-Hydroxybenzotriazole (HOBt) which is used to suppress racemization and improve the efficiency of amide synthesis, followed by the treatment of trifluoroacetic acid (TFA) for 30 min. Then the compound **5a**–**f** was obtained. The reaction of compound **3** with chloroformate in the presence of triethylamine resulted in the compound **6a**–**b**.

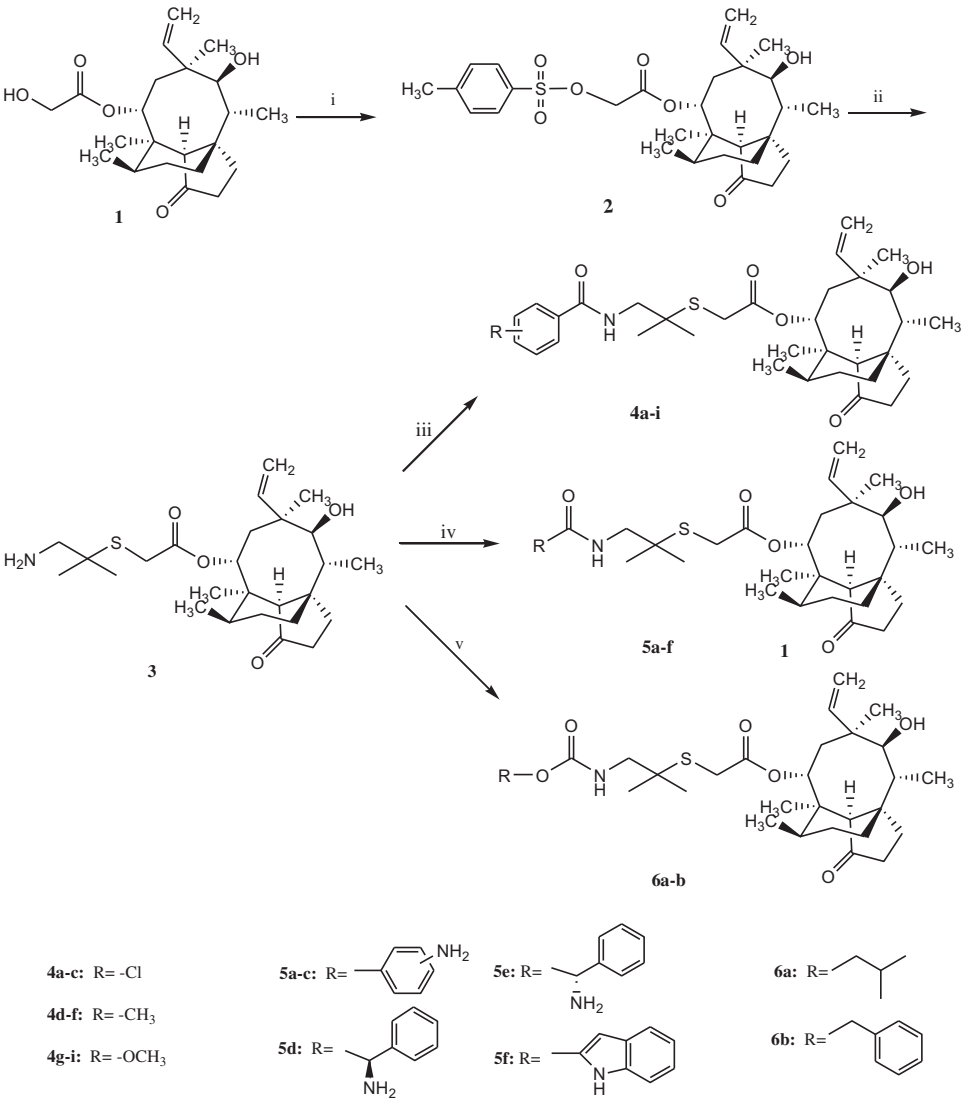
All the formed amides were treated by distilled water and saturated $NaHCO_3$ or/and NH_4Cl washing, followed by purification with column chromatography. All derivatives were fully characterized by means of IR, ^1H NMR and ^{13}C NMR spectral analysis.

Compound **3** is the most important intermediate. This compound was synthesized in strong alkaline environment but was not stable in such conditions for the carbonyl group at C21 position might be attacked by the terminal amine, giving six-membered ring amide [22]. So the controls of reaction time and temperature were very necessary. The compound **3** was purified by column chromatography according to some reports [22,23]. But we obtained this compound with good yield and convenience by recrystallization with ethyl acetate.

Compounds **4a**–**i** also can be obtained by the condensation method with N,N′-dicyclohexylcarbodiimide (DCC) as condensation agent. But the by-product 1,3-dicyclohexylurea (DCU) which chemical polarity is close to the target compounds is very difficult to remove by purification with column chromatography.

2.2. Biological evaluation

All the synthesized pleuromutilin derivatives **4a**–**i**, **5a**–**f**, and **6a**–**b** were screened for their *in vitro* antibacterial activity against



Scheme 1. Reagent and condition: (i) TsCl, NaOH, H_2O, t-butyl methyl ether, reflux, 1 h; (ii) $HSC(CH_3)_2CH_2NH_2 \cdot HCl$, EtONa, EtOH, 0 °C, 2.5 h; (iii) aromatic acyl chloride, $N(CH_2CH_3)_3$, CH_2Cl_2, 0 °C, 3 h; (iv) amino acid derivative, DCC, HOBt, CH_2Cl_2, rt, 15h, then TFA, CH_2Cl_2, rt, 30 min; (v): Chloroformate, $N(CH_2CH_3)_3$, CH_2Cl_2, 0 °C or rt, 4.5 h.

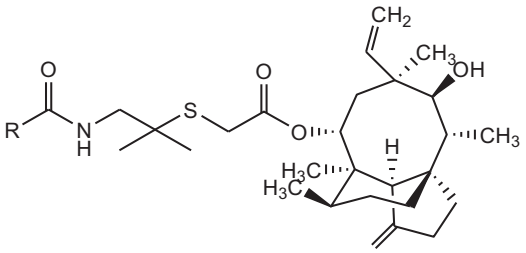
MRSA, MRSE, *E. coli*, and *S. agalactia* by agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS). Minimum inhibitory concentration (MIC) is defined as the minimum concentration of the compound required to completely inhibit the bacterial growth. The determination of MIC values was performed in triplicate at pH 7.40. The MICs of the synthesized compounds **4a**–**i**, **5a**–**f**, and **6a**–**b** along with tiamulin and valnemulin which were used as reference drugs are depicted in Table 1. The MICs of 17 new pleuromutilin derivatives *in vitro* against MRSA, MRSE, *E. coli*, and *S. agalactia* ranged from 8 to 0.25 μg/mL, 32 to 0.5 μg/mL, 32 to 1 μg/mL, and 32 to 0.5 μg/mL respectively.

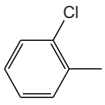
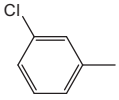
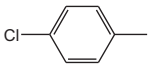
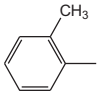
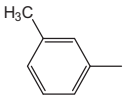
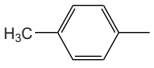
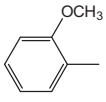
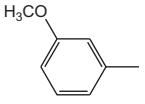
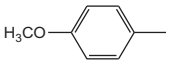
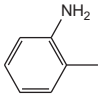
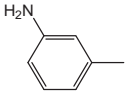
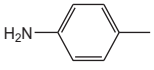
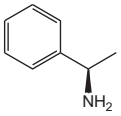
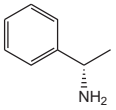
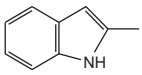
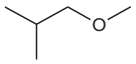
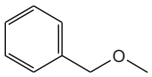
Antibacterial activity for all the synthesized compounds was evaluated against the above mentioned four bacterial strains. Oxford cup assay was carried out and the zones of inhibition for different concentrations of the synthetic compounds were measured and the results are given in Table 2. Also tiamulin and valnemulin were used as reference drugs.

Among all the pleuromutilin derivatives examined, three compounds **4i**, **5a**, **5c** showed good antibacterial activity, while the others showed moderate antibacterial activity as indicated by MIC values and the zones of inhibition. Compounds **4i**, **5a** and **5c** inhibited the growth of pathogen particularly MRSA and MRSE, even better than the reference drug tiamulin at the same concentration. However, all the synthesized compounds showed lower antibacterial activity than valnemulin. From MIC values and the zones of inhibition it was observed that the substituents, especially chlorine substituent, in the benzene ring of ortho para showed stronger antibacterial activity than that in the benzene ring of meta. Also it was observed that the stronger conjugation effect of substituents, the more excellent antibacterial activity. For example, compounds **4g**–**i** and **5a**–**c** showed stronger antibacterial activity than compound **4a**–**c**.

The compounds **4a**, **5a**, **5c**, **6a** and **6b** have been reported by Zhang and coworkers [22–24]. Here we synthesized these compounds again for the supplements to compounds **4b**, **4c** and **5b** and compared their antibacterial activities. Furthermore we supplemented their ^{13}C NMR spectrum data and MIC except **6b** which was reported its MIC for different bacterial strains.

Table 1
Antibacterial activity of the novel pleuromutilin derivatives.



Compound no.	R	MIC (μg/mL)			
		MRSA	MRSE	*E. coli*	*S. agalactia*
4a		0.5	1	4	8
4b		8	16	8	32
4c		1	0.5	2	4
4d		0.25	16	2	0.5
4e		1	16	8	2
4f		0.25	16	4	0.5
4g		0.25	8	4	2
4h		2	32	8	4
4i		0.25	2	1	0.5
5a		1	0.5	2	0.5
5b		2	1	8	0.5
5c		0.25	0.5	2	0.5
5d		2	16	8	16
5e		0.25	4	4	8
5f		2	16	16	8
6a		2	8	16	4
6b		4	32	32	16
Tiamulin		0.5	2	2	2
Valnemulin		0.125	0.5	0.5	0.5

3. Conclusions

We have synthesized a series of novel pleuromutilin derivatives containing thioether moiety by three methods of acylation reaction under very mild conditions. All these new derivatives have been investigated for their antibacterial activity *in vitro* against some Gram-positive bacteria, the investigation of MIC and antibacterial activity revealed that all the synthesized compounds showed moderate to good inhibitory characteristics. Among all the 17 compounds screened, compound **4i**, **5a** and **5c** with stronger conjugation effect of methoxy or amino in the in the benzene ring of ortho para were resulted as most active antibacterial agents against MRSA, MRSE, *E. coli* and *S. agalactia*.

4. Experimental section

4.1. General

Melting points were determined on a Tianda Tianfa YRT-3 apparatus (China) with open capillary tubes and are uncorrected. IR spectra were obtained on a Thermo Nicolet NEXUS-670 spectrometer and recorded as KBr thin film and absorptions are reported in cm^{-1}. NMR spectra were recorded on a Bruker-400 MHz spectrometers in appropriate solvents. Chemical shifts (δ) were expressed in parts per million (ppm) relative to the tetramethylsilane. Multiplicities of NMR signals are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad), etc. ^{13}C NMR spectra were recorded on 100 MHz spectrometers. All reactions were monitored by TLC on 0.2 mm thick silica gel GF254 pre-coated plates. After elution, plate was visualized under UV illumination at 254 nm for UV active materials. Further visualized was achieved by staining with 0.05% $KMnO_4$ aqueous solution or 0.5% phosphomolybdic acid. Column chromatography was carried out on silica gel (200–300 mesh). The products were eluted in appropriate solvent mixture under air pressure. Concentration and evaporation of the solvent after reaction or extraction was carried out on a rotary evaporator. All reagents were purchased from Aladdin (China) and used without further purification.

4.2. 14-O-(p-toluene sulfonyloxyacetyl) mutilin (**2**)

40% sodium hydroxide solution (5 mL, 50 mmol) was added dropwise to a mixture of pleuromutilin (7.57 g, 20 mmol) and p-toluenesulfonyl chloride (4.2 g, 22 mmol) in t-butyl methyl ether (20 mL) and water (5 mL). The mixture was stirred vigorously under reflux for 1 h, then diluted with water (50 mL) and stirred under an

Table 2
The zone of inhibition for MRSA, MRSE, *E. coli*, and *S. agalactia* in mm.

Compound	MRSA (μg/mL)			MRSE (μg/mL)			*E. coli* (μg/mL)			*S. agalactia* (μg/mL)		
	320	160	80	320	160	80	320	160	80	320	160	80
4a	14.16	12.19	14.11	13.36	12.20	11.40	14.48	11.91	11.31	10.80	11.23	10.55
4b	13.50	13.11	11.87	11.93	10.62	9.45	15.68	10.65	11.95	12.17	13.91	10.44
4c	14.62	13.79	11.75	12.45	12.67	8.72	12.57	12.88	11.77	14.00	12.05	10.77
4d	12.27	11.85	10.28	13.77	12.88	10.70	15.00	13.65	12.08	12.40	10.87	10.00
4e	13.09	13.21	11.20	12.41	10.75	9.90	15.52	13.74	11.30	13.62	12.97	13.01
4f	12.61	11.49	10.41	12.05	10.35	10.91	16.59	15.07	9.80	12.26	10.95	9.82
4g	12.38	12.06	9.50	13.54	12.50	10.77	17.42	15.50	12.40	13.71	12.98	11.79
4h	12.81	12.98	11.80	12.50	11.69	11.38	14.30	13.34	12.18	13.42	12.40	12.63
4i	19.72	18.35	15.29	18.33	15.25	14.41	18.50	17.07	15.69	16.58	15.92	16.01
5a	19.56	18.92	16.55	17.36	16.02	15.32	13.75	13.68	13.07	11.88	11.75	12.02
5b	10.22	10.77	9.56	11.21	9.72	9.63	10.68	12.46	9.94	9.66	9.80	8.86
5c	22.25	21.60	20.65	17.89	18.13	17.31	18.83	17.76	18.09	11.45	11.62	10.95
5d	10.25	9.98	9.41	10.85	10.02	10.90	10.84	11.31	9.20	11.03	10.61	10.11
5e	10.28	10.11	9.82	11.21	10.63	10.29	10.66	9.89	10.04	11.19	10.43	10.68
5f	8.52	8.77	9.05	9.00	8.84	10.35	10.80	9.52	8.87	9.25	9.62	9.31
6a	10.88	9.56	9.64	11.10	10.96	10.60	10.32	9.55	9.91	10.20	9.01	9.85
6b	12.69	11.53	10.40	10.98	10.16	11.00	14.66	15.31	11.69	11.48	10.55	10.70
Tiamulin	18.26	17.64	16.52	16.41	15.59	14.50	18.31	16.52	15.21	12.32	11.43	11.07
Valnemulin	25.76	22.89	20.43	27.46	25.95	22.55	24.50	23.71	19.54	15.79	14.51	12.75

ice bath for 15 min, followed by washing with water (50 mL) and cold t-butyl methyl ether (20 mL). Filtration afforded the title compound as white solid (9.8, 93%). It was used in the next step without further purification. mp: 147–148 °C; 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 0.63 (d, 3H, $J = 6.8$ Hz), 0.87 (d, 3H, $J = 6.8$ Hz), 1.11–1.15 (m, 1H), 1.22–1.26 (s, 5H), 1.33–1.36 (m, 1H), 1.41–1.44 (m, 1H), 1.46–1.50 (m, 5H), 1.63–1.65 (dd, 2H, $J_1 = 10$ Hz, $J_2 = 7.2$ Hz), 2.01–2.08(m, 3H), 2.21–2.29 (m, 3H), 2.45 (s, 3H), 3.34 (d, 1H, $J = 6.4$ Hz), 4.48 (s, 2H), 5.17–5.21 (d, 1H, $J = 8.8$ Hz), 5.31–5.34 (d, 1H, $J = 6.4$ Hz), 5.75–5.78 (d, 1H, $J = 4.2$ Hz), 6.43 (q, 1H, $J = 17.2$ Hz, 10.8 Hz); 7.35–7.37 (d, 2H, $J = 4.0$ Hz), 7.80–7.82 (d, 2H, $J = 4.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.7, 164.8, 145.2, 138.6, 132.5, 129.9, 127.9, 117.2, 74.4, 70.2, 64.9, 57.9, 45.3, 44.4, 43.9, 41.7, 36.4, 35.9, 34.3, 30.2, 26.7, 26.3, 24.7, 21.6, 16.4, 14.6, 11.4.

4.3. 14-O-[(1-amino-2-methylpropane-2-yl) thioacetyl] mutilin (**3**)

Metallic sodium (0.69 g, 30 mmol) was cut into small pieces and added portion wise to absolute ethanol (150 mL). After metallic sodium had been dissolved completely at room temperature, the solution was filtered and 1-amino-2-methyl-2-propanethiol hydrochloride (2.12 g, 15 mmol) was added. The mixture was stirred for 0.5 h at room temperature, followed by freezing to 0 °C and compound 2 (7.99 g, 15 mmol) was added. The mixture was stirred in an ice bath for 2.5 h, evaporated under reduced pressure to 50 mL, extracted with ethyl acetate (100 mL) and washed with water (200 mL). The organic layer was separated and dried with anhydrous Na_2SO_4 overnight. The organic solvent was evaporated in vacuum and the residue was purified by recrystallization with ethyl acetate or column chromatography (silica gel, ethyl acetate:ethanol 10:1 v/v). the product was obtained (5.03 g, 72%). mp: 154–155 °C; IR (free base, KBr): 3351, 2956, 2864, 1734, 1721, 1634, 1456, 1373, 1274, 1209, 1112, 1033, 982, 955, 941, 916 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.73 (d, 3H, $J = 7.2$ Hz), 0.87 (d, 3H, $J = 7.2$ Hz), 1.09–1.16 (m, 1H), 1.23 (s, 6H), 1.30–1.38 (m, 2H), 1.45 (s, 1H), 1.52–1.53 (m, 7H), 1.55–1.60 (m, 1H), 1.63–1.69 (m, 2H), 1.74–1.78(q, 1H, $J = 0.8$ Hz), 2.04–2.10 (q, 2H), 2.18–2.25(m, 2H), 2.32–2.59 (q, 1H, $J = 6.8$ Hz), 3.09 (s, 2H), 3.13–3.17 (t, 2H, $J = 1.6$ Hz), 3.35 (d, 1H, $J = 6.4$ Hz), 5.17–5.22 (q, 1H, $J = 1.6$ Hz), 5.31–5.34 (q, 1H, $J = 1.2$ Hz), 5.74(d, 1H, $J = 8.4$), 6.44–6.51 (q, 1H, $J_1 = 11.2$ Hz, $J_2 = 10.8$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.9, 169.4, 139.0, 117.1, 74.6, 69.3, 58.2, 51.7, 48.5, 45.4, 44.7, 43.9, 41.8, 36.7, 35.9, 34.4, 31.2, 30.4, 26.8, 26.3, 26.2, 24.8, 16.8, 14.9, 11.4.

4.4. General procedure for the synthesis of compounds **4a–i**

A mixture of modified benzoic acid (5.0 mmol) and $SOCl_2$ (10 mL) was heated under reflux for 5 h. The excess $SOCl_2$ was evaporated in vacuum and the resulting oil was cooled to room temperature. To the cooled oil dichloromethane (60 mL), triethylamine (2.5 g, 24.7 mmol) and compound 3 (1.6 g, 3.5 mmol) were added and stirred at 0 °C for 3 h. The mixture was quenched with saturated aqueous NH_4Cl and washed with water, followed by separation of organic layer. The aqueous layer was extracted with dichloromethane and the combined organic layer extracts were washed with water again. The obtained organic solvent was dried with anhydrous Na_2SO_4 overnight. After evaporation in vacuum of the organic solvent, the residue was purified by column chromatography (silica gel, petroleum ether:ethyl acetate 1:1 v/v) to afford the desired compounds.

4.4.1. 14-O-[(2-chlorobenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**4a**)

White solid; yield: 66%; mp: 66–68 °C; IR (KBr): 3417, 2928, 2889, 1728, 1651, 1594, 1537, 1469, 1385, 1282, 1202, 1184, 1117, 1017, 980, 939, 917, 751 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.70 (d, 3H, $J = 7.2$ Hz), 0.87 (d, 3H, $J = 7.2$ Hz), 1.12–1.14 (m, 4H), 1.24–1.29 (t, 2H), 1.35 (s, 7H), 1.42–1.44 (d, 5H), 1.64–1.76 (m, 4H), 2.03–2.07 (d, 2H), 2.21–2.28 (d, 3H), 3.14–3.24 (m, 2H), 3.33 (s, 1H), 3.43–3.50 (t, 2H, $J = 6.8$ Hz), 5.08–5.18 (m, 2H), 5.69(d, 1H), 6.34–6.41 (q, 1H, $J_1 = 10.4$ Hz, $J_2 = 10.$ Hz); 7.06 (s, 1H), 7.34–7.42 (m, 2H, $J = 7.2$ Hz), 7.65 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.9, 169.8, 166.7, 138.8, 135.4, 131.2, 130.9, 130.3, 129.9, 126.9, 117.2, 74.5, 69.9, 58.1, 48.1, 47.1, 45.4, 44.8, 43.9, 41.7, 36.7, 35.9, 34.4, 31.5, 30.4, 26.8, 26.5, 26.4, 24.8, 16.8, 14.8, 11.5.

4.4.2. 14-O-[(3-chlorobenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**4b**)

White solid; yield: 74%; mp: 92–94 °C; IR (KBr): 3323, 3084, 2924, 2863, 1718, 1649, 1567, 1538, 1454, 1369, 1292, 1238, 1144, 1117, 1015, 979, 940, 917, 883, 753, 681, 610 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.71 (d, 3H, $J = 7.2$ Hz), 0.87 (d, 3H, $J = 7.2$ Hz),

1.14 (s, 4H), 1.29–1.32 (d,7H), 1.45 (s, 4H), 1.60–1.63 (d, 1H), 1.65–1.68 (t, 3H), 1.75–1.78 (d, 1H), 2.06–2.18 (m, 2H), 2.20–2.25(m, 2H), 2.29–2.34 (q, 1H, J = 6.0 Hz), 3.16–3.24 (d, 2H), 3.26–3.31 (m, 1H), 3.35 (d, 1H, J = 6.4), 3.45–3.50 (q, 1H), 5.09 (s, 1H), 5.13 (d, 1H, J = 4.0), 5.76(d, 1H, J = 8.4), 6.38–6.45 (q, 1H, J_1 = 10.4 Hz, J_2 = 10.8 Hz), 7.38–7.42 (t, 2H), 7.48–7.50 (m, 1H), 7.64–7.67 (t, 1H, J = 5.6 Hz), 7.79–7.93 (m, 1H), 7.94 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 170.8, 165.8, 138.7, 136.3, 134.6, 131.4, 129.8, 127.5, 125.3, 117.2, 74.5, 70.3, 58.0, 47.7, 45.4, 44.9, 43.9, 41.7, 36.6, 35.9, 34.4, 31.5, 30.3, 26.8, 26.4, 26.2, 24.8, 16.9, 14.8, 11.5.

4.4.3. 14-O-[(4-chlorobenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (4c)

White solid; yield: 67%; mp: 68–70 °C; IR (KBr): 3395, 3081, 2929, 2864, 1727, 1651, 1596, 1539, 1486, 1455, 1384, 1284, 1190, 1147, 1116, 1014, 980, 938, 916, 846, 757, 678, 607 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.69 (d, 3H, J = 7.2 Hz), 0.89 (m, 6H, J = 7.2 Hz), 1.13–1.18 (m, 4H), 1.25 (s, 4H), 1.29 (s, 3H), 1.30–1.51 (m, 4H), 1.61–1.69 (m, 2H), 1.75–1.79 (q, 1H, J = 2.8 Hz), 2.06–2.12 (q, 2H), 2.16-2.31(m, 3H), 3.09 (s, 2H), 3.16–3.29 (m, 3H), 3.33–3.37 (t, 1H), 3.46–3.51 (q, 1H, J = 6.4), 5.08–5.15 (t, 2H), 5.73(d, 1H, J = 8.8), 6.36–6.44 (q, 1H, J_1 = 11.2 Hz, J_2 = 10.8 Hz), 7.43–7.45 (d, 2H, J = 8.4 Hz), 7.52–7.55 (t, 1H, J = 6.0 Hz), 7.86–7.88 (d, 1H, J = 8.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.7, 170.6, 165.9, 138.7, 137.6, 133.9, 128.7, 128.6, 117.2, 74.5, 70.3, 58.0, 47.7, 47.6, 44.9, 43.9, 41.7, 36.6, 35.9, 34.4, 31.6, 30.3, 26.8, 26.4, 26.2, 24.8, 16.9, 14.8, 11.5.

*4.4.4. 14-O-[(2-methylbenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**4d**)*

White solid; yield: 79%; mp: 133–134 °C; IR (KBr): 3459, 3330, 2953, 2883, 1730, 1650, 1601, 1539, 1485, 1455, 1406, 1371, 1301, 1284, 1185, 1147, 1115, 1037, 985, 940, 915, 771, 744, 723, 667 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.69 (d, 3H, J = 7.2 Hz), 0.86 (d, 3H, J = 7.2 Hz), 1.07–1.18 (m, 4H), 1.21–1.27 (m, 2H), 1.33 (d, 7H), 1.41 (d, 3H), 1.43–1.53 (m, 2H), 1.59–1.67 (m, 2H), 1.73–1.77(q, 1H, J = 2.4 Hz), 2.06–2.08 (m, 2H), 2.12–2.32(m, 3H), 2.49 (s, 3H), 3.13–3.24 (q, 2H, J = 16.0 Hz), 3.31–3.36 (q, 2H), 3.44–3.49 (q, 1H, J = 6.4), 5.09–5.16 (t, 2H), 5.69(d, 1H, J = 8.8), 6.34–6.41 (q, 1H, J_1 = 11.2 Hz, J_2 = 10.8 Hz), 6.79–6.82 (t, 1H), 7.20–7.24 (t, 2H), 7.30–7.34 (t, 1H), 7.48 (d, 1H, J = 7.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 169.9, 169.8, 138.7, 136.3, 136.2, 131.0, 129.8, 126.9, 125.7, 117.1, 74.5, 69.9, 58.0, 47.5, 47.4, 45.3, 44.8, 43.9, 41.7, 36.6, 35.9, 34.4, 31.5, 30.3, 26.8, 26.4, 26.3, 24.8, 20.1, 16.8, 14.8, 11.4.

*4.4.5. 14-O-[(3-methylbenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**4e**)*

White solid; yield: 74%; mp: 67–69 °C; IR (KBr): 3396, 3328, 2928, 2861, 1728, 1650, 1628, 1613, 1573, 1539, 1505, 1456, 1417, 1385, 1285, 1189, 1117, 1018, 980, 916, 751 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.72 (d, 3H, J = 7.2 Hz), 0.87 (d, 3H, J = 6.8 Hz), 1.09–1.13 (d, 4H), 1.23–1.31 (m, 7H), 1.38–1.45 (t, 3H, J = 14.4), 1.49–1.78 (m, 6H), 2.03–2.32 (m, 6H), 2.40 (d, 3H), 3.15–3.30 (m, 2H), 3.34 (d, 2H, J = 6.0), 3.45–3.50 (q, 1H, J = 6.4), 5.10–5.14 (t, 2H), 5.73(d, 1H, J = 8.4), 6.37–6.45 (q, 1H, J_1 = 11.6 Hz, J_2 = 11.2 Hz), 7.24–7.27 (t, 2H), 7.38 (t, 1H), 7.80 (d, 1H, J = 8.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 170.3, 167.1, 141.7, 138.7, 131.6, 129.1, 127.1, 117.2, 74.5, 70.1, 58.0, 47.7, 47.4, 45.3, 44.9, 43.9, 41.7, 36.6, 35.9, 34.4, 33.8, 31.5, 30.3, 26.8, 26.3, 26.2, 25.6, 24.9, 21.4, 16.8, 14.8, 11.5.

4.4.6. 14-O-[(4-methylbenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (4f)

White solid; yield: 77%; mp: 63–66 °C; IR (KBr): 3403, 2928, 2854, 1732, 1651, 1615, 1539, 1506, 1456, 1417, 1386, 1286, 1189, 1149, 1117, 1019, 980, 917, 836, 752, 667 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.73 (d, 3H, J = 6.8 Hz), 0.88 (d, 3H, J = 6.8 Hz), 1.15 (s, 4H), 1.25–1.33 (m, 8H), 1.38–1.53 (q, 5H), 1.61–1.79 (m, 4H), 2.05–2.33 (m, 5H), 2.42 (d, 3H), 3.15–3.35 (m, 4H), 3.47-3.52 (q, 1H, J = 6.4), 5.11–5.16 (t, 2H), 5.77(d, 1H, J = 8.4), 6.39–6.46 (q, 1H, J = 11.2 Hz), 7.34 (d, 2H), 7.41 (s, 1H), 7.68–7.74(q, 2H, J_1 = 8.0 Hz, J_1 = 6.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 170.4, 167.4, 138.7, 138.3, 134.5, 132.1, 128.4, 127.9, 124.1, 117.2, 74.5, 70.1, 58.1, 47.7, 47.5, 45.4, 44.9, 43.9, 41.8, 36.6, 35.9, 34.4, 31.6, 30.4, 26.8, 26.4, 26.3, 24.8, 21.4, 16.9, 14.8, 11.5.

*4.4.7. 14-O-[(2-methoxybenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**4g**)*

White solid; yield: 81%; mp: 60–62 °C; IR (KBr): 3389, 2931, 2878, 1732, 1650, 1600, 1537, 1483, 1463, 1373, 1285, 1241, 1183, 1116, 1021, 980, 917, 758, 656 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.70 (d, 3H, J = 5.6 Hz), 0.85 (d, 3H, J = 6.0 Hz), 1.09–1.13 (t, 3H), 1.21–1.39 (m, 9H), 1.43–1.44 (t, 4H), 1.49–1.76 (m, 5H), 2.01–2.07 (m, 3H), 2.15–2.30 (m, 3H), 3.17–3.25 (t, 2H), 3.33 (t, 1H, J = 8.0), 3.40–3.52 (m, 2H), 3.98 (q, 3H), 5.08–5.21 (q, 2H), 5.73(d, 1H, J = 7.2), 6.38–6.46 (m, 1H), 7.40–7.44 (q, 1H), 8.14–8.16 (q, 1H, J_1 = 2.0 Hz, J_1 = 2.4 Hz), 8.48(s, 1H); ^{13}C NMR(100 MHz, $CDCl_3$) δ 216.9, 169.1, 165.3, 157.6, 138.8, 132.6, 132.1, 121.5, 127.1, 117.0, 111.2, 74.4, 69.4, 58.1, 55.9, 47.9, 46.8, 45.3, 44.6, 43.8, 41.7, 36.6, 35.9, 34.3, 31.5, 30.3, 26.7, 26.5, 26.2, 24.7, 16.7, 14.8, 11.4.

*4.4.8. 14-O-[(3-methoxybenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**4h**)*

White solid; yield: 78%; mp: 59–61 °C; IR (KBr): 3400, 2931, 2857, 1729, 1651, 1583, 1537, 1485, 1463, 1372, 1287, 1244, 1189, 1117, 1037, 980, 917, 752, 689 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.71 (d, 3H, J = 6.8 Hz), 0.87 (d, 3H, J = 6.4 Hz), 1.08–1.13 (d, 4H), 1.23–1.27 (m, 2H), 1.30–1.31 (d, 7H), 1.45 (d, 4H), 1.48–1.54 (t, 2H), 1.60–1.65 (t, 2H), 1.74–1.78 (d, 1H), 2.03–2.11 (m, 3H), 2.18–2.31 (m, 3H), 3.16–3.34 (m, 4H), 3.46–3.51 (q, 1H), 3.86 (t, 3H), 5.09–5.14 (t, 2H), 5.74 (d, 1H, J = 8.0), 6.37–6.44 (m, 1H, J_1 = 11.2 Hz, J_2 = 11.6 Hz), 7.04–7.06 (d, 1H), 7.34–7.38 (q, 1H), 7.45–7.49 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 170.4, 166.9, 159.8, 138.6, 136.0, 129.5, 118.9, 117.7, 117.3, 112.3, 74.5, 70.1, 58.0, 55.3, 47.7, 47.5, 45.3, 44.9, 43.9, 41.7, 36.6, 35.9, 34.4, 31.5, 30.3, 26.8, 26.4, 26.2, 24.8, 16.8, 14.8, 11.5.

*4.4.9. 14-O-[(4-methoxybenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**4i**)*

White solid; yield: 83%; mp: 60–63 °C; IR (KBr): 3406, 2931, 2885, 1732, 1646, 1607, 1540, 1505, 1456, 1417, 1373, 1286, 1255, 1185, 1179, 1116, 1028, 979, 938, 916, 845, 767, 610 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.71 (t, 3H, J = 6.0 Hz), 0.87 (t, 3H, J = 5.2 Hz), 1.13–1.14 (d, 4H), 1.24–1.38 (m, 9H), 1.44–1.46 (t, 4H), 1.52–1.78 (m, 4H), 2.03–2.06 (m, 3H), 2.19–2.31 (m, 3H), 3.15–3.36 (m, 4H), 3.46–3.49 (q, 1H), 3.86 (t, 3H), 5.11–5.15 (m, 2H), 5.73(s, 1H), 6.37–6.46 (m, 1H), 6.93–6.97 (q, 2H), 7.34 (s, 1H), 7.87–7.89 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 170.4, 166.7, 162.2, 138.7, 128.9, 126.8, 117.3, 113.7, 74.5, 70.2, 60.3, 58.1, 55.4, 47.8, 47.5, 45.4, 44.9, 43.9, 41.8, 36.6, 35.9, 34.4, 31.5, 30.4, 26.9, 26.4, 26.2, 24.8, 16.9, 14.8, 14.2, 11.5.

*4.5. General procedure for the synthesis of compounds **5a–f***

A suspension of amino acid derivative (5 mmol) was dissolved in a mixture of tetrahydrofuran (50 mL) and water (20 mL). 1 N NaOH (6 mL) was added followed by adding 1.1 g tert–butoxycarbonyl (5 mmol) dropwise at room temperature. After stirring for 4 h, the tetrahydrofuran was evaporated in vacuum from the reaction mixture. The residue was added ethyl acetate (50 mL) and 5% citric acid (30 mL). The organic layer was separated, washed with water (20 mL), dried with anhydrous Na_2SO_4 and rotary evaporated to

dryness. Crude residue was used into next reaction without purification.

A mixture of the above N-Boc protected amino acids (3.7 mmol), 1.63 g compound 3 (3.5 mmol), 0.72 g N,N′-dicyclohexylcarbodiimide (3.5 mmol), 0.47 g 1-Hydroxybenzotriazole (3.5 mmol) and 60 mL dichloromethane was stirred at room temperature for 15 h. The mixture was filtered to remove dicyclohexylurea which was produced by the reaction and the filtrate was washed with saturated aqueous $NaHCO_3$ and water. The solvent was evaporated in vacuum and the residue was treated with a mixture of 10 mL trifluoroacetic acid and 10 mL dichloromethane at room temperature for 30 min. The reaction mixture was quenched with 25% aqueous $NaHCO_3$ (50 mL) and washed with water, dried with anhydrous Na_2SO_4 overnight and rotary evaporated to dryness. Crude residue thus obtained was purified over silica gel column chromatography (petroleum ether:ethyl acetate 1:1 v/v) afford the desired compounds.

4.5.1. 14-O-[(2-aminobenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**5a**)

White solid; yield: 81%; mp: 70–72 °C; IR (KBr): 3342, 2929, 2871, 1717, 1633, 1584, 1532, 1455, 1372, 1292, 1189, 1159, 1117, 1019, 980, 936, 916, 746 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.72 (t, 3H, J = 7.2 Hz), 0.86 (t, 3H, J = 6.8 Hz), 1.11–1.15 (q, 4H), 1.12–1.25 (q, 4H), 1.29–1.38 (q, 7H), 1.44–1.52 (q, 4H), 1.62–1.78 (m, 6H), 2.04–2.30 (m, 6H), 3.15–3.34 (m, 4H), 3.39–3.44 (q, 1H, J = 6.4 Hz), 3.68–3.72 (q, 2H, J = 6.8 Hz), 5.11–5.19 (m, 2H), 5.72(d, 1H), 6.40–6.45 (q, 1H, J_1 = 11.2 Hz, J_2 = 10.8 Hz), 6.66–6.99 (m, 2H), 7.19–7.27 (m, 2H), 7.52–7.54 (q, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.9, 170.2, 169.2, 148.8, 138.7, 132.2, 127.4, 117.3, 117.2, 116.6, 116.0, 74.5, 70.1, 58.2, 58.0, 47.6, 47.1, 45.4, 44.8, 43.9, 41.7, 36.6, 35.9, 34.4, 31.5, 30.3, 26.8, 26.4, 26.3, 24.7, 18.3, 16.8, 14.8, 11.4.

4.5.2. 14-O-[(3-aminobenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**5b**)

White solid; yield: 55%; mp: 77–79 °C; IR (KBr): 3380, 2931, 2873, 1726, 1666, 1586, 1537, 1487, 1454, 1372, 1285, 1201, 1138, 1017, 980, 938, 917, 748, 722 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.71 (d, 3H, J = 6.8 Hz), 0.86 (d, 3H, J = 6.4 Hz), 1.13 (s, 4H), 1.24–1.30 (m, 9H), 1.37–1.45 (t, 5H), 1.52–1.77 (m, 4H), 2.04–2.09 (t, 2H), 2.18–2.31 (m, 3H), 3.15–3.28(q, 3H), 3.35 (s, 1H), 3.45 (br, 1H), 5.09–5.13 (t, 2H), 5.72(d, 1H), 6.36–6.43 (q, 1H, J_1 = 11.6 Hz, J_2 = 11.2 Hz), 7.03 (br, 1H), 7.28 (d, 1H), 7.37–7.53 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.9, 170.4, 138.7, 130.9, 129.6, 117.2, 74.6, 70.2, 60.4, 58.0, 47.7, 47.6, 45.4, 44.8, 43.9, 41.7, 36.6, 35.9, 34.8, 31.6, 30.5, 30.3, 26.8, 26.3, 26.2, 24.8, 19.1, 16.9, 14.8, 11.5.

4.5.3. 14-O-[(4-aminobenzamide-2-methylpropane-2-yl) thioacetate] Mutilin (**5c**)

White solid; yield: 65%; mp: 86–85 °C; IR (KBr): 3389, 2929, 2872, 1725, 1633, 1606, 1538, 1506, 1456, 1385, 1287, 1187, 1117, 1018, 980, 917, 842, 768 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.72 (d, 3H, J = 6.8 Hz), 0.87 (d, 3H, J = 7.2 Hz), 1.14 (s, 4H), 1.25–1.31 (q, 9H), 1.45 (s, 4H), 1.50–1.79 (m, 5H), 2.05–2.24 (m, 5H), 2.29–2.33 (t, 1H), 3.15–3.28(m, 3H), 3.35 (s, 1H), 3.44–3.49 (q, 1H, J = 6.4 Hz), 4.01 (br, 1H), 5.11–5.18 (t, 2H), 5.73 (d, 1H), 6.39–6.46 (q, 1H, J_1 = 10.8 Hz, J_2 = 11.2 Hz), 6.67–6.69 (d, 2H), 7.16–7.19 (t, 1H), 7.71–7.74 (d, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 170.2, 166.9, 149.6, 138.7, 128.8, 124.1, 117.3, 114.1, 74.5, 70.1, 58.1, 53.8, 47.8, 47.4, 45.4, 44.9, 43.9, 41.8, 36.6, 35.9, 34.4, 31.5, 30.4, 29.2, 26.8, 26.4, 26.3, 24.8, 16.9, 14.8, 11.5.

4.5.4. 14-O-[D(−)-phenylglycinamide-2-methylpropane-2-yl) thioacetate] Mutilin (**5d**)

White solid; yield: 79%; mp: 59–61 °C; IR (KBr): 3373, 2891, 1731, 1667, 1519, 1455, 1415, 1385, 1282, 1190, 1117, 1018, 981, 954, 917, 699 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.72 (d, 3H, J = 7.2 Hz), 0.90 (d, 3H, J = 6.8 Hz), 1.11–1.18 (q, 10H), 1.24–1.39 (m, 5H), 1.44 (d, 4H), 1.48–1.56 (t, 2H), 1.61–1.69 (q, 2H), 1.78 (d, 1H), 2.04 (s, 3H), 2.08 (d, 2H), 2.14–2.26 (m, 2H), 2.31–2.34 (t, 1H), 3.04–3.30(m, 9H), 3.36 (d, 1H), 4.59 (s, 1H), 5.16–5.26 (q, 2H), 5.72(d, 1H), 6.39–6.46 (q, 1H, J_1 = 10.8 Hz, J_2 = 11.2 Hz), 7.29-7.36 (m, 3H), 7.43–7.45 (d, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.9, 169.8, 138.9, 128.8, 128.0, 126.9, 117.2, 74.6, 69.7, 58.1, 47.5, 47.1, 45.4, 44.8, 43.9, 41.8, 36.7, 35.9, 34.4, 31.4, 30.4, 26.8, 26.3, 26.2, 24.8, 16.8, 14.9, 11.5.

4.5.5. 14-O-[L(−)-phenylglycinamide-2-methylpropane-2-yl) thioacetate] Mutilin (**5e**)

White solid; yield: 79%; mp: 62–64 °C; IR (KBr): 3376, 22930, 1731, 1669, 1522, 1456, 1417, 1387, 1282, 1190, 1117, 1019, 981, 954, 917, 699 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.70 (d, 3H, J = 6.4 Hz), 0.89 (d, 3H, J = 6.4 Hz), 1.15–1.23 (t, 14H), 1.44–1.54 (t, 5H), 1.63–1.79 (q, 4H), 1.99–2.09 (t, 3H), 2.23–2.32 (t, 7H), 3.06–3.25(m, 4H), 3.35 (d, 1H), 4.56 (s, 1H), 5.14–5.26 (q, 2H), 5.72(d, 1H), 6.39–6.46 (q, 1H, J_1 = 10.8 Hz, J_2 = 11.2 Hz), 7.27–7.33 (q, 3H), 7.43–7.44 (d, 2H), 7.63 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.9, 173.0, 169.6, 138.9, 128.7, 127.9, 126.8, 117.1, 74.5, 69.6, 59.9, 58.1, 47.4, 47.0, 45.4, 44.7, 43.8, 41.7, 36.6, 35.9, 34.4, 31.4, 30.3, 29.6, 26.8, 26.3, 26.2, 24.8, 16.8, 14.8, 11.5.

4.5.6. 14-O-[(2-Indoleamide-2-methylpropane-2-yl) thioacetate] Mutilin (**5f**)

White solid; yield: 71%; mp: 116–119 °C; IR (KBr): 3327, 2927, 2851, 1731, 1627, 1573, 1549, 1456, 1419, 1374, 1308, 1282, 1189, 1116, 1017, 979, 938, 946, 919, 705, 756, 747, 681, 617 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.77 (d, 3H, J = 7.2 Hz), 0.88 (d, 3H, J = 7.2 Hz), 1.7 (s, 3H), 1.33–1.35 (d, 7H), 1.49 (s, 4H), 1.53–1.79 (m, 6H), 2.05–2.26 (m, 4H), 2.31–2.36 (t, 1H), 3.20–3.27 (t, 2H), 3.32–3.38 (m, 2H), 3.49–3.54 (t, 1H), 5.15–5.24 (q, 2H), 5.80(d, 1H), 6.46–6.53 (q, 1H, J_1 = 11.2 Hz, J_2 = 10.8 Hz), 7.10 (d, 1H), 7.13–7.17 (t. 1H), 7.31 (d, 1H), 7.46 (d, 1H), 7.65–7.69 (t, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.7, 170.8, 138.8, 130.9, 127.8, 124.4, 121.9, 120.6, 117.4, 111.9, 102.5, 74.6, 70.4, 58.1, 47.7, 47.6, 45.4, 44.9, 44.0, 41.8, 36.7, 36.0, 34.4, 31.6, 30.4, 26.9, 26.4, 26.3, 24.8, 16.9, 14.9, 14.2, 11.5.

4.6. General procedure for the synthesis of compounds **6a–b**

Chloroformate (3.7 mmol) was added dropwise to a solution of compound 3 (1.6 g, 3.5 mmol) and triethylamine (0.81 g, 8 mmol) in dichloromethane (60 mL) and was stirred at 0 °C or room temperature for 4.5 h. Then the reaction mixture was washed with saturated aqueous NH_4Cl and water and dried with anhydrous Na_2SO_4 overnight. The solvent was evaporated in vacuum and the residue was chromatographed on silica gel (petroleum ether:ethyl acetate 2:1 v/v) to afford a pure product.

4.6.1. 14-O-[1-N-carbamate-isobutyl-2-methylpropane-2-yl] thioacetate] Mutilin (**6a**)

White solid; yield: 89%; mp: 75–77 °C; IR (KBr): 3481, 3440, 2959, 1719, 1698, 1519, 1456, 1416, 1385, 1367, 1286, 1232, 1189, 1119, 1038, 982, 942, 917 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.72 (t, 3H, J = 6.0 Hz), 0.88 (d, 3H, J = 6.4 Hz), 0.91–0.94 (q, 6H), 1.09–1.12 (d, 1H), 1.63 (s, 3H), 1.25 (s, 6H), 1.31–1.38 (d, 2H), 1.44 (d, 4H), 1.47–1.55 (q, 2H), 1.61–1.66 (t, 2H), 1.75–1.78 (d, 1H), 1.89–1.93 (t, 1H), 2.06–2.09 (d, 2H), 2.16–2.24 (m, 2H), 2.34 (s, 1H), 3.09–3.15(q, 4H), 3.33–3.35 (d 1H), 3.84 (d, 2H), 5.16–5.21 (d, 1H), 5.31–5.39 (t, 2H), 5.74(d, 1H), 6.42–6.49 (m, 1H, J_1 = 11.2 Hz, J_2 = 11.2 Hz), ^{13}C NMR(100 MHz, $CDCl_3$) δ 216.8, 169.6, 157.0, 138.9, 117.2, 74.6, 71.0, 69.7, 58.1, 49.4, 47.2, 45.4, 44.8, 43.9, 41.7, 36.7, 35.9, 34.4, 31.3, 30.4, 28.0, 26.8, 26.3, 26.2, 26.1, 24.8, 19.0, 16.8, 14.8, 11.4.

4.6.2. 14-O-[1-N-carbamate-benzyl-2-methylpropane-2-yl] thioacetate] Mutilin (**6b**)

White solid; yield: 78%; mp: 60–63 °C; IR (KBr): 3418, 2931, 1727, 1519, 1455, 1417, 1385, 1282, 1251, 1140, 1117, 1016, 981, 917,698, 668 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.71 (d, 3H, $J = 6.8$ Hz), 0.87 (d, 3H, $J = 6.8$ Hz), 1.12–1.17 (t, 5H), 1.26–1.33 (t, 7H), 1.38–1.42 (t, 2H), 1.45–1.50 (t, 5H), 1.55 (s, 2H), 1.61–1.77 (m, 4H), 2.04–2.10 (d, 2H), 2.14–2.25 (m, 2H), 2.31–2.34 (t, 1H), 3.09–3.24(m, 4H), 3.32–3.36 (q 1H), 5.11–5.13 (d, 2H), 5.17 (s, 1H), 5.26–5.29 (d, 1H), 5.54 (s, 1H), 5.72–5.74 (d, 1H), 6.40–6.48 (q, 1H, $J_1 = 11.2$ Hz, $J_2 = 10.8$ Hz), 7.31–7.37 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 169.6, 156.6, 138.8, 136.6, 128.4128.1, 117.2, 74.6, 69.7, 66.7, 58.1, 49.5, 47.1, 45.8, 45.4, 44.8, 43.8, 41.7, 36.7, 35.9, 34.4, 31.3, 30.4, 26.8, 26.3, 26.2, 26.1, 24.8, 16.8, 14.8, 11.5, 8.6.

4.7. Evaluation of the antibacterial activity

4.7.1. MIC of the compounds by agar dilution method

The minimum inhibitory concentration (MIC) values were determined using agar dilution method according to NCCLS. 12800 μg synthesized compounds were weighed accurately and dissolved in about 5 mL ethanol. Then distilled water was added to the solution to 10 mL. Tiamulin fumarate and valnemulin hydrochloride as contrast drugs were dissolved in 10 mL distilled water. Then all the solutions were diluted with distilled water by two fold. 2 mL of the 2-fold serial dilutions of each test compound/drug were incorporated into 18 mL hot Mueller-Hintion agar medium. Inoculum of MRSA, MRSE, *E. coli*, and *S. agalactiae* which were all separated from the clinic were prepared from blood slants and adjusted to approximate 10^5–10^6 cfu/mL with sterile saline (0.90% NaCl). A 10 μL amount of bacterial suspension was spotted into Mueller-Hintion agar plates containing 2-fold serial dilutions of compounds/drugs. The plates were incubated at 36.5 °C for 48 h. The MIC is defined as the minimum concentration of compound to give complete inhibition of bacterial growth. The same procedure was repeated in triplicate.

4.7.2. Oxford cup assay

Oxford cup assay was performed to evaluate the rate of inhibition in the growth of bacteria. Inoculum was prepared in 0.9% saline using McFarland standard and spread uniformly on nutrient agar plates. All the compounds were prepared as the same as MIC test by agar dilution method and the resulting solution with varying concentration was added to the Oxford cups which were placed at equidistance on the above agar surface. The zone of inhibition for each concentration was measured after 24 h incubation at 37 °C.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.01.048.

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