Accepted Article

Received Date : 07-Feb-2016

Revised Date : 18-May-2016

Accepted Date : 31-May-2016

Article type : Research Article

Design, synthesis and structure-activity relationship studies of novel pleuromutilin derivatives having a piperazine ring

Jian Luo, Qiu-E Yang, Yuan-Yuan Yang, You-Zhi Tang*, Ya-Hong Liu*

Guangdong Provincial Key Laboratory of Veterinary Pharmaceutics Development and Safety Evaluation, College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China

Correspondence should be addressed:

College of Veterinary Medicine, South China Agricultural University, No. 483 Wushan Road, Tianhe District, Guangzhou 510642, China

You-Zhi Tang,

Fax: +86 20 85280665; Email: *youzhitang@scau.edu.cn*

or Ya-Hong Liu,

Fax: +86 20 85284896; Email: *gale@scau.edu.cn*

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/cbdd.12799

This article is protected by copyright. All rights reserved.

Accepted Article

Abstract

A series of novel pleuromutilin derivatives possessing piperazine moieties were synthesized under mild conditions. The in vitro antibacterial activities of these derivatives against *Staphylococcus aureus* and *Escherichia coli* were tested by the agar dilution method. Structure-activity relationship (SAR) studies resulted in compounds 11b, 13b and 14a with the most potent in vitro antibacterial activity among the series (MIC = 0.0625-0.125 μg/ml). The binding of compounds 11b, 13b and 14a to the *Escherichia coli* ribosome was investigated by molecular modeling, and it was found that there is a reasonable correlation between the binding free energy and the antibacterial activity.

Introduction

The rate of new antibiotics reach the market is declining, and at the same time, the rate of multi-drug resistant strains is increasing(1). Therefore, the discovery and development of new antibiotics with a novel mechanism of action has become more and more important to the medical community(2). Natural products or semisynthetic derivatives of natural products usually play a vital role for developing new antibacterial agents (3, 4).

Pleuromutilin (1, Fig. 1), a 5-6-8 tricycle diterpene natural product with a modest antibacterial activity, was first isolated from two basidiomycete species in 1951(5, 6). Pleuromutilin selectively inhibits bacterial protein synthesis by binding to the ribosomes, but does not bind to mammalian ribosomes(7). Further studies have shown that this class of antibiotics interact with the 23S rRNA of the 50S bacterial ribosome subunit and displays lack of cross-resistance with antibiotics currently in market(2, 8, 9). This new mechanism of action of pleuromutilin derivatives have let them lead compounds in the discovery and development of novel antibacterial agents for the treatment of the problem of bacterial resistance(1, 2, 10). Throughout the 1970s, chemists at Sandoz reported initial structure-

This article is protected by copyright. All rights reserved.

activity relationship (SAR) studies on a series of semisynthetic pleuromutilin analogous (11-13). Further studies have shown that modification of the C14 position of pleuromutilin could led to compounds with improved antimicrobial activity(14). Additionally, the de novo syntheses of analogues of pleuromutilin, which allow the core of the pleuromutilin to be varied, have been reported recently (15, 16).

More than 3000 semisynthetic pleuromutilin analogous with C14 glycol ester side chains have been synthesized and studied(10). Luckily, three of them were marketed by pharmaceutical companies. Tiamulin (2, Fig. 1) and valnemulin (3, Fig. 1) were approved as therapeutic agents for pig and poultry in 1979 and 1999, respectively(17). Although retapamulin (4, Fig. 1) was approved by the Food and Drug Administration (FDA) in 2007 for human use(18), it was limited to the topical treatment of skin infections. The sulfide linkage in these three pleuromutilin analogues yield potent in vitro antibacterial activities, as well as limited oral bioavailability in vivo of these derivatives for their strong hydrophobic nature (7).

In order to overcome this problem, along with previous SAR studies (1, 7, 19), we have focused our efforts on the development of novel pleuromutilin antibiotics with amine substituents incorporated into the C14 side chain (20). Then, tiamulin is used as a lead compound. The aim was first of all to make an isometric exchange of the sulfur in the linker with nitrogen in order to decrease the hydrophobicity of the linker. Considering piperazine ring plays a vital role for increasing antibacterial activities of quinolones (21, 22). We envisaged that the development of pleuromutilin derivatives with piperazine ring as linker and various benzene ring as substituents incorporated into the C14 side chain may increase hydrogen bonding and ∏-∏ stacking interactions and thereby increase their antibacterial properties.

This article is protected by copyright. All rights reserved.

Accepted Article

For the present sutdy, we report the design, synthesis, and SAR studies of novel pleuromutilin derivatives with one piperazine ring as linker with similar substituents incorporated into the C14 side chain. To explore the antibacterial mechanisms of these derivatives, several compounds were chosen for molecular docking studies.

Experimental

Materials

Pleuromutilin (>90% pure) was obtained from Great Enjoyhood Biochemical Co Ltd (Daying, China). Piperazine derivatives were purchased from J&K Scientific Ltd. The other reagents were all of analytical grade and purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Melting points were determined on a Shenguang X-4 apparatus (China) and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were measured on Bruker AV-400 or Bruker AV-600 spectrometer in $CDCl_3$. Chemical shift values (δ) were given in p.p.m. and tetramethylsilane was used as internal standard. Fourier transform infrared (FTIR) were obtained with a Nicolet 6700 Research FTIR spectrometer using KBr pellets. Mass spectra were recorded with Agilent 6430 and LTQ-Orbitrap mass spectrometer (Thermo Fisher) using the electro spray ionization (ESI) method.

Synthesis

A general synthesis strategy based on the usual 22-*O*-tosylpheuromutilin and piperazine derivatives were used (Scheme 1).

22-*O*-tosylpleuromutilin (1a)

A solution of pleuromutilin 1 (10.0g, 26.4 mmol) in pyridine (30.0 mL) was stirred at 0 ℃ in a three-necked round bottom flask, and *p*-toluenesulfonyl chloride (5.5 g, 29.0 mmol) was added. The solution was stirred at 0 ℃ for 3h. $CHCl_3$ (50 mL) and Ice-cold water (50 mL)

This article is protected by copyright. All rights reserved.

Accepted Article

were added to the solution. The organic phase was washed with a 2 M aqueous solution of H_2SO_4, a saturated aqueous solution of $NaHCO_3$and water, respectively. Then the organic phase was dried with anhydrous Na_2SO_4 for 2 h and evaporated in vacuum. The residue was precipitated from isopropanol to give a white solid (10.3 g, 73.0%) (20, 23).

The synthetic route of pleuromutilin derivatives having a piperazine ring

A solution of compound 1a (1.3 g, 2.4 mmol) in acetonitrile (10 mL) was refluxed for 0.5h. NaI and Na_2CO_3 were added and the solution was refluxed for 2 h and then gave the iodide 22-deoxypleuromutilin. A solution of piperazine derivatives in acetonitrile were added in above mixture. The pleuromutilin derivatives having a piperazine ring were obtained by the nucleophilic attack of piperazine derivatives on iodide 22-deoxypleuromutilin. After completion of the reaction, water (50 mL) was added to the solution, which was then extracted with $CHCl_3$ (50 mL). The organic phase was washed with water for twice, dried (Na_2SO_4) and concentrated under reduced pressure to give the crude product. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (2:1) as eluent to give a pure product.

22-(4-(2-Methoxy-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (11a)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 64.0%; m.p.: 55.0-57.4℃;^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm)6.93 (4H, m), 6.54 (1H, dd, *J* 10.8, 17.2, H19), 5.81 (1H, d, *J* 8.8, H14), 5.35(1H, d, *J* 10.8, H20), 5.21 (1H, d, *J* 17.2, H20), 3.86 (3H, s), 3.36 (1H, dd, *J* 6.8, 10.8, H11), 3.17 (6H, m, H22), 2.75 (4H, m), 2.22 (5H, m, H2, H4, H10, H13),1.78 (1H, dd, *J* 2.0, 14.4, H6), 1.66 (5H, m, H1, H7, H8,),1.46(3H, s, H15), 1.34 (3H, m, H8, H13, 11-OH), 1.13 (1H, m, H8),1.17 (3H, s, H18), 0.88 (3H, d, *J* 6.8 H17), 0.75 (3H, d, *J* 6.8, H16);^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.08 (C3),169.14 (C21), 152.29, 141.26, 139.14 (C19), 122.99, 121.04, 118.31, 117.29 (C20), 111.28, 74.61 (C11), 68.27 (C14), 60.16, 58.22

This article is protected by copyright. All rights reserved.

Accepted Article

(C4), 55.39, 53.42, 50.35 (C22), 45.48 (C9), 45.02 (C13), 43.98 (C12), 41.81 (C5), 36.78 (C6), 36.07 (C10), 34.49 (C2), 30.47 (C8), 26.86 (C7), 26.36 (C18), 24.89 (C1), 16.76 (C16), 14.93 (C15), 11.50 (C17); IR (KBr, cm^{-1}) 3504, 2926, 1728, 1500, 1450, 1286, 1240, 1211, 1034, 918, 753; HR-MS (ESI): Calcd for $C_{33}H_{48}N_2O_5$ ($M\text{-}H^+$): 553.3636; Found: 553.3632.

22-(4-(3-Methoxy-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (11b)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 78.0%; m.p.: 55.0-57.4℃; ^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.16 (1H, m), 6.49 (4H, m, H19), 5.81 (1H, d, *J* 8.8, H14), 5.35 (1H, d, *J* 10.8, H20), 5.20 (1H, d, *J* 17.2, H20), 3.78 (3H, s),3.23 (6H, m, H11, H22), 2.71 (4H, d, *J* 22.4), 2.21 (6H, m, H2, H4, H10, H13, 11-OH), 1.64 (6H, m, H1, H7, H8, H6), 1.45 (3H, s, H15),1.34 (3H, m, H8, H13)1.17 (3H, s, H18),0.88 (3H, d, *J* 6.8 H17),0.75 (3H, d, *J* 6.8, H16);^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.04 (C3), 168.98 (C21), 160.59, 152.55, 139.11 (C19), 129.79,117.29 (C20), 108.93, 104.50, 102.61, 74.61 (C11), 68.38 (C14),59.94, 58.20 (C4), 55.18, 52.95,48.85 (C22), 45.48 (C9), 45.05 (C13), 43.97 (C12), 41.80 (C5), 36.76 (C6), 36.07 (C10), 34.48 (C2), 30.46 (C8), 26.85 (C7), 26.38 (C18), 24.87 (C1), 16.76 (C16), 14.92 (C15), 11.50 (C17); IR (KBr, cm^{-1}) 3504, 2938, 1727, 1598, 1495, 1450, 1381, 1294, 1256, 1204, 1176, 976, 825, 765, 688; HR-MS (ESI): Calcd for $C_{33}H_{48}N_2O_5$ ($M\text{-}H^+$): 553.3636; Found: 553.3630.

22-(4-(4-Methoxy-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (11c)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 58.0%; m.p.: 55.0-57.4℃; ^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 6.90 (2H), 6.84 (2H, d, *J* 8.8), 6.53 (1H, dd, *J* 10.8, 17.2, H19), 5.81 (1H, d, *J* 8.4, H14), 5.35 (1H, dd, *J* 1.6, 11.2, H20),5.21 (1H, dd, *J* 1.6, 17.6, H20), 3.77（3H, s）3.36 (1H, dd, *J* 6.4, 10.8, H11), 3.24 (1H, d, *J* 17.2, H22),3.11 (5H, m, H22), 2.73 (4H, d, *J* 23.2),2.21 (5H, m, H2, H4, H10, H13), 1.78 (1H, dd, *J* 2.8, 14.4, H6), 1.59 (5H, m,

This article is protected by copyright. All rights reserved.

Accepted Article

H1, H7, H8,),1.46 (3H, s, H15), 1.30 (2H, m, H8, H13), 1.17 (3H, s, H18), 0.88 (3H, d, *J* 7.2 H17), 0.75 (3H, d, *J* 6.8, H16); ^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.05 (C3), 171.11,169.04 (C21), 151.17,139.13 (C19), 118.34, 117.29 (C20), 114.48, 74.61 (C11), 68.35 (C14), 59.98, 58.21 (C4), 55.57, 53.13, 50.48 (C22), 45.48 (C9), 45.05 (C13), 43.98 (C12), 41.81 (C5), 36.77(C6), 36.07 (C10), 34.48 (C2), 30.47 (C8), 26.86 (C7), 26.37 (C18), 24.87 (C1), 16.76 (C16), 14.92 (C15), 11.50 (C17); IR (KBr, cm^{-1}) 3444, 2936, 1734, 1511, 1454, 1383, 1285, 1244, 1201, 1153, 1116, 1019, 918, 823; HR-MS (ESI): Calcd for $C_{33}H_{48}N_2O_5$ ($M\text{-}H^+$): 553.3636; Found: 553.3633.

22-(4-(2-Methyl-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (12a)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 64.0%;m.p.: 55.0-57.4℃; ^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.16 (2H), 7.00 (2H), 6.54 (1H, dd, *J* 11.2, 17.6, H19), 5.82 (1H, d, *J* 8.4, H14), 5.35 (1H, d, *J* 11.2, H20), 5.21 (1H, d, 17.6, H20), 3.37 (1H, dd, *J* 6.4, 10.4, H11), 3.26 (1H, d, *J* 17.2, H22), 3.13 (1H, d, *J* 16.8, H22), 2.98 (4H), 2.73 (4H, d, *J* 27.6), 2.382 (1H, t, *J* 6.8, H2), 2.29 (3H, s), 2.15 (4H, m, H2, H4, H10, H13), 1.45 (9H, m, H1, H6, H7, H8, H13, 11-OH),1.47 (3H, s, H15),1.17 (3H, s, H18),0.88 (3H, d, *J* 6.8, H17), 0.76 (3H, d, *J* 6.4, H16); ^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.08 (C3), 169.14 (C21), 151.42,139.15 (C19), 132.64, 131.03, 126.59, 123.22, 119.12, 117.29 (C20), 74.62 (C11), 68.26 (C14),60.03,58.23 (C4), 53.46,51.48 (C22), 45.49 (C9), 45.08 (C13),43.97 (C12), 41.81 (C5), 36.79 (C6),36.09 (C10), 34.49 (C2), 30.48 (C8),26.86 (C7), 26.39 (C18), 24.88 (C1), 17.83,16.76 (C16), 14.94 (C15), 11.51 (C17); IR (KBr, cm^{-1}) 3498, 2950, 1727, 1491, 1452, 1421, 1402, 1381, 1281, 1240, 1208, 1172, 1149, 1123, 1037, 1021, 916, 819, 763, 724; HR-MS (ESI): Calcd for $C_{33}H_{48}N_2O_4$ ($M\text{-}H^+$): 537.3687; Found: 537.3684.

This article is protected by copyright. All rights reserved.

Accepted Article

22-(4-(3-Methyl-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (12b)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 72.0%; m.p.: 55.0-57.4℃;^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.15 (1H, t, *J* 7.6), 6.71 (3H, m), 6.53 (1H, dd, *J* 10.8, 17.2, H19), 5.81 (1H, d, *J* 8.4, H14),5.35 (1H, dd, *J* 1.2, 10.8, H20), 5.21 (1H, dd, *J* 1.2, 17.6, H20), 3.36 (1H, dd, *J* 6.4, 10.4, H11),3.24 (5H, m, H22), 3.10 (1H, d, *J* 17.2, H22), 2.71 (4H, d, *J* 22.4),2.36 (1H, t, *J* 7.2, H2), 2.31 (3H, s),2.15 (4H, m, H2, H4, H10, H13), 1.53 (9H, m, H1, H6, H7, H8, H13, 11-OH),1.46 (3H, s, H15), 1.17 (3H, s, H18), 0.88 (3H, d, *J* 7.2, H17),0.75 (3H, d, *J* 6.8, H16); ^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.05 (C3), 169.01 (C21), 151.25,139.12 (C19), 138.82, 128.97, 120.72,117.30 (C20), 117.06, 113.28, 74.61 (C11), 68.38 (C14),59.99,58.21 (C4), 53.07,49.02 (C22), 45.48 (C9), 45.06 (C13),43.98 (C12), 41.81 (C5), 36.77 (C6),36.08 (C10), 34.48 (C2), 30.47 (C8),26.86 (C7), 26.38 (C18), 24.87 (C1), 21.76,16.76 (C16), 14.92 (C15), 11.50 (C17); IR (KBr, cm^{-1}) 3556, 2934, 1735, 1602, 1494, 1453, 1363, 1294, 1190, 1152, 1116, 1016, 939, 844, 773, 693; HR-MS (ESI): Calcd for $C_{33}H_{48}N_2O_4$ ($M\text{-}H^+$): 537.3687; Found: 537.3682.

22-(4-(4-Methyl-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (12c)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 58.0%; m.p.: 55.0-57.4℃;^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.07 (2H, d, *J* 8.0), 6.84 (2H, d, *J* 7.0), 6.53 (1H, dd, *J* 10.8, 17.2, H19),5.81 (1H, d, *J* 8.8, H14), 5.35 (1H, dd, *J* 1.6, 11.2, H20), 5.20 (1H, dd, *J* 1.2, 17.6, H20), 3.36 (1H, dd, *J* 6.4, 10.0, H11), 3.23 (5H, m, H22), 3.10 (1H, d, *J* 17.2, H22), 2.72 (4H, d, *J* 22.8), 2.36 (1H, t, *J* 6.8, H2), 2.27 (3H, s), 2.14 (4H, m, H2, H4, H10, H13), 1.78 (1H, dd, *J* 2.8, 14.4, H6), 1.59 (4H, m, H1, H7),1.46 (3H, s, H15),1.33 (3H, m, H8, H13, 11-OH), 1.17 (3H, s, H18),1.12 (1H, m, H8),0.88 (3H, d, *J* 6.8 H17), 0.75 (3H, d, *J* 6.8, H16);^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.05 (C3), 169.01 (C21), 149.11, 139.12 (C19),

This article is protected by copyright. All rights reserved.

Accepted Article

129.66, 117.30 (C20), 116.54, 74.61 (C11), 68.36 (C14), 59.96, 58.21(C4), 53.05, 49.53 (C22), 45.48 (C9), 45.05 (C13), 43.98 (C12), 41.81 (C5), 36.77 (C6), 36.08 (C10), 34.48 (C2), 31.58, 30.47 (C8), 26.86 (C7), 26.38 (C18), 24.87 (C1), 20.42, 16.76 (C16), 14.92 (C15), 11.50 (C17); IR (KBr, cm^{-1})3563, 2933, 1734, 1615, 1515, 1453, 1412, 1383, 1303, 1236, 1196, 1152, 1115, 1019, 915, 811; HR-MS (ESI): Calcd for $C_{33}H_{48}N_2O_4$ ($M\text{-}H^+$): 537.3687; Found: 537.3680.

22-(4-(2-Hydroxy-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (13a)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 92.0%; m.p.: 55.0-57.4℃;^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.17 (1H, dd, *J* 1.6, 8.0), 7.08 (1H, m), 6.94 (1H, dd, *J* 1.2, 8.0), 6.86 (1H, m), 6.53 (1H, dd, *J* 10.8, 17.2, H19), 5.82 (1H, d, *J* 8.4, H14), 5.36 (1H, dd, *J* 1.2, 10.8, H20), 5.21 (1H, dd, *J* 1.2, 17.2, H20),3.37 (1H, dd, *J* 6.8, 10.0, H11), 3.28 (1H, d, *J* 16.8, H22), 3.15 (1H, d, *J* 17.2, H22), 2.95 (4H, m), 2.77 (4H, d, *J* 22.0), 2.37 (1H, t, *J* 6.8, H2), 2.16 (5H, m, H2, H4, H10, H13), 1.78 (1H, dd, *J* 2.8, 14.4, H6),1.60 (4H, m, H1, H7), 1.46 (3H, s, H15), 1.32 (3H, m, H8, H13, 11-OH), 1.18 (3H, s, H18), 1.13 (1H, m, H8), 0.89 (3H, d, *J* 7.2, H17), 0.75 (3H, d, *J* 6.8, H16); ^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.03 (C3), 171.13，169.02 (C21), 151.48,139.12 (C19), 126.57, 121.53, 120.09, 117.31 (C20), 114.08,74.62 (C11), 68.41 (C14), 60.38,59.77, 58.21 (C4), 53.48, 52.34 (C22),45.48 (C9), 45.06 (C13), 43.98 (C12), 41.81 (C5), 36.75 (C6),36.09 (C10), 34.47 (C2), 30.46 (C8),26.85 (C7), 26.40 (C18), 24.87 (C1),16.74 (C16), 14.92 (C15), 11.50 (C17); IR (KBr, cm^{-1}) 3419, 2937, 1734, 1589, 1439, 1455, 1375, 1283, 1250, 1195, 1152, 1116, 1019, 927, 756; HR-MS (ESI): Calcd for $C_{32}H_{46}N_2O_5$ ($M\text{-}H^+$): 539.3479; Found: 539.3477.

This article is protected by copyright. All rights reserved.

Accepted Article

22-(4-(3-Hydroxy-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (13b)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield:76.0%; m.p.: 55.0-57.4℃;^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.09 (1H, t, *J* 8.0),6.47 (3H, m), 6.32 (1H, dd, *J* 2.0, 8.0, H19), 5.81 (1H, d, *J* 8.4, H14), 5.45 (1H, br),5.34 (1H, dd, *J* 1.2, 10.8, H20), 5.20 (1H, dd, *J* 1.2, 17.2, H20), 3.36 (1H, dd, *J* 6.4, 10.8, H11), 3.23(5H, m, H22), 3.11(1H, d, *J* 17.2, H22), 2.71 (4H, d, *J* 22.0), 2.36 (1H, t, *J* 6.8, H2),2.16 (4H, m, H2, H4, H10, H13),1.78 (1H, dd, *J* 2.8, 14.4, H6), 1.60 (4H, m, H1, H7), 1.45 (3H, s, H15),1.33 (3H, m, H8, H13, 11-OH), 1.17 (3H, s, H18), 1.12 (1H, m, H8), 0.88 (3H, d, *J* 6.8, H17), 0.74 (3H, d, *J* 6.8, H16);^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.22 (C3), 169.00 (C21), 156.70, 152.63,139.05 (C19), 129.98, 117.36 (C20), 108.62,106.81, 103.22, 74.64 (C11), 68.46 (C14), 59.89, 58.22 (C4), 52.90(C22), 48.74,45.48 (C9), 45.05 (C13), 43.97 (C12), 41.81 (C5), 36.76 (C6),36.06 (C10), 34.49 (C2), 30.47 (C8),26.85 (C7), 26.37 (C18), 24.86 (C1),16.77 (C16), 14.93 (C15), 11.52 (C17); IR (KBr, cm^{-1}) 3375, 2935, 2862, 1731, 1599, 1453, 1193, 976, 866, 756, 689; HR-MS (ESI): Calcd for $C_{32}H_{46}N_2O_5$ ($M\text{-}H^+$): 539.3479; Found: 539.3477.

22-(4-(4-Hydroxy-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (13c)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield:64.0%; m.p.: 55.0-57.4℃;^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 6.862-6.841 (2H, d, *J* 8.4), 6.76 (2H, d, *J* 8.8), 6.55 (1H, dd, *J* 11.2, 17.6, H19), 5.81 (1H, d, *J* 8.4, H14), 5.34 (1H, dd, *J* 1.2, 10.8, H20), 5.22 (1H, dd, *J* 1.2, 17.6, H20), 5.15 (1H, br), 3.36 (1H, dd, *J* 6.4, 10.8, H11), 3.24 (1H, d, *J* 16.8, H22), 3.11 (5H, m, H22), 2.75 (4H, d, *J* 22.0), 2.36 (1H, t, *J* 6.8, H2), 2.17 (4H, m, H2, H4, H10, H13), 1.78 (1H, dd, *J* 2.8, 14.4, H6), 1.61 (4H, m, H1, H7), 1.45 (3H, s, H15), 1.32 (3H, m, H8, H13, 11-OH), 1.17 (3H, s, H18), 1.12 (1H, m, H8), 0.88 (3H, d, *J* 7.2, H17), 0.74 (3H, d, *J* 7.2, H16); ^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.18 (C3), 171.22, 69.05 (C21),

This article is protected by copyright. All rights reserved.

Accepted Article

150.10,139.08 (C19), 118.68, 117.32 (C20), 115.91,74.63 (C11), 68.40 (C14), 60.43,59.92, 58.220\ (C4), 53.08, 50.63 (C22),45.48 (C9), 45.04 (C13), 43.97 (C12), 41.81 (C5), 36.76 (C6),36.06 (C10), 34.49 (C2), 30.46 (C8),26.85 (C7), 26.36 (C18), 24.86 (C1),16.76 (C16), 14.93 (C15), 11.51 (C17); IR (KBr, cm^{-1}) 3382, 2931, 1732, 1589, 1493, 1454, 1375, 1283, 1250, 1152, 1116, 1019, 927, 812, 754; HR-MS (ESI): Calcd for $C_{32}H_{46}N_2O_5$ ($M\text{-}H^+$): 539.3479; Found: 539.3483.

22-(4-(2-Nitro-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (14a)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 52.0%; m.p.: 55.0-57.4℃;^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.75 (1H, d, *J* 8.0), 7.47 (1H, t, *J* 7.6), 7.15 (1H, d, *J* 8.4), 7.05 (1H, t, *J* 7.6), 6.52 (1H, dd, *J* 10.8, 17.2, H19), 5.81 (1H, d, *J* 8.4, H14), 5.34 (1H, d, *J* 11.2, H20), 5.21 (1H, dd, *J* 17.6, H20),3.36 (1H, dd, *J* 6.8, 10.8, H11), 3.24 (1H, d, *J* 16.8, H22), 3.13 (5H, m, H22),2.71 (4H, d, *J* 22.8), 2.36 (1H, t, *J* 6.8, H2), 2.15 (4H, m, H2, H4, H10, H13), 1.78 (1H, d, *J* 14.4, H6), 1.60 (4H, m, H1, H7), 1.46 (3H, s, H15), 1.31 (3H, m, H8, H13, 11-OH), 1.17(3H, s, H18), 1.13 (1H, m, H8), 0.88 (3H, d, *J* 6.8, H17), 0.74 (3H, d, *J* 6.8, H16); ^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.01 (C3), 169.00 (C21), 145.92, 143.65,139.10 (C19), 133.47, 125.80, 121.99, 121.10, 117.31 (C20), 74.61 (C11), 68.41 (C14), 59.87,58.20 (C4), 52.92,51.49 (C22),45.49 (C9), 45.05 (C13), 43.97 (C12), 41.80 (C5), 36.75 (C6),36.07 (C10), 34.47 (C2), 30.46 (C8),26.85 (C7), 26.38 (C18), 24.87 (C1),16.76 (C16), 14.91 (C15), 11.50 (C17); IR (KBr, cm^{-1})3500, 2931, 1727, 1601, 1527, 1452, 1382, 1358, 1286, 1209, 1151, 1124, 1036, 1019, 916; HR-MS (ESI): Calcd for $C_{32}H_{45}N_3O_6$ ($M\text{-}H^+$): 568.3381; Found: 568.3380.

This article is protected by copyright. All rights reserved.

Accepted Article

22-(4-(3-Nitro-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (14b)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 62.0%; m.p.: 55.0-57.4℃;^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.68 (2H, m), 7.37 (1H, t, *J* 8.4), 7.17 (1H, dd, J 2.0, 8.0), 6.52 (1H, dd, *J* 11.2, 17.2, H19), 5.82 (1H, d, *J* 8.4, H14), 5.35 (1H, dd, *J* 1.6, 11.2, H20),5.21 (1H, dd, *J* 1.6, 17.2, H20), 3.25 (7H, m, H11, H22), 2.75 (4H, d, *J*19.2), 2.36 (1H, t, *J* 6.8, H2), 2.15 (4H, m, H2, H4, H10, H13), 1.79 (1H, dd, *J* 2.8, 14.4, H6), 1.59 (4H, m, H1, H7),1.46 (3H, s, H15), 1.32 (2H, m, H8, H13), 1.18 (3H, s, H18),1.13 (1H, m, H8), 0.89 (3H, d, *J* 6.8, H17), 0.75 (3H, d, *J* 6.8, H16); ^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.00 (C3), 168.88 (C21), 151.66, 149.30,139.09 (C19), 129.72, 121.06,117.31 (C20), 113.75, 109.76, 74.61 (C11), 68.53 (C14),59.78,58.19 (C4), 52.51,48.16 (C22), 45.47 (C9), 45.07 (C13),43.98 (C12), 41.80 (C5), 36.73 (C6),36.08 (C10), 34.47 (C2), 30.45 (C8),26.85 (C7), 26.34 (C18), 24.87 (C1),16.77 (C16), 14.91 (C15), 11.50 (C17); IR (KBr, cm^{-1}) 3554, 2926, 1732, 1616, 1525, 1455, 1343, 1293, 1241, 1201, 1175, 1114, 1016, 934, 866, 847, 759, 736, 667; HR-MS (ESI): Calcd for $C_{32}H_{45}N_3O_6$ ($M\text{-}H^+$): 568.3381; Found: 568.3378.

22-(4-(4-Nitro-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (14c)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 72.0%; m.p.: 55.0-57.4℃; ^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 8.13 (2H, d, *J* 9.6), 6.82 (2H, d, *J* 9.2), 6.51 (1H, dd, *J* 11.2, 17.6, H19),5.82 (1H, d, *J* 8.4, H14),5.35 (1H, dd, *J* 1.2, 10.8, H20), 5.21 (1H, dd, *J* 1.2, 17.2, H20), 3.11 (11H, m, H11, H22), 2.35 (1H, t, *J* 6.8, H2),2.15 (4H, m, H2, H4, H10, H13), 1.78 (1H, dd, *J* 2.8, 14.4, H6), 1.58 (5H, m, H1, H7, 11-OH), 1.45 (3H, s, H15), 134 (2H, m, H8, H13), 1.18 (3H, s, H18),1.13 (1H, m, H8), 0.89 (3H, d, *J* 7.2, H17), 0.74 (3H, d, *J* 6.8, H16); ^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 216.92 (C3), 168.88 (C21), 151.66, 149.30, 139.07 (C19), 125.96, 121.06, 117.32 (C20), 112.85, 109.76, 74.60 (C11), 68.53 (C14), 59.16,58.16

This article is protected by copyright. All rights reserved.

Accepted Article

(C4), 52.19,46.82 (C22), 45.46 (C9), 45.05 (C13), 43.98 (C12), 41.78 (C5), 36.68 (C6),36.09 (C10), 34.45 (C2), 30.42 (C8),26.85 (C7), 26.41 (C18), 24.86 (C1),16.77 (C16), 14.88 (C15), 11.49 (C17); IR (KBr, cm^{-1}) 3453, 2932, 1131, 1597, 1503, 1453, 1383, 1323, 1241, 1203, 1153, 1116, 1013, 920, 829, 755, 692, 666; HR-MS (ESI): Calcd for $C_{32}H_{45}N_3O_6$($M\text{-}H^+$): 568.3381; Found: 568.3382.

22-(4-(2-Chloro-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (15a)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 36.0%; m.p.:213.0-213.4℃;^{1}H-NMR spectrum (600 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.35 (1H, dd, *J* 1.2, 8.0), 7.22 (1H, dt, *J* 1.2, 7.8), 7.057 (1H, dd, *J* 1.8, 13.8), 6.97 (1H, dt, *J* 1.2, 7.8), 6.54 (1H, dd, *J* 11.4, 17.4, H19), 5.82 (1H, d, *J* 8.4, H14), 5.35 (1H, dd, *J* 1.8, 10.8, H20), 5.21 (1H, dd, *J* 1.2, 17.4, H20), 3.37 (1H, dd, *J* 5.4, 10.8, H11), 3.26 (1H, d, *J* 16.8, H22), 3.13 (5H, m, H22), 2.79(2H, d, *J* 4.2), 2.72 (2H, d, *J* 4.2), 2.37 (1H, m, H2), 2.18 (4H, m, H2, H4, H10, H13), 1.78 (1H, d, *J* 3.0, 14.4, H6), 1.60 (4H, m, H1, H7), 1.47 (3H, s, H15), 1.36 (2H, m, H8, H13), 1.18 (3H, s, H18), 1.13 (1H, m, H8), 0.89 (3H, d, *J* 6.6, H17), 0.76 (3H, d, *J* 7.2, H16); ^{13}C NMR (150 MHz, d_1-$CDCl_3$):δ (ppm) 217.00 (C3), 169.08 (C21), 149.22, 139.72 (C19), 130.61, 128.83, 127.59, 123.72, 120.46, 117.23 (C20), 74.61 (C11), 68.33 (C14), 60.00,58.21 (C4), 53.21,50.95 (C22), 45.48 (C9), 45.08 (C13), 43.98 (C12), 41.81 (C5), 36.77 (C6),36.09 (C10), 34.47 (C2), 30.47 (C8),26.86 (C7), 26.43 (C18), 24.87 (C1),16.74 (C16), 14.93 (C15), 11.49 (C17); IR (KBr, cm^{-1}) 3500, 2953, 1727, 1480, 1292, 1211, 1151, 1020, 916, 766. HR-MS (ESI): Calcd for $C_{32}H_{45}ClN_2O_4$ ($M\text{-}H^+$) : 557.3141; Found: 557.3145.

22-(4-(3-Chloro-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (15b)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 60.0%; m.p.:65.1-65.8℃;^{1}H-NMR spectrum (600 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.15 (1H, t, *J* 7.8), 6.86 (1H, t, *J* 1.8), 6.80(1H, dd, *J* 1.8, 7.8), 6.77

This article is protected by copyright. All rights reserved.

Accepted Article

(1H, dd, *J* 2.4, 8.4), 6.52 (1H, dd, *J* 10.8, 17.4, H19), 5.81 (1H, d, *J* 8.4, H14), 5.35 (1H, dd, *J* 1.8, 11.4, H20), 5.21 (1H, dd, *J* 1.8, 17.4, H20), 3.37 (1H, dd, *J* 6.6, 10.8, H11), 3.25 (5H, m, H22), 3.11 (1H, d, *J* 16.8, H22), 2.74 (2H, m), 2.67(2H, m), 2.37 (1H, m, H2), 2.23 (2H, m, H10, H13), 2.09 (2H, m, H2, 11-OH), 2.04(1H, s, H4), 1.78 (1H, dd, *J* 3.0, 14.4, H6), 1.61 (4H, m, H1, H7), 1.46 (3H, s, H15), 1.37 (1H, m, H8), 1.31 (1H, d, *J* 16.2, H13), 1.18 (3H, s, H18), 1.13 (1H, m, H8), 0.89 (3H, d, *J* 6.6, H17), 0.75 (3H, d, *J* 7.2, H16); ^{13}C NMR (150 MHz, d_1-$CDCl_3$):*δ* (ppm) 216.99 (C3), 168.92 (C21), 152.21, 139.15 (C19), 134.95, 130.02, 119.31, 117.23 (C20), 115.82, 113.89, 74.60 (C11), 68.46(C14), 59.86,58.12 (C4), 52.74,48.45 (C22), 45.47 (C9), 45.09 (C13), 43.98 (C12), 41.80 (C5), 36.74 (C6),36.08 (C10), 34.46 (C2), 30.45 (C8),26.85 (C7), 26.44 (C18), 24.87 (C1),16.74 (C16), 14.91 (C15), 11.49 (C17); IR (KBr, cm^{-1}) 3457, 2936, 1734, 1594, 1454, 1384, 1200, 1152, 1016, 939. HR-MS (ESI): Calcd for $C_{32}H_{45}ClN_2O_4$ ($M\text{-}H^+$): 557.3141; Found: 557.3140.

22-(4-(4-Chloro-phenyl)-piperazin-1-yl)-22-deoxypleuromutilin (15c)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 72.0%; m.p.: 55.0-57.4℃;^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): *δ* (ppm) 7.19 (2H, d, *J* 8.8), 6.82 (2H, d, *J* 8.8), 6.52 (1H, dd, *J* 10.8, 17.2, H19),5.81 (1H, d, *J* 8.4, H14), 5.34 (1H, dd, *J* 1.2, 11.2, H20),5.21 (1H, dd, *J* 1.2, 17.6, H20), 3.36 (1H, dd, *J* 6.4, 10.4, H11), 3.22 (5H, m, H22), 3.10 (1H, d, *J* 16.8, H22), 2.70 (4H, m), 2.36 (1H, t, *J* 7.2, H2), 2.15 (4H, m, H2, H4, H10, H13), 1.800-1.756 (1H, dd, *J* 2.8, 14.4, H6), 1.59 (5H, m, H1, H7, 11-OH), 1.45(3H, s, H15), 1.34 (2H, m, H8, H13), 1.17 (3H, s, H18), 1.12 (1H, m, H8), 0.88 (3H, d, *J* 6.8, H17), 0.74 (3H, d, *J* 6.8, H16);^{13}C NMR (100 MHz, d_1-$CDCl_3$):*δ* (ppm) 217.04 (C3), 169.01 (C21), 149.85, 139.12 (C19),128.95, 124.59, 117.29 (C20), 74.60 (C11), 68.39 (C14), 61.30, 59.93,58.20 (C4), 52.85,48.98 (C22), 45.47 (C9), 45.06 (C13), 43.97 (C12), 41.80 (C5), 36.75 (C6),36.07 (C10), 34.47 (C2), 30.46 (C8),26.85 (C7), 26.39 (C18), 24.86 (C1),16.76 (C16), 14.92 (C15), 11.50 (C17); IR (KBr,

This article is protected by copyright. All rights reserved.

Accepted Article

cm^{-1}) 3557, 2936, 1732, 1596, 1496, 1451, 1384, 1292, 1234, 1202, 1153, 1115, 1018, 916, 818, 754; HR-MS (ESI): Calcd for $C_{32}H_{45}ClN_2O_4$ (M-H$^+$): 557.3141; Found: 557.3142.

22-(4-phenyl-piperazin-1-yl)-22-deoxypleuromutilin (16)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 50.1%; m.p.:76.0-76.6℃; ^{1}H-NMR spectrum (600 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 7.19 (2H), 6.85 (2H), 6.79 (1H), 6.46 (1H, dd, *J* 11.4, 17.4, H19), 5.74 (1H, d, *J* 8.4, H14), 5.27 (1H, d, *J* 11.4, H20), 5.13 (1H, dd, 17.4, H20), 3.29 (1H, dd, *J* 6.6, 9.6, H11), 3.17 (5H，H22), 3.04 (1H, d, *J* 17.4, H22), 2.66 (4H, m), 2.29 (1H, t, *J* 6.6, H2), 2.10 (4H, m, H2, H4, H10, H13), 1.71 (1H, dd, *J* 3.0, 14.4, H6), 1.51 (5H, m, H1, H7, H8),1.28 (3H, m, H13, 11-OH), 1.39 (3H, s, H15), 1.10 (3H, s, H18), 0.81 (3H, d, *J* 17.2, H17), 0.68 (3H, d, *J* 7.2, H16); ^{13}C NMR (150 MHz, d_1-$CDCl_3$):δ (ppm) 217.01 (C3), 168.94 (C21), 151.16, 139.15 (C19), 129.13, 119.87, 117.26 (C20), 116.20, 74.62 (C11), 68.44 (C14), 59.92, 58.21 (C4), 52.99, 48.97 (C22), 45.48 (C9), 45.08 (C13), 43.99 (C12), 41.82 (C5), 36.76 (C6), 36.09 (C10), 34.47 (C2), 31.58 (C8), 30.470, 26.89 (C7), 26.42 (C18), 24.88 (C1), 16.76 (C16), 14.93 (C15), 11.49 (C17); IR (KBr, cm^{-1}) 3452, 2935, 1732, 1599, 1453, 1202, 1153, 1017, 917, 759, 692; HR-MS (ESI): Calcd for $C_{32}H_{46}N_2O_4$ (M-H$^+$): 523.3530; Found: 523.3524.

22-(4-Methyl-piperazin-1-yl)-22-deoxypleuromutilin (17)

Prepared according to the above general preparation of pleutromutilin derivatives **in scheme 1**. Light pink powder; yield: 32.0%; m.p.: 55.0-57.4℃; ^{1}H-NMR spectrum (400 MHz; d_1-$CDCl_3$; TMS): δ (ppm) 6.52 (1H, dd, *J* 11.2, 17.6, H19), 5.79 (1H, d, *J* 8.4, H14),5.33 (1H, dd, *J* 1.2, 10.8, H20), 5.20 (1H, dd, *J* 1.2, 17.6, H20), 3.35 (1H, dd, *J* 6.8, 8.8, H11),3.17 (1H, d, *J* 17.2, H22),3.04 (1H, d, *J* 17.2, H22), 2.60 (8H, m) 2.36 (1H, d, *J* 5.4, H4), 2.33 (3H, s), 2.14 (4H, m, H2, H10, H13),1.61 (6H, m, H1, H6, H7, H8,),1.44 (3H, s, H15),1.30 (1H, m, H8),1.29(1H, d, *J* 12, H13),1.16 (3H, s, H18), 0.88 (3H, d, *J* 7.2 H17), 0.73 (3H, d, *J* 6.8,

This article is protected by copyright. All rights reserved.

Accepted Article

H16);^{13}C NMR (100 MHz, d_1-$CDCl_3$):δ (ppm) 217.06 (C3), 169.08 (C21), 139.13 (C19), 117.26 (C20), 74.60 (C11), 68.29 (C14), 59.93, 58.20 (C4), 54.71, 52.79 (C22), 45.81, 45.49 (C9), 45.02 (C13), 43.97 (C12), 41.79 (C5), 36.75 (C6), 36.05 (C10), 34.48 (C2), 30.46 (C8), 26.84 (C7), 26.35 (C18), 24.86 (C1), 16.73 (C16), 14.91 (C15), 11.50 (C17); IR (KBr, cm^{-1}) 3503, 2930, 1727, 1480, 1452, 1292, 1209, 1151, 1123, 1038, 916, 765; HR-MS (ESI): Calcd for $C_{27}H_{44}N_2O_4$ ($M\text{-}H^+$): 461.3374; Found: 461.3373.

Minimal inhibitory concentration (MIC) testing

MIC values of these novel pleuromutilin derivatives, and pleuromutilin were determined by agar dilution in accordance with the "*Clinical and Laboratory Standards Institute*"(CLSI, 2008).The following reference strains were used for quality control: *Staphylococcus aureus* ATCC 29213.

Preparation of stock and working solutions of pleuromutilin derivatives

Stock solutions of these compounds were prepared in N,N-Dimethylformamide (DMF) at the concentrations of 5120 μg/ml. The working solutions (256 μg/ml) were obtained by diluting stock solutions in sterile Mueller Hinton broth.

Preparation solution of bacteria

After all three strains were all recovered, single colony selected was incubated on Mueller Hinton agar respectively, followed by incubation for 18-24 h at 37℃. Then, inocula were prepared by transferring several colonies of bacteria to saline. The suspensions were mixed for 15 s and then corrected to 0.5 McF arland standard using saline. Further dilutions in saline were made to get the required working suspensions (10^5 CFU/ml).

The determination of MIC

The test was performed in 96-well plate. All dates were tested in duplicate in each plate. 100 μl of Mueller Hinton broth was added into all the wells of the 96-well plate. 100 μl of the working solutions (256 μg/ml) of compounds were added into the wells in rows A to H in

This article is protected by copyright. All rights reserved.

Accepted Article

column 1 and well mixed. Then 100 µl of the mixture in column 1 was inhaled to add into the wells in column 2, well mixed. The similar operation was repeated until the 10th row of wells was filled. 100 µl of excess medium was discarded from the wells in column 10. 100 µl of the bacteria solution was added to the wells in rows A to H in columns 1 to 10, well mixed. Two columns served as drug-free controls (no cultures were added in one column and drugs replaced by blank solvent in the other column). Tiamulin was used as positive controls against bacteria. The final concentration of DMF in the first well column was 1.25%. Initially, preliminary analyses were conducted with 1.25% (v/v) DMF/MHB and this did not affect neither the growth of the tested bacteria nor the determination of MIC. The concentration of drugs in each row of well were 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 µg/ml respectively. Each 96-well plate was covered and incubated for 18-24 h at 37℃. The MIC value was defined as the lowest concentration of the sample which inhibits the visible growth of test bacteria.

Molecular Modeling

For all these compounds bearing a same pleuromutilin scaffold, similarity-based molecular docking was performed to analyze the different activities of compounds. It has been revealed that tiamulin and valnemulin bind to domain V of 23S rRNA in the peptidyl transferase center (PTC) of *Escherichia coli* ribosomes (24). Thus, the peptidyl transferase center (PTC) ribosome model was constructed that consists of all residues within 30 Åfrom the PTC binding site. Because of the lack of crystal structures of *Escherichia coli* in complex with pleuromutilin analogues, the PTC model was constructed based on the crystal structure of *Deinococcus radiodurans* in complex with tiamulin (PDB ID: 1XBP) (9, 25). AutoDockTools were used for docking study. Briefly, the receptor used for docking was extracted from the crystal structure of 1XBP in which the tiamulin was removed. The binding site of tiamulin in 1XBP was set as docking position. The compounds were prepared with Avogadro 1.1.1 (25),

This article is protected by copyright. All rights reserved.

Accepted Article

with a 5000 steps Steepest Descent as well as 1000 steps Conjugate Gradients geometry optimization using MMFF94 force field.

Results and Discussion

Chemistry

All those pleuromutilin derivatives were prepared as shown in Scheme 1 from 22-*O*-tosylpheuromutilin (1a) and piperazine derivatives following the method reported by Hirokawa et. al. (7). In brief, KI was added into the reaction solution reaction to transfer the 22-O-tosylpheuromutilin into iodide compound, which is much easier to substitute than the tosyl group. Actually, in addition to the Scheme 1, all those compounds could be synthesized directly from 1a with much longer time reactions. However, this method was given up for lower yield.

All those compounds were purified by silica column chromatography. Melt points of those compounds were measured using the Shenguang X-4 apparatus (China) apparatus and are uncorrected, which were used to ensure the purity of those compounds. The structures of those compounds were characterized by FT-IR, ^{1}H-NMR, ^{13}C-NMR and HR-MS.

Antibacterial activity

The antibacterial actives of those pleuromutilin derivatives were evaluated against *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* which was isolated from GuangDong Province in 2013 and *Escherichia coli* ATCC25922 using agar dilution method. The Minimum inhibitory concentrations (MIC) of those 17 new pleuromutilin derivatives, 11a-c, 12a-c, 13a-c, 14a-c, 15a-c 16 and 17, as well as tiamulin used as positive control, were determined in duplicated at pH 7.40. The results of these studies are listed in Table 1.

The activities of compounds 16 and 17 were evaluated (bioactivities: 16>17) at first. This observation suggests that the replacement of the methyl on the nitrogen atom of the piperazine ring with benzene ring increase the electron densities of C14 side chain of

This article is protected by copyright. All rights reserved.

Accepted Article

pleuromutilin derivatives, which resulted in compound 16 possessing higher antibacterial activities compared with compound 17. A series of novel pleuromutilin derivatives containing thiadiazole moieties with substituted phenyl have been synthesized by Shang et. al.(26). The substitution position in the benzene ring attached on the thiadiazole appeared to have influence on the antibacterial activity of these pleuromutilin derivatives. For the potent activity exhibited by the benzene ring in the pleuromutilin derivatives, as well as the results from Shang et. al.(26), further research on benzene ring was carried on.

Using the piperazine derivatives 16 and 17 as lead compounds, our research focused on the substitution pattern on the benzene ring attached on the piperazine ring of those pleuromutilin derivatives. Both electron withdrawing and donating groups on the *ortho*-, *meta*-, and *para*-position of the benzene ring were introduced to explore the SAR (Table 1). It can be observed that most compounds exhibited relatively moderate antibacterial activities against both *Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus*(Guangdong isolated). It could also be obtained that all these 17 compounds displayed pretty weak activities against *E. coli*.

The *ortho*- substituted benzene derivatives with CH_3O- and CH_3- (compounds 11a and 12a) exhibited much less potency compared to compounds 16 and 17. The *ortho*-OH substituted compound 13a showed the same antibacterial activities as 16 and tiamulin against ATCC 29213 and *Staphylococcus aureus* (Guangdong isolated).The *ortho*-NO_2 substituted derivative 14a was the most active compound in this series. However, replacement of the nitro group in compound 14a with chlorine group at the same position, which resulted the compound 15a dramatically reduced the antibacterial activity.

The *meta*- substituted benzene derivatives with CH_3O-, CH_3- and NO_2- substituents showed enhanced antibacterial activities (11b, 13b and 14b compared with 16). Compounds 11b and 13b displayed superior antibacterial activities to that of compound 16 and tiamulin against

This article is protected by copyright. All rights reserved.

Accepted Article

ATCC 29213 and *Staphylococcus aureus* (Guangdong isolated). Compound 14b displayed higher antibacterial activity than tiamulin against ATCC 29213 as well as the same activity as tiamulin against *Staphylococcus aureus* (Guangdong isolated). The *meta*- substituted benzene derivatives with CH_3- and Cl- (compounds 12b and 15b) showed less potency compared to tiamulin.

Generally, all the *para*- substituted benzene derivatives bearing methoxyl, methyl, hydroxyl, nitro and chlorine substituents (11c, 12c, 13c, 14c and 15c) showed moderate antibacterial activities. Compound 14c, bearing nitro group in the *para*-position showed higher activity compared to other *para*-position substituted derivatives. The *para*-position appeared to have less influence on the antibacterial activity of these pleuromutilin derivatives bearing a phenyl piperazine.

Molecular Docking Study

Three tested pleuromutilin derivatives with excellent antibacterial activities, 11b, 13b and 14a, were selected for molecular docking investigations. Compound 11b was found to form a strong hydrogen bond with ribosome in the docking studies. As shown in Fingure 2, the oxygen atom of 11b (on C3) formed one H-bond with the hydroxyl group of G2484 (O/OH distance: 1.9Å). The binding free energy of 11b with ribosome was calculated to be -12.99 kcal/mol. Compound 13b, with the same antibacterial activity as 11b, demonstrated a similar binding mode to that of 11b. From Figure 3, two strong hydrogen bond were observed through the interaction of 13b with G2044 (O/NH, 1.7Å) and G2484 (H/OH, 2.6Å). Although the binding free energy of 13b with ribosome (-12.12 kcal/mol) was higher than that of 11b, the antibacterial activity of 13b was the same as that of 11b. One possible reason was that the replacement of the methoxyl substituent in 11b with a hydroxy substituent on the benzene ring gave 13b that yield better solubility.

This article is protected by copyright. All rights reserved.

Accepted Article

The molecular docking results of 14a are shown in Fig. 4. The binding free energy of 14a with ribosome was calculated to be -6.94 kcal/mol. Only one hydrogen bond was observed through the interaction of 14a with G2044 (O/NH, 1.7Å). However, there are 9 residues around 14a with distance less than 3Åin this docking mode. The interaction between those residues and 14a might be the main reason that why 14a possessed the best antibacterial activities than all the other derivatives in this paper.

Conclusions

A series of 17 novel pleuromutilin derivatives with piperazine as a linker including various phenyl substituents into the C14 side chain has been reported. The obtained MIC values revealed that all the synthesized derivatives possessed excellent antibacterial activity. SAR studies have shown that a benzene substituent bearing *meta*-hydroxy, *meta*-methoxy and *ortho*-nitro groups lead to increased antibacterial activity in vitro. Among the derivatives prepared, compounds 11b, 13b and 14a exhibited the most potent activity. Docking studies were performed on these three compounds. The results showed that the binding free energies were -12.99, -12.12 and -6.94 kcal/mol, respectively. The discovery of compounds 11b, 13b and 14a and the SAR, and docking studies reported here will provide insight into further optimization of pleuromutilin derivatives.

Acknowledgements

This work was supported by the Pearl River S&T Noval Program of Guangzhou (No. 2014J2200042) and the Natural Science Foundation of Guangdong Province (No. S2012030006590).

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Figure Captions

Scheme 1 Reagent and conditions: (i) *p*-toluenesulfonyl chloride, pyridine, 0℃, 3h, 63.5%; (ii) NaI, K_2CO_3, acetonitrile, reflux; (iii) piperazine derivatives, acetonitrile, reflux.

Table 1 MIC (μg/ml) for *Staphylococcus aureus* (*S. aureus*) ATCC 29213, *Staphylococcus aureus*(S. aureus) (Guangdong isolated) and*Escherichia coli* (*E. coli*) ATCC25922.

Figure 1 Structure of pleuromutilin (1), tiamulin (2), valnemulin (3) and retapamulin (4).

Figure 2 Result of the docking of 11b (green) into the PTC model binding site. Residue numbers shown are according to *E. coli* 23s RNA numbering.

Figure 3 Result of the docking of 13b (yellow) into the PTC model binding site. Residue numbers shown are according to *E. coli* 23s RNA numbering.

Figure 4 Result of the docking of 14a (purple) into the PTC model binding site. Residue numbers shown are according to *E. coli* 23s RNA numbering.

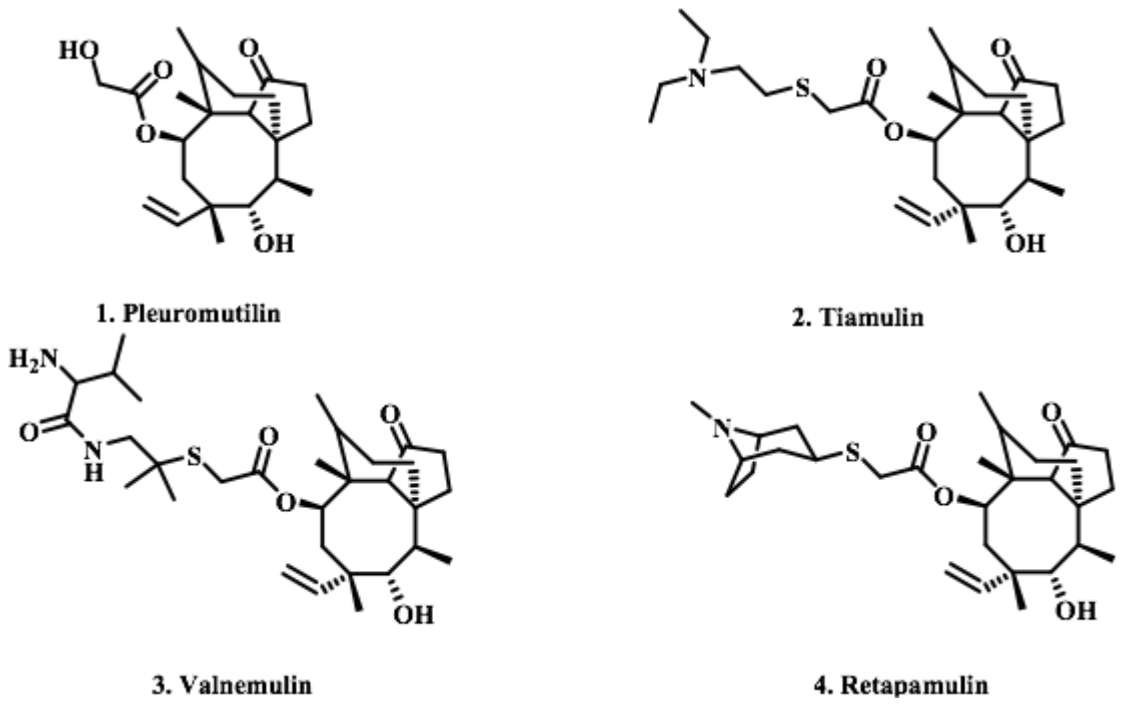
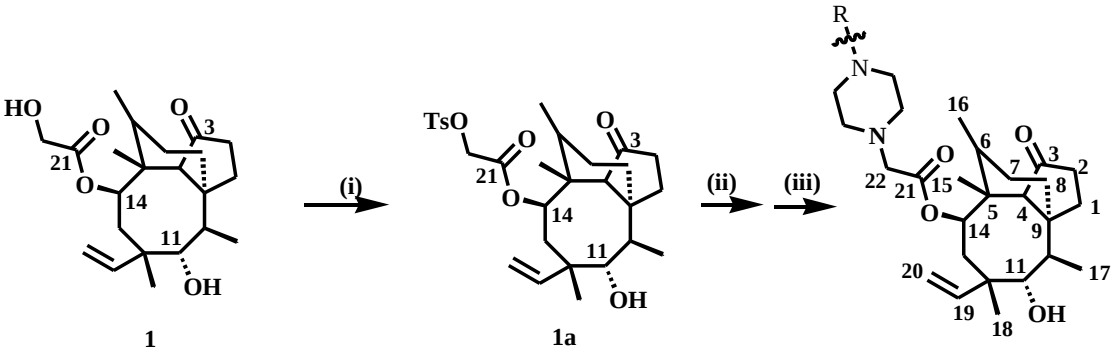


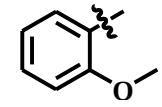
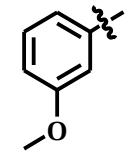
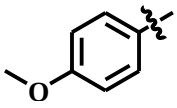
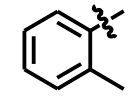
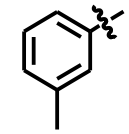
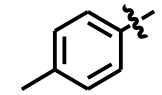
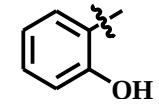
Figure 1

This article is protected by copyright. All rights reserved.



1 **1a**

Scheme 1

Compounds	R	*S. aureus* ATCC 29213	*S. aureus* (Guangdong isolated)	*E. coli* ATCC25922
11a		**64**	**64**	**>128**
11b		**0.125**	**0.125**	**>128**
11c		**0.5**	**0.5**	**>128**
12a		**64**	**64**	**>128**
12b		**0.5**	**0.5**	**>128**
12c		**2**	**2**	**>128**
13a		**0.25**	**0.25**	**>128**

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

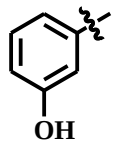
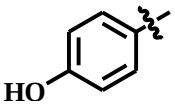
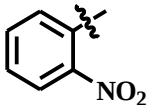
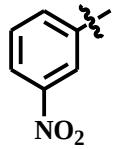
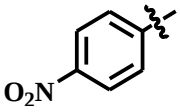
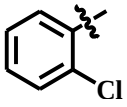
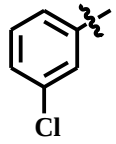
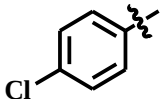
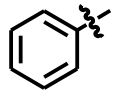
13b		**0.125**	**0.125**	**>128**
13c		**2**	**2**	**>128**
14a		**0.0625**	**0.0625**	**>128**
14b		**0.125**	**0.25**	**>128**
14c		**0.25**	**0.5**	**>128**
15a		**1**	**1**	**>128**
15b		**0.5**	**0.5**	**>128**
15c		**1**	**1**	**>128**
16		**0.25**	**0.25**	**>128**
17		**1**	**8**	**>128**
Tiamulin		**0.25**	**0.25**	**128**

Table 1

This article is protected by copyright. All rights reserved.

Accepted Article

References

1. Dreier I, Hansen LH, Nielsen P, Vester B (2014) A click chemistry approach to pleuromutilin derivatives. Part 3: Extended footprinting analysis and excellent MRSA inhibition for a derivative with an adenine phenyl side chain. *Bioorg Med Chem Lett;***24**: 1043-6.
2. Ling C, Fu L, Gao S, Chu W, Wang H, Huang Y, et al. (2014) Design, Synthesis, and Structure-Activity Relationship Studies of Novel Thioether Pleuromutilin Derivatives as Potent Antibacterial Agents. *J Med Chem;***57**: 4772-95.
3. Aviles E, Prudhomme J, Le Roch KG, Franzblau SG, Chandrasena K, Mayer AMS, et al. (2015) Synthesis and preliminary biological evaluation of a small library of hybrid compounds based on Ugi isocyanide multicomponent reactions with a marine natural product scaffold. *Bioorg Med Chem Lett;***25**: 5339-43.
4. Li X, Ma S (2015) Advances in the discovery of novel antimicrobials targeting the assembly of bacterial cell division protein FtsZ. *Eur J Med Chem;***95**: 1-15.
5. Kavanagh F, Hervey A, Robbins WJ (1951) Antibiotic Substances From Basidiomycetes: VIII. Pleurotus Multilus (Fr.) Sacc. and Pleurotus Passeckerianus Pilat. *Proc Natl Acad Sci;***37**: 570-4.
6. Kavanagh F, Hervey A, Robbins WJ (1952) Antibiotic Substances from Basidiomycetes: IX. Drosophila Subtarata. (Batsch Ex Fr.) Quel. *Proc Natl Acad Sci;***38**: 555-60.
7. Hirokawa Y, Kinoshita H, Tanaka T, Nakamura T, Fujimoto K, Kashimoto S, et al. (2009) Pleuromutilin derivatives having a purine ring. Part 3: Synthesis and antibacterial activity of novel compounds possessing a piperazine ring spacer. *Bioorg Med Chem Lett;***19**: 175-9.
8. Hodgin LA, Hogenauer G (1974) The mode of action of pleuromutilin derivatives. Effect on cell-free polypeptide synthesis. *European journal of biochemistry / FEBS;***47**: 527-33.
9. Schlunzen F, Pyetan E, Fucini P, Yonath A, Harms JM (2004) Inhibition of peptide bond formation by pleuromutilins: the structure of the 50S ribosomal subunit from Deinococcus radiodurans in complex with tiamulin. *Mol Microbiol;***54**: 1287-94.
10. Fazakerley NJ, Procter DJ (2014) Synthesis and synthetic chemistry of pleuromutilin. *Tetrahedron;***70**: 6911-30.
11. Egger H, Reinshagen H (1976) New pleuromutilin derivatives with enhanced antimicrobial activity.II.Structure-activity correlations. *The Journal of Antibiotics;***29**: 923-7.
12. Egger H, Reinshagen H (1976) New pleuromutilin derivatives with enhanced antimicrobial activity. I. Synthesis. *The Journal of Antibiotics;***29**: 915-22.
13. Riedl K (1976) Studies on pleuromutilin and some of its derivatives. *The Journal of Antibiotics;***29**: 132-9.
14. Choudary B, Giles RG, Jovic F, Lewis N, Moore S, Urquhart M (2012) Synthesis of the Pleuromutilin Antibiotic SB-268091: A New Practical and Efficient Synthesis of Quinuclidine-4-thiol. *Org Process Res Dev;***16**: 1927-39.
15. Ruscoe RE, Fazakerley NJ, Huang H, Flitsch S, Procter DJ (2016) Copper-Catalyzed Double Additions and Radical Cyclization Cascades in the Re-Engineering of the Antibacterial Pleuromutilin. *Chem-Eur J;***22**: 116-9.

This article is protected by copyright. All rights reserved.

Accepted Article

16. Fazakerley NJ, Helm MD, Procter DJ (2013) Total Synthesis of (+)-Pleuromutilin. *Chem-Eur J;***19**: 6718-23.

17. Uccello DP, Miller SM, Dieterich NA, Stepan AF, Chung S, Farley KA, et al. (2011) The synthesis of C-13 functionalized pleuromutilins via C-H amidation and subsequent novel rearrangement product. *Tetrahedron Lett;***52**: 4247-51.

18. Novak R, Shlaes DM (2010) The pleuromutilin antibiotics: A new class for human use. *Current Opinion in Investigational Drugs;***11**: 182-91.

19. Dreier I, Kurnar S, Sondergaard H, Rasmussen ML, Hansen LH, List NH, et al. (2012) A Click Chemistry Approach to Pleuromutilin Derivatives, Part 2: Conjugates with Acyclic Nucleosides and Their Ribosomal Binding and Antibacterial Activity. *J Med Chem;***55**: 2067-77.

20. Tang Y, Luo J, Chen X, Wang B, Shen X, Liu J (2013) Synthesis and in vitro antibacterial activity of four novel pleuromutilin derivatives. *Journal of the Chilean Chemical Society;***58**: 1537-40.

21. Emami S, Ghafouri E, Faramarzi MA, Samadi N, Irannejad H, Foroumadi A (2013) Mannich bases of 7-piperazinylquinolones and kojic acid derivatives: Synthesis, in vitro antibacterial activity and in silico study. *Eur J Med Chem;***68**: 185-91.

22. Mentese MY, Bayrak H, Uygun Y, Mermer A, Ulker S, Karaoglu SA, et al. (2013) Microwave assisted synthesis of some hybrid molecules derived from norfloxacin and investigation of their biological activities. *Eur J Med Chem;***67**: 230-42.

23. Lolk L, Pohlsgaard J, Jepsen AS, Hansen LH, Nielsen H, Steffansen SI, et al. (2008) A click chemistry approach to pleuromutilin conjugates with nucleosides or acyclic nucleoside derivatives and their binding to the bacterial ribosome. *J Med Chem;***51**: 4957-67.

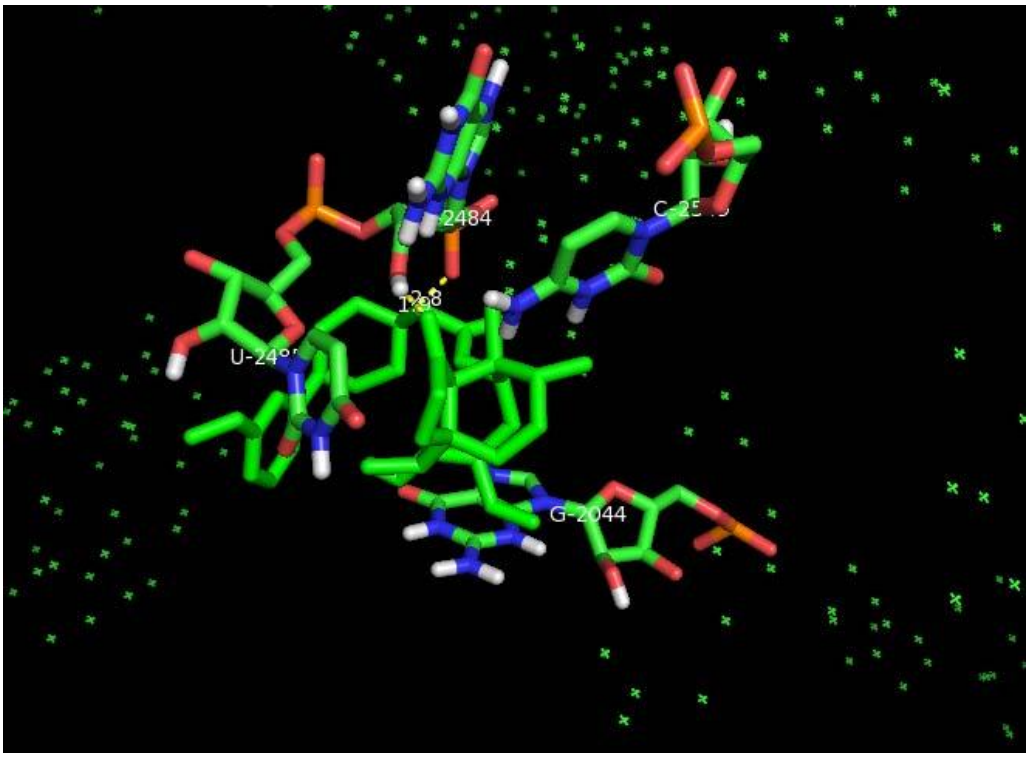
24. Poulsen SM, Karlsson M, Johansson LB, Vester B (2001) The pleuromutilin drugs tiamulin and valnemulin bind to the RNA at the peptidyl transferase centre on the ribosome. *Mol Microbiol;***41**: 1091-9.

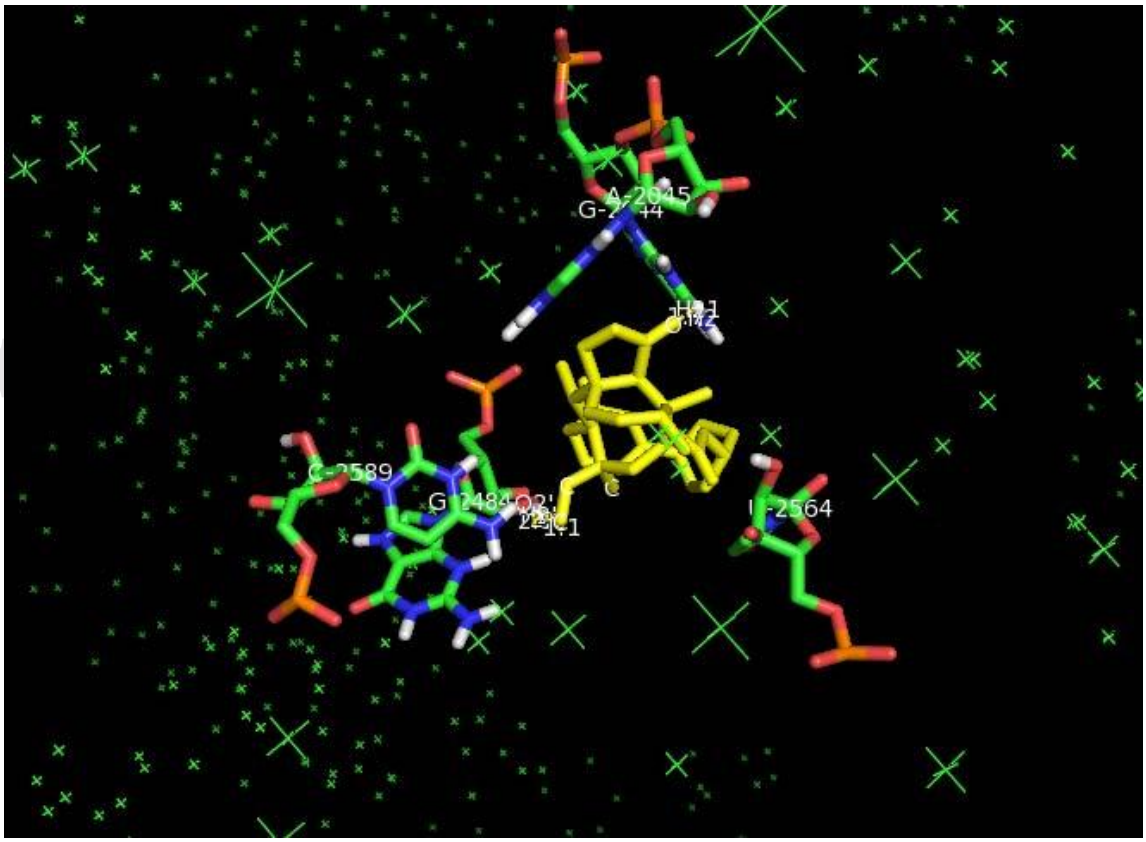
25. Hanwell MD, Curtis DE, Lonie DC, Vandermeersch T, Zurek E, Hutchison GR (2012) Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. *J Cheminformatics;***4**:

26. Shang R, Pu X, Xu X, Xin Z, Zhang C, Guo W, et al. (2014) Synthesis and Biological Activities of Novel Pleuromutilin Derivatives with a Substituted Thiadiazole Moiety as Potent Drug-Resistant Bacteria Inhibitors. *J Med Chem;***57**: 5664-78.

This article is protected by copyright. All rights reserved.

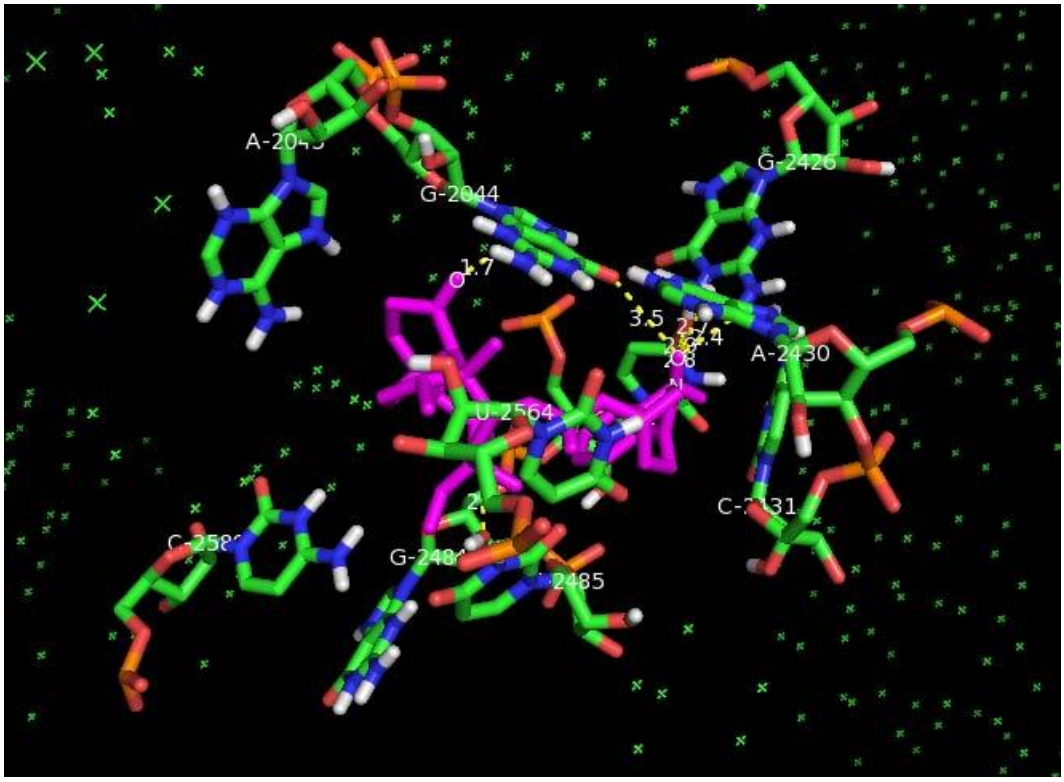
Accepted Article





This article is protected by copyright. All rights reserved.

Accepted Article



This article is protected by copyright. All rights reserved.