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A New Combination of a Pleuromutilin Derivative and Doxycycline for Treatment of Multidrug-Resistant *Acinetobacter baumannii*

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Abstract

Multidrug-resistant (MDR) *Acinetobacter baumannii* is one of the most difficult Gram-negative bacteria to treat and eradicate. In a cell-based screening of pleuromutilin derivatives against a drug sensitive *A. baumannii* strain, new molecules (**2–4**) exhibit bacteriostatic activity with 3.13 µg/mL concentration and **1** shows bactericidal activity with an MBC of 6.25 µg/mL. The pleuromutilin derivative **1** displays strong synergistic effects with doxycycline in a wide range of concentrations. A 35/1 ratio of **1** and doxycycline (**1**-Dox35/1) kills drug susceptible *A. baumannii* with the MBC of 2.0 µg/mL and an MDR *A. baumannii* with the MBC of 3.13 µg/mL. *In vitro* anti-*Acinetobacter* activity of **1**-Dox35/1 is superior to that of clinical drugs such as tobramycin, tigecycline, and colistin. The efficacy of **1**-Dox35/1 is evaluated in a mouse septicemia model; treatment of the infected C57BL/6 mice with **1**-Dox35/1 protects from lethal infection of *A. baumannii* with an ED₅₀ value of <2.0 mg/kg.

TOC Image

Keywords

Acinetobacter baumannii; Multidrug resistant; Gram-negative bacteria; Pleuromutilin derivatives; Tetracycline; Doxycycline; Synergistic effect; *In vivo* efficacy

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Supporting Information. Some assay data, copies of NMR spectra, HPLC chromatogram of new compounds, and assay procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>

INTRODUCTION

Acinetobacter baumannii is one of the most common healthcare-associated Gram-negative bacteria that causes a variety of diseases, ranging from pneumonia to serious blood infections, and affects people with compromised immune systems.^{1,2} A significant increase in the incidence of multidrug-resistant (MDR) strains has raised the profile of this emerging opportunistic pathogen.^{3,4} Due to its ability to survive on artificial surfaces and to resist desiccated conditions, *A. baumannii* survives in food, water, or soil, and it can be spread by direct contact.⁵ *A. baumannii* infections are commonly implicated in nosocomial infections, and in some countries high isolation rates of drug resistant *A. baumannii* have been reported from patients in the intensive care unit (ICU).⁶ Lately, high incidence of MDR *A. baumannii* infections have been reported in veterans in Iraq and Afghanistan.^{7,8} *A. baumannii* targets mucous membranes and injured skin area, causing necrotizing infections that lead to septicemia and death.¹ Mortality rates from *A. baumannii* septicemia were reported to be 34.0–43.4% in the ICU and 16.3% outside the ICU.⁹ Clinically isolated *A. baumannii* strains exhibit a wide array of drug resistance mechanisms including a variety of efflux mechanisms that show resistance to all commonly prescribed antibacterial drugs.¹⁰ The therapeutic strategies for treating these highly resistant organisms are associated with significant toxicity with the limited number of antibacterial agents (e.g. sulbactam, imipenem-cilastatin, meropenem, doripenem, amikacin, tobramycin, polymyxins (or colistin), tigecycline, and minocycline).¹ Although a significant number of studies has been devoted to optimizing the use of currently available agents or identifying any combination thereof, new chemical entities have not been developed for the treatment of MDR *A. baumannii* infections since the introduction of tigecycline in 2005. Thus, the need for new agents to treat infections caused by *A. baumannii* is urgent.

Since the discovery of diverse structures of bacterial protein biosynthesis inhibitors (e.g. aminoglycosides, tetracyclines, chloramphenicol, linezolid, lincosamides, spectinomycin, streptogramins, mupirocin, and macrolides), rRNA is still an attractive antibacterial drug target.

Pleuromutilin, a strong binder of the 50S rRNA subunit, was first isolated in 1952 from two *Basidiomycete* spp. (*Pleurotus mutilus* and *Pleurotus passeckerianus*). Two pleuromutilin derivatives, tiamulin and valnemulin, were successfully developed as therapeutic agents for veterinary use.^{11–14} Tiamulin is a prophylactic and therapeutic agent for swine dysentery, and shows activity against anaerobic bacteria, intestinal spirochetes and *Mycoplasma* spp.¹⁵ Several other semisynthetic pleuromutilin derivatives have been designed for human use. Retapamulin is the first antibacterial agent of the pleuromutilin class, which shows excellent *in vivo* antibacterial activity. Retapamulin was approved in 2007 by FDA for the topical treatment of the impetigo and secondarily infected traumatic lesions of skin infections caused by Gram-positive bacteria such as *S. aureus* and *S. pyogenes*.¹³ Recently, significant advances were made in development of pleuromutilin to be systemic or orally bioavailable antibiotics for MRSA infections. BC-3781 (Nabriva Therapeutics) entered Phase II clinical studies, demonstrating a therapeutic potential for the treatment of bacterial skin and lung infections including community-acquired bacterial pneumonia.^{16,17} As represented by

BC-3781 and other effective derivatives, thioether analogues of the C14-side chain promise the increase of antibacterial activity (Figure 1).^{18–28}

In our discovery program for effective protein biosynthesis inhibitors against Gram-negative bacteria, we identified that several pleuromutilin derivatives exhibited bacteriostatic and/or bactericidal activity against *A. baumannii*. Here, we wish to report that the analogue **1** showed significant synergistic effects with doxycycline against *A. baumannii* and a combination of **1** and doxycycline was effective in an *in vivo* lethal infection model.

RESULTS AND DISCUSSION

Chemistry and SAR

New drugs for drug resistant Gram-negative organisms are clearly a major unmet clinical need for new antibiotic agents. In order to identify new pleuromutilin derivatives that expand the spectrum of activity against Gram-negative organisms, we have generated a 50-membered library whose structure contained the methoxyamine-oxime (**A**), hydroxyamine-oxime (**B**), or 3-hydroxy (**C**) core structure; those structures were further diversified by reduction of the double bond (C16-position), amide-formations, and reductive aminations (see supporting information). The generated molecules were evaluated in the growth inhibitory assay against an *A. baumannii* strain (ATCC19606). We identified four anti-*Acinetobacter* pleuromutilin analogues (**1–4**) which exhibited MIC values less than 12.5 µg/mL (Figure 2).

In order to confirm anti-*Acinetobacter* activity of **1–4**, these molecules were resynthesized. Their syntheses are illustrated in Scheme 1. The C3-carbonyl group of pleuromutilin (**5**) was reduced with NaBH₄ to form the *secondary* alcohol with *R*-configuration exclusively.²⁹ The *primary* alcohol of the C14-side chain was tosylated with TsCl and DMAP to afford **6** in 90% overall yield. Thioether formation of **6** with 1-amino-2-methylpropane-2-thiol was accomplished according to a reported condition,^{19,20} yielding the free amine **7** in 90% yield. Amide-forming reaction of **7** with Boc-D-Val-OH under Glyceroacetone-Oxyma, EDCI, and NaHCO₃ in DMF-H₂O followed by deprotection of the Boc group with 4*N*HCl afforded **1** in 85% overall yield.^{30–32} The dipeptide analogues **2** and **3** were synthesized by coupling with the corresponding Boc-protected amino acid and subsequent Boc-deprotection. Reductive amination of **1** with *N*-Boc-2-aminoacetaldehyde followed by deprotection of the Boc group afforded **4** in 80% yield. The analogues synthesized in Scheme 1 were purified via reverse-phase HPLC, and their MIC values were determined against drug sensitive *A. baumannii*. The analogues **3–4** exhibited the MIC and MBC values of 3.13 and >12.5 µg/mL, respectively, suggesting that they are bacteriostatic molecules. On the other hand, analogue **1** displayed the MIC and MBC value of 3.13 and 6.25 µg/mL, respectively, indicating its bactericidal activity (Table 1). *In vitro* bacterial growth inhibitory activity of **1** was comparable to anti-acinetobacter drugs such as tobramycin and colistin (polymyxin E).

The identified molecules **1–4** are the C3-reduced analogues of valnemulin (Figure 1). Interestingly, **1** exhibited superior MIC and MBC levels to those of valnemulin. The analogue **1** killed an MDR strain, *A. baumannii* (ATCC BAA-1800) at 6.25–12.5 µg/mL

concentrations, albeit tobramycin and colistin did not kill the same strain at the concentrations effective against a drug-susceptible strain (entry 2 in Table 1). Based on bacterial growth inhibitory assays of the pleuromutilin analogues against batteries of Gram-negative and -positive bacteria, the following structure-activity relationship (SAR) was realized. Alkylation or acylation of the D-valine amino group decreases bactericidal activity, although modification with a variety of functional groups (R in Figure 3) is possible to retain the bacteriostatic activity. Hydrogenation of the C12-vinyl group increases activity against Gram-positive bacteria. The C3-hydroxy group improves not only bactericidal activity against *A. baumannii*, but also biopharmaceutic and pharmacokinetic properties such as water-solubility (1.5 times greater than valnemulin)³³ and metabolic stability (*vide infra*).

Synergistic effect of **1** with doxycycline

The synergistic or antagonistic activities of **1** were assessed *in vitro* via micro dilution broth checkerboard technique.^{34–36} In the checkerboard analyses of a combination of **1** and anti-*Acinetobacter* drugs (tobramycin, gentamicin, tigecycline, minocycline, doxycycline, rifampicin, and polymyxin), **1** displayed strong synergistic effects with only doxycycline in a wide range of concentrations. Table 2 summarizes the results of FIC index analyses for a combination of **1** plus doxycycline and valnemulin plus doxycycline that showed synergistic combination ($\Sigma\text{FIC} < 0.5$). The FIC index range of 0.16 to 0.50 was observed for 8 combinations of two molecules out of 96 different concentrations (entries 1–8 in Table 2). Compared to a wide range of synergistic effects observed for **1**, valnemulin showed synergistic effect only at two combinations with doxycycline (entry 9). It was demonstrated that 60/1, 35/1, and 2/1 ratio of **1** and doxycycline (**1**-Dox 60/1, **1**-Dox 35/1, and **1**-Dox 2/1) killed a drug susceptible *A. baumannii* with the MBC of 0.78 $\mu\text{g/mL}$ (entries 3–5 in Table 1). These combinations killed the rifampicin (32xMIC)- and **1** (16xMIC)-resistant strains at 0.78 and 3.13 $\mu\text{g/mL}$ concentration in 24h. Significantly, **1**-Dox 60/1, **1**-Dox 35/1, and **1**-Dox 2/1 killed MDR *A. baumannii* (ATCC BAA-1800) with much lower concentrations (MBC of 3.13–6.25 $\mu\text{g/mL}$) than the MBC values of the individual molecules (MBC >25 and 12.5 $\mu\text{g/mL}$ for Dox and **1**, respectively).

In vitro metabolic stability and toxicity of **1**

Despite widespread use of valnemulin in veterinary fields, its metabolic profile has not been reported until recently. Several groups reported that the half-lives of valnemulin in plasma *in vivo* and *ex-vivo* are relatively short (1.3–2.9 h), suggesting challenges in its application for systemic antimicrobial therapy.^{37–39} In our *in vitro* metabolic stability testing,⁴⁰ we observed a striking difference in half-life ($t_{1/2}$) between **1** and valnemulin in rat liver microsomes; $t_{1/2}$ of valnemulin was 0.58 min., on the other hand, $t_{1/2}$ of **1** was >60 min. Thus, *in vitro* half-life was significantly extended by reduction of the C3-carbonyl group of valnemulin. *In vitro* cytotoxicity against mammalian cells (e.g. Vero cells) of **1** (IC_{50} 45.3 $\mu\text{g/mL}$) was 2.85 times less toxic than that of valnemulin (IC_{50} 14.9 $\mu\text{g/mL}$) (Figure 4).^{41–43}

In vivo effect of **1**-Dox 35/1

As summarized above, the favorable *in vitro* physicochemical properties of **1** over valnemulin were realized. In addition, it was determined that **1** binds to the rat plasma

protein with 76.9% (PPB), providing insights into the *in vivo* efficacy of **1** in infected animal models.⁴⁴ The *in vivo* efficacy of **1** and **1**-Dox 35/1 was evaluated in a mouse septicemia model using the C57BL/6 mice and *A. baumannii* (ATCC19606) strain (at a dose that lead to 75% of death).^{45,46} One hour after the infection, the molecules (**1**, **1**-Dox 35/1, or tobramycin) were administered intraperitoneally (IP) at a single-dose (from 2 to 60 mg/kg) (Figure 5). At the 10, 20, and 60 mg/kg **1**-Dox 35/1 dose, 100% of the mice survived, while all mice in the control group succumbed to infection in two days. The reference molecule, tobramycin survived the mice at 6.0 mg/kg dose IP. 100% of the mice in the group administered 4.0 mg/kg **1**-Dox 35/1 were rescued mortality of the *A. baumannii* infection. At the 2.0 mg/kg **1**-Dox 35/1 dose, 60% of the mice survived. Similarly, **1** showed effectiveness in the same *in vivo* studies, but required higher dosage than that with **1**-Dox35/1.

A resistance mechanism of *A. baumannii* against **1**

It is well established that the pleuromutilin derivatives target the peptidyl transfer center of the 50S ribosomal protein L3 (rplC), inhibiting protein biosynthesis.^{47–54} Previously, other groups reported multiple mutations in *rplC* of *S. aureus* that can define a region of *rplC* capable of causing decreased susceptibility of the pleuromutilin derivative in *S. aureus*.^{48,49} In order to identify a potential mechanism of resistance to **1**, we isolated the chromosomal DNA from the resistant mutant (**1**^R, 16xMBC) and its parental wild-type control *A. baumannii* (ATCC19606). The *rplC* gene fragment was amplified using *A. baumannii* *rplC* specific primers and sequenced.^{55,56} The DNA sequencing results were blasted against *rplC* DNA sequence of *A. baumannii* in the NIH genetic sequence database. The DNA sequence alignment revealed a C456A single nucleotide mutation, which corresponded to N152K mutation in the protein sequence of RplC (Figure 6). Interestingly, **1**-Dox 35/1 and **1**-Dox 2/1 effectively killed *A. baumannii* mutant **1**^R with the MBC value of 3.13–6.26 µg/mL (*vide supra*). Any other tetracyclines such as minocycline, tigecycline, and demeclocycline did not exhibit the same effect as observed with doxycycline (Dox) against *A. baumannii* mutant **1**^R.

Spontaneous mutation frequency

The frequency that an *A. baumannii* strain spontaneously developed resistance to **1**-Dox 35/1 was evaluated by applying the culture of *A. baumannii* (ATCC19606) strain to agar media containing **1**-Dox 35/1 at concentrations 4- and 8-fold the MIC on agar media. There was no colony on the plate containing 4xMIC after 48h incubation when 1×10⁹ CFU bacteria were plated. However, two colonies were identified on the plate containing 8xMIC when applied 1×10¹⁰ CFU bacteria. Two strains isolated in these experiments did not grow on the agar plates containing **1**-Dox35/1 at the 4x and 8xMIC concentrations. Thus, calculated spontaneous resistance mutation frequency of **1**-Dox35/1 is less than 1×10⁻¹⁰ for the ATCC19606 strain. On the other hand, **1** alone showed spontaneous resistant mutants for the same strain with the mutation frequency of 1×10⁻⁸ at 4xMIC concentration.

CONCLUSION

This paper describes the identification of a new pleuromutilin derivative **1** that displays a strong synergistic effect with doxycycline (Dox). Combinations of **1** and Dox at a series of

concentrations (i.e. **1**-Dox 60/1, **1**-Dox 35/1, and **1**-Dox 2/1) exhibit bactericidal activity against drug-sensitive and -resistant *A. baumannii* at 1.56–6.25 µg/mL concentrations. *In vitro* bactericidal activity of **1**-Dox35/1 against *A. baumannii* is superior to that of clinically utilized drugs such as tobramycin, tigecycline, and colistin. Structurally, **1** is a C3-hydroxy analogue of valnemulin. Despite such a simple chemical modification, *in vitro* half-life of **1** ($t_{1/2}$ >60 min.) in rat microsomes is significantly extended compared to that of valnemulin ($t_{1/2}$ 0.58 min.), which promises *in vivo* efficacy of **1** and combinations of **1** and Dox in infected animal models. *In vitro* cytotoxicity of **1** was much lower than that of valnemulin. The *in vivo* efficacy of **1**-Dox35/1 was demonstrated via a mouse septicemia model using the infected C57BL/6 mice with *A. baumannii*; the ED₅₀ value of **1**-Dox 35/1 was determined to be <2 mg/kg (a single-dose). The gene analyses of a **1**-mutant *A. baumannii* strain revealed a single mutation in the *rplC* gene, which confers the binding site of **1** in bacterial 50S ribosome subunit. Although the mode of action of **1**-Dox 35/1 is far from completely understood, **1**-Dox 35/1 is effective against a resistant strain **1**^R. We will identify target and off-target mutations of resistant strain against **1**-Dox 35/1 by whole-genome sequencing analyses.

The pleuromutilin analogue **1** exhibited permeability across Caco-2 epithelial monolayers (Papp rate coefficient >1, data not shown) with moderate levels of efflux (an efflux ratio of approximately 5). Pharmacokinetics and oral bioavailability of a combination of **1** and Dox will be evaluated using preclinical animal models, and these data including detailed evaluation on *in vivo* efficacy will be reported elsewhere.

EXPERIMENTAL SECTION

Chemistry. General Information

All chemicals were purchased from commercial sources and used without further purification unless otherwise noted. THF, CH₂Cl₂, and DMF were purified via Innovative Technology's Pure-Solve System. All reactions were performed under an Argon atmosphere. All stirring was performed with an internal magnetic stirrer. Reactions were monitored by TLC using 0.25 mm coated commercial silica gel plates (EMD, Silica Gel 60F₂₅₄). TLC spots were visualized by UV light at 254 nm, or developed with ceric ammonium molybdate or anisaldehyde or copper sulfate or ninhydrin solutions by heating on a hot plate. Reactions were also monitored by using SHIMADZU LCMS-2020 with solvents: A: 0.1% formic acid in water, B: acetonitrile. Flash chromatography was performed with SiliCycle silica gel (Purasil 60 Å, 230–400 Mesh). Proton magnetic resonance (¹H-NMR) spectral data were recorded on 400, and 500 MHz instruments. Carbon magnetic resonance (¹³C-NMR) spectral data were recorded on 100 and 125 MHz instruments. For all NMR spectra, chemical shifts (δH, δC) were quoted in parts per million (ppm), and *J* values were quoted in Hz. ¹H and ¹³C NMR spectra were calibrated with residual undeuterated solvent (CDCl₃: δH = 7.26 ppm, δC = 77.16 ppm; CD₃CN: δH = 1.94 ppm, δC = 1.32 ppm; CD₃OD: δH = 3.31 ppm, δC = 49.00 ppm; DMSO-*d*₆: δH = 2.50 ppm, δC = 39.52 ppm; D₂O: δH = 4.79 ppm) as an internal reference. The following abbreviations were used to designate the multiplicities: s = singlet, d = doublet, dd = double doublets, t = triplet, q = quartet, quin = quintet, hept = heptet, m = multiplet, br = broad. Infrared (IR) spectra were recorded on a

Perkin-Elmer FT1600 spectrometer. HPLC analyses were performed with a Shimadzu LC-20AD HPLC system. All compounds were purified by reverse HPLC to be 95% purity.

(3*R*,3*aR*,4*R*,5*R*,7*S*,8*S*,9*R*,9*aS*,12*R*)-3,8-dihydroxy-4,7,9,12-tetramethyl-7-vinyldecahydro-4,9*a*-propanocyclopenta[8]annulen-5-yl 2-((1-((*R*)-2-amino-3-methylbutanamido)-2-methylpropan-2-yl)thio)acetate (1)

To a stirred solution of **7** (1.65 g, 3.52 mmol), Boc-D-Val-OH (1.15 g, 5.28 mmol), NaHCO₃ (2.96 g, 35.2 mmol) and Glyceroacetone-Oxyma (1.20 g, 5.28 mmol) in DMF-H₂O (9/1, 17.6 mL) was added EDCI (3.38 g, 17.6 mmol). The reaction mixture was stirred for 15h at rt, quenched with aq. sat. NaHCO₃, and extracted with EtOAc. The combined organic extract was washed with 1*N*HCl, brine, dried over Na₂SO₄ and concentrated *in vacuo*. To a stirred solution of the crude product (0.30 g, 0.46 mmol) in dioxane (0.5 mL) was added a 4*N* solution of HCl/dioxane (1.14 mL). The reaction mixture was stirred for 1h at rt, and all volatiles were evaporated *in vacuo*. The crude mixture was purified by C18 reverse-phase HPLC [column: HYPERSIL GOLD™ (175 Å, 12 µm, 250 × 10 mm), solvents: 50:50 MeOH : H₂O, flow rate: 2.0 mL/min, UV: 220 nm] to afford **1** (0.23 g, 0.40 mmol, 88%, retention time: 25 min): TLC (CHCl₃/MeOH 90:10) *R_f* = 0.30; [α]_D²² -0.201 (*c* = 1.61, MeOH); IR (thin film) ν_{max} = 3395 (br), 2957, 2875, 1716, 1658, 1525, 1463, 1370, 1285, 1144, 1020, 1005, 932 cm⁻¹; ¹H NMR (400 MHz, Methanol-*d*₄) δ 6.40 – 6.31 (m, 1H), 5.62 (d, *J* = 9.2 Hz, 1H), 5.17 (q, *J* = 1.7 Hz, 1H), 5.15 – 5.12 (m, 1H), 4.49 (t, *J* = 5.3 Hz, 1H), 3.38 – 3.34 (m, 2H), 3.29 (d, *J* = 6.8 Hz, 2H), 3.26 – 3.21 (m, 2H), 2.36 (ddd, *J* = 11.8, 7.2, 4.2 Hz, 1H), 2.22 – 2.10 (m, 2H), 2.03 (quind, *J* = 6.9, 5.2 Hz, 1H), 1.92 (dddd, *J* = 14.7, 13.0, 10.5, 5.2 Hz, 2H), 1.72 – 1.57 (m, 4H), 1.50 – 1.35 (m, 3H), 1.33 (q, *J* = 3.8 Hz, 1H), 1.27 (s, 3H), 1.26 (s, 3H), 1.25 (s, 3H), 1.13 (s, 3H), 1.01 (d, *J* = 6.9 Hz, 3H), 0.95 (d, *J* = 6.8 Hz, 3H), 0.86 (d, *J* = 7.0 Hz, 3H), 0.70 (d, *J* = 7.2 Hz, 3H); ¹³C NMR (101 MHz, MeOD) δ 176.36, 171.35, 141.52, 116.22, 77.39, 76.08, 73.26, 61.58, 52.21, 47.65, 47.18, 46.72, 45.95, 42.56, 37.23, 37.13, 34.59, 33.32, 33.14, 32.90, 32.51, 28.98, 28.00, 26.97, 26.91, 19.87, 17.84, 17.68, 17.53, 12.51; HRMS (ESI+) *m/z* calcd for C₃₁H₅₅N₂O₅S [M + H] 567.3832, found: 567.3866.

(3*R*,3*aR*,4*R*,5*R*,7*S*,8*S*,9*R*,9*aS*,12*R*)-3,8-dihydroxy-4,7,9,12-tetramethyl-7-vinyldecahydro-4,9*a*-propanocyclopenta[8]annulen-5-yl 2-((1-((*R*)-2-((*S*)-2,5-diaminopentanamido)-3-methylbutanamido)-2-methylpropan-2-yl)thio)acetate (2)

To a stirred solution of **1** (73.2 mg, 0.13 mmol), Boc-L-Orn(Boc)-OH (63.2 mg, 0.19 mmol), NaHCO₃ (109 mg, 1.29 mmol) and Glyceroacetone-Oxyma (59.3 mg, 0.26 mmol) in DMF-H₂O (9/1, 0.65 mL) was added EDCI (124 mg, 0.65 mmol). The reaction mixture was stirred for 8h at rt, quenched with aq. sat. NaHCO₃, and extracted with EtOAc. The combined organic extract was washed with 1*N*HCl, brine, dried over Na₂SO₄ and concentrated *in vacuo*. To a stirred solution of the crude product (45.6 mg, 0.051 mmol) in dioxane (0.2 mL) was added a 4*N* solution of HCl/dioxane (0.25 mL). The reaction mixture was stirred for 2h at rt, and all volatiles were evaporated *in vacuo*. The crude mixture was purified by C18 reverse-phase HPLC [column: HYPERSIL GOLD™ (175 Å, 12 µm, 250 × 10 mm), solvents: 50:50 MeOH:H₂O, flow rate: 2.0 mL/min, UV: 220 nm] to afford **2** (33.0 mg, 0.047 mmol, 93%, retention time: 13 min): TLC (CHCl₃/MeOH 90:10) *R_f* = 0.10; [α]_D²² +0.091 (*c* = 0.11, MeOH); IR (thin film) ν_{max} = 3438 (br), 3202 (br), 2968, 1658,

1427, 1287, 1148, 1018, 724 cm⁻¹; ¹H NMR (400 MHz, Methanol-*d*₄) δ 8.23 (d, *J* = 6.6 Hz, 1H), 6.35 (dd, *J* = 17.0, 11.3 Hz, 1H), 5.62 (d, *J* = 9.9 Hz, 1H), 5.19 – 5.14 (m, 1H), 5.13 (s, 1H), 4.49 (t, *J* = 5.7 Hz, 1H), 4.22 (d, *J* = 6.6 Hz, 1H), 4.01 (t, *J* = 6.1 Hz, 1H), 3.77 – 3.71 (m, 1H), 3.61 – 3.56 (m, 1H), 2.96 (t, *J* = 7.6 Hz, 2H), 2.41 – 2.29 (m, 1H), 2.21 – 2.05 (m, 3H), 1.99 – 1.85 (m, 4H), 1.76 – 1.57 (m, 6H), 1.56 – 1.38 (m, 5H), 1.35 – 1.29 (m, 2H), 1.26 (s, 3H), 1.26 (s, 3H), 1.25 (s, 3H), 1.13 (s, 3H), 1.05 (s, 3H), 1.03 (s, 3H), 0.86 (d, *J* = 6.4 Hz, 3H), 0.70 (d, *J* = 7.7 Hz, 3H); ¹³C NMR (101 MHz, MeOD) δ 173.61, 171.56, 169.98, 141.62, 116.18, 77.39, 76.02, 73.57, 73.31, 72.45, 62.18, 61.02, 53.82, 52.19, 47.78, 47.19, 45.94, 42.56, 40.29, 37.22, 37.16, 34.59, 32.91, 32.52, 32.18, 31.74, 28.06, 28.04, 27.02, 26.81, 22.54, 19.91, 18.88, 17.80, 17.54, 12.53; HRMS (ESI+) *m/z* calcd for C₃₆H₆₅N₄O₆S [M + H] 681.4625, found: 681.4602.

(3*R*,3*aR*,4*R*,5*R*,7*S*,8*S*,9*R*,9*aS*,12*R*)-3,8-dihydroxy-4,7,9,12-tetramethyl-7-vinyldecahydro-4,9*a*-propanocyclopenta[8]annulen-5-yl 2-((1-((*R*)-2-(2-aminoacetamido)-3-methylbutanamido)-2-methylpropan-2-yl)thio)acetate (3)

To a stirred solution of **1** (73.2 mg, 0.13 mmol), Boc-Gly-OH (33.9 mg, 0.19 mmol), NaHCO₃ (108 mg, 1.29 mmol) and Glyceroacetone-Oxyma (43.3 mg, 0.19 mmol) in DMF-H₂O (9/1, 0.65 mL) was added EDCI (124 mg, 0.65 mmol). The reaction mixture was stirred for 12h at rt, quenched with aq. sat. NaHCO₃, and extracted with EtOAc. The combined organic extract was washed with 1*N*HCl, brine, dried over Na₂SO₄ and concentrated *in vacuo*. To a stirred solution of the crude product (39.7 mg, 0.055 mmol) in dioxane (0.2 mL) was added 4*N*HCl/dioxane (0.3 mL). The reaction mixture was stirred for 2h at rt, and all volatiles were evaporated *in vacuo*. The crude mixture was purified by C18 reverse-phase HPLC [column: HYPERSIL GOLD™ (175 Å, 12 µm, 250 × 10 mm), solvents: 80:20 MeOH : H₂O, flow rate: 2.0 mL/min, UV: 220 nm] to afford **3** (32.6 mg, 0.052 mmol, 95%, retention time: 11 min): [α]_D²² +0.538 (*c* = 1.08, MeOH); IR (thin film) ν_{max} = 3421 (br), 3320 (br), 3078 (br), 2961, 2877, 1715, 1661, 1541, 1463, 1456, 1388, 1370, 1279, 1146, 1020, 1004, 935 cm⁻¹; ¹H NMR (400 MHz, Methanol-*d*₄) δ 6.35 (dd, *J* = 17.2, 11.4 Hz, 1H), 5.62 (d, *J* = 9.4 Hz, 1H), 5.17 (s, 1H), 5.16 – 5.12 (m, 1H), 4.49 (t, *J* = 5.5 Hz, 1H), 4.26 (d, *J* = 6.6 Hz, 1H), 3.66 (s, 1H), 3.35 (s, 1H), 3.30 – 3.25 (m, 2H), 2.40 – 2.31 (m, 1H), 2.20 – 2.09 (m, 3H), 1.96 (dd, *J* = 13.9, 4.3 Hz, 1H), 1.92 – 1.86 (m, 1H), 1.73 – 1.56 (m, 5H), 1.49 – 1.37 (m, 3H), 1.34 – 1.28 (m, 3H), 1.26 (s, 3H), 1.25 (s, 3H), 1.25 (s, 3H), 1.13 (s, 3H), 1.01 (d, *J* = 6.5 Hz, 3H), 0.99 (d, *J* = 6.5 Hz, 3H), 0.86 (d, *J* = 7.0 Hz, 3H), 0.70 (d, *J* = 7.2 Hz, 3H); ¹³C NMR (101 MHz, MeOD) δ 171.51, 141.55, 116.24, 77.41, 76.07, 73.31, 60.60, 52.22, 47.19, 45.96, 42.58, 37.23, 37.16, 34.59, 33.32, 32.91, 31.94, 29.11, 29.04, 28.99, 28.04, 26.96, 26.85, 19.89, 18.49, 17.82, 17.50, 12.52; HRMS (ESI+) *m/z* calcd for C₃₃H₅₈N₃O₆S [M + H] 624.4046, found: 624.4081.

(3*R*,3*aR*,4*R*,5*R*,7*S*,8*S*,9*R*,9*aS*,12*R*)-3,8-dihydroxy-4,7,9,12-tetramethyl-7-vinyldecahydro-4,9*a*-propanocyclopenta[8]annulen-5-yl 2-((1-((*R*)-2-((2-aminoethyl)amino)-3-methylbutanamido)-2-methylpropan-2-yl)thio)acetate (4)

To a stirred solution of **1** (100 mg, 0.17 mmol) and *N*-Boc-2-aminoacetaldehyde (53.1 mg, 0.33 mmol) in MeOH (0.83 mL) was added sodium cyanoborohydride (21.0 mg, 0.33 mmol) at 0°C. After being stirred for 2h at rt, quenched with aq. sat. NaHCO₃, and extracted with CHCl₃. The combined organic extract was dried over Na₂SO₄ and concentrated *in vacuo*. To

a stirred solution of the crude product (27.3 mg, 0.038 mmol) in dioxane (0.2 mL) was added a 4*N*HCl/dioxane (0.4 mL). The reaction mixture was stirred for 3h at rt, and all volatiles were evaporated *in vacuo*. The crude mixture was purified by C18 reverse-phase HPLC [column: HYPERSIL GOLD™ (175 Å, 12 µm, 250 × 10 mm), solvents: 50:50 MeOH : H₂O, flow rate: 2.0 mL/min, UV: 220 nm] to afford **4** (21.6 mg, 0.035 mmol, 93%, retention time: 27 min): TLC (CHCl₃/MeOH 90:10) *R_f*= 0.20; [α]_D²² 0.031 (*c* = 0.033, MeOH); IR (thin film) ν_{max} = 3430 (br), 2959, 2931, 2878, 1716, 1676, 1563, 1555, 1542, 1463, 1457, 1287, 1130, 1020, 1005, 933 cm⁻¹; ¹H NMR (500 MHz, Methanol-*d*₄) δ 6.37 – 6.31 (m, 1H), 5.62 (d, *J* = 9.3 Hz, 1H), 5.16 (d, *J* = 4.8 Hz, 1H), 5.13 (s, 1H), 4.49 (t, *J* = 5.5 Hz, 1H), 3.70 – 3.64 (m, 1H), 3.46 (d, *J* = 14.0 Hz, 1H), 3.34 (d, *J* = 2.9 Hz, 2H), 3.27 (d, *J* = 14.1 Hz, 3H), 3.23 – 3.16 (m, 1H), 2.40 – 2.33 (m, 1H), 2.29 – 2.21 (m, 1H), 2.19 – 2.11 (m, 2H), 1.98 – 1.87 (m, 2H), 1.72 – 1.61 (m, 3H), 1.61 – 1.57 (m, 1H), 1.48 – 1.36 (m, 2H), 1.35 – 1.32 (m, 1H), 1.29 (s, 6H), 1.24 (s, 3H), 1.15 (d, *J* = 6.7 Hz, 3H), 1.13 (s, 3H), 1.11 (d, *J* = 6.7 Hz, 3H), 0.86 (d, *J* = 7.0 Hz, 3H), 0.69 (d, *J* = 7.2 Hz, 3H); ¹³C NMR (101 MHz, MeOD) δ 171.49, 141.64, 116.18, 77.40, 76.02, 73.45, 68.59, 52.20, 47.80, 47.19, 46.71, 45.98, 45.83, 42.59, 37.21, 37.19, 34.60, 33.31, 32.90, 32.74, 31.99, 29.00, 28.05, 27.11, 19.07, 18.76, 17.80, 17.52, 12.51; HRMS (ESI+) *m/z* calcd for C₃₃H₆₀N₃O₅S [M + H] 610.4254, found 610.4245.

Pleuromutilin (5)

Pleuromutilin (**5**) (~85%) was purchased from Nanjing Pharmatechs Co., Ltd., and used after purification by silica gel column chromatography (hexanes/EtOAc 67:33 to 50:50): TLC (hexanes/EtOAc 50:50) *R_f*= 0.40; [α]_D²¹ +0.960 (*c* = 4.41, CHCl₃); IR (thin film) ν_{max} = 3448 (br), 2984, 2936, 2884, 2865, 1731, 1455, 1415, 1375, 1283, 1232, 1217, 1154, 1097, 1016, 978, 933, 916, 754 cm⁻¹; ¹H NMR (400 MHz, Chloroform-*d*) δ 6.49 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.36 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.21 (dd, *J* = 17.4, 1.6 Hz, 1H), 4.07 (d, *J* = 17.1 Hz, 1H), 4.01 (d, *J* = 17.1 Hz, 1H), 3.36 (d, *J* = 6.5 Hz, 1H), 2.43 (brs, 1H), 2.34 (quin, *J* = 7.0 Hz, 1H), 2.25 (ddd, *J* = 10.3, 5.4, 2.2 Hz, 1H), 2.22 (quin, *J* = 9.2 Hz, 1H), 2.09 (quin, *J* = 8.7 Hz, 2H), 1.78 (dq, *J* = 14.3, 2.8 Hz, 1H), 1.72 – 1.61 (m, 2H), 1.55 (td, *J* = 13.8, 3.5 Hz, 1H), 1.49 (d, *J* = 3.3 Hz, 1H), 1.47 – 1.45 (m, 1H), 1.43 (s, 3H), 1.38 (dq, *J* = 14.4, 3.8 Hz, 1H), 1.32 (d, *J* = 16.1 Hz, 1H), 1.17 (s, 3H), 1.12 (dd, *J* = 13.9, 4.5 Hz, 1H), 0.89 (d, *J* = 7.0 Hz, 3H), 0.70 (d, *J* = 7.0 Hz, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 216.88, 172.16, 138.79, 117.38, 74.54, 69.75, 61.29, 58.05, 45.40, 44.69, 43.98, 41.80, 36.57, 36.01, 34.40, 30.36, 26.80, 26.29, 24.81, 16.61, 14.75, 11.52; HRMS (ESI+) *m/z* calcd for C₂₂H₃₅O₅ [M + H] 379.2484, found: 379.2438.

(3*R*,3*aR*,4*R*,5*R*,7*S*,8*S*,9*R*,9*aS*,12*R*)-3,8-dihydroxy-4,7,9,12-tetramethyl-7-vinyldecahydro-4,9*a*-propanocyclopenta[8]annulen-5-yl 2-(tosyloxy)acetate (6)

To a stirred solution of **5** (7.48 g, 19.8 mmol) in MeOH (80 mL) was added NaBH₄ (1.50 g, 39.5 mmol) at 0°C. After 8h, the reaction mixture was quenched with aq. sat. NH₄Cl. After 12h, the reaction mixture was extracted with EtOAc and the combined organic phase was dried over Na₂SO₄ and concentrated *in vacuo*. To a stirred solution of the crude product in CH₂Cl₂ (100 mL) was added TsCl (4.51 g, 23.7 mmol) and DMAP (3.61 g, 29.6 mmol) at 0°C. After 7h at 0°C, the reaction mixture was quenched with 1*N*HCl and extracted with EtOAc. The combined organic extract was washed with aq. sat. NaHCO₃, dried over

Na₂SO₄, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (hexanes/EtOAc 60:40) to yield **6** (10.5 g, 19.7 mmol, 99%): TLC (hexanes/EtOAc 50:50) R_f = 0.50; $[\alpha]_D^{21}$ -0.124 (c = 0.66, CHCl₃); IR (thin film) $\nu_{\max}$ = 3562 (br), 2946, 2878, 1755, 1734, 1453, 1370, 1293, 1224, 1190, 1176, 1118, 1096, 1042, 1020, 1005, 925, 839, 814, 765, 719, 663 cm⁻¹; ¹H NMR (400 MHz, Chloroform-*d*) δ 7.81 (d, J = 8.4 Hz, 2H), 7.36 – 7.33 (m, 2H), 6.45 (dd, J = 17.4, 11.0 Hz, 1H), 5.62 (d, J = 9.5 Hz, 1H), 5.31 (dd, J = 11.0, 1.6 Hz, 1H), 5.16 (dd, J = 17.4, 1.6 Hz, 1H), 4.55 (q, J = 5.1 Hz, 1H), 4.47 (s, 2H), 3.15 (dd, J = 11.1, 6.3 Hz, 1H), 2.45 (s, 3H), 2.26 – 2.19 (m, 1H), 2.14 – 2.05 (m, 2H), 1.99 – 1.90 (m, 1H), 1.84 (td, J = 13.4, 5.2 Hz, 1H), 1.73 – 1.69 (m, 1H), 1.63 (tdd, J = 16.1, 8.2, 4.1 Hz, 2H), 1.51 – 1.47 (m, 1H), 1.47 – 1.43 (m, 1H), 1.43 – 1.39 (m, 1H), 1.39 – 1.34 (m, 1H), 1.29 (d, J = 3.8 Hz, 1H), 1.19 (s, 3H), 1.13 (s, 3H), 0.80 (d, J = 7.0 Hz, 3H), 0.61 (d, J = 7.2 Hz, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 216.73, 164.87, 145.23, 139.21, 132.62, 129.89 (2C), 128.11 (2C), 116.84, 74.81, 72.18, 65.16, 50.84, 45.93, 44.97, 44.86, 41.28, 36.34, 35.60, 34.27, 32.46, 31.71, 27.55, 26.23, 21.70, 17.47, 16.88, 12.16; HRMS (ESI+) m/z calcd for C₂₉H₄₃O₇S [M + H] 535.2730, found: 535.2742.

(3*R*,3*aR*,4*R*,5*R*,7*S*,8*S*,9*R*,9*aS*,12*R*)-3,8-dihydroxy-4,7,9,12-tetramethyl-7-vinyldecahydro-4,9*a*-propanocyclopenta[8]annulen-5-yl 2-((1-amino-2-methylpropan-2-yl)thio)acetate (7**)**

To a stirred solution of **6** (10.5 g, 19.7 mmol), 1-amino-2-methylpropane-2-thiol hydrochloride (5.58 g, 39.4 mmol) and ⁿBu₄NBr (0.64 g, 1.97 mmol) in THF (80 mL) was added 1*N*NaOH (79.2 mL). After 4h at 50°C, the reaction mixture was extracted with CHCl₃. The combined organic extract was dried over Na₂SO₄, concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (hexanes/EtOAc 50:50 to CHCl₃/MeOH 75:25) to give **7** (8.75 g, 18.7 mmol, 95%): TLC (CHCl₃/MeOH 90:10) R_f = 0.20; $[\alpha]_D^{21}$ -0.062 (c = 0.87, CHCl₃); IR (thin film) $\nu_{\max}$ = 3421 (br), 2955, 2876, 1721, 1462, 1371, 1283, 1219, 1121, 1020, 1005, 932, 772 cm⁻¹; ¹H NMR (400 MHz, Chloroform-*d*) δ 6.51 (dd, J = 17.4, 11.0 Hz, 1H), 5.60 (d, J = 9.4 Hz, 1H), 5.32 (dd, J = 11.0, 1.7 Hz, 1H), 5.16 (dd, J = 17.4, 1.7 Hz, 1H), 4.55 (t, J = 5.5 Hz, 1H), 3.16 (d, J = 6.4 Hz, 1H), 3.13 (s, 2H), 2.61 (s, 2H), 2.26 – 2.18 (m, 1H), 2.15 (t, J = 6.8 Hz, 1H), 2.08 (dd, J = 15.8, 9.3 Hz, 1H), 2.00 – 1.91 (m, 1H), 1.85 (td, J = 13.7, 4.5 Hz, 1H), 1.74 – 1.58 (m, 4H), 1.51 (d, J = 5.2 Hz, 1H), 1.48 – 1.41 (m, 2H), 1.41 – 1.33 (m, 1H), 1.24 (s, 6H), 1.23 (s, 3H), 1.14 (s, 3H), 0.80 (d, J = 7.0 Hz, 3H), 0.71 (d, J = 7.0 Hz, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 169.38, 139.56, 116.66, 74.93, 71.25, 51.68, 51.01, 48.44, 46.00, 45.20, 44.86, 41.24, 36.62, 35.61, 34.31, 32.63, 31.76, 31.33, 27.66, 26.30, 26.25 (2C), 17.64, 17.21, 12.16; HRMS (ESI+) m/z calcd for C₂₆H₄₆NO₄S [M + H] 468.3148, found: 468.3181.

Minimum Inhibitory Concentration Assays

A single colony of *A. baumannii* (ATCC 19606) was grown on a Tryptic soy agar plate.⁵⁷ Seed cultures and larger cultures were obtained using Tryptic soy agar broth. The flasks were incubated overnight in a shaking incubator at 37 °C with a shaking speed of 200 r.p.m. and cultured to mid-log phase (OD ~0.5). The OD was monitored at 600 nm using a 96-well microplate reader. The inhibitors were dissolved in DMSO (a final concentration of 1 mg per 100 μ L). This concentration was used as the stock solution for all studies. Bacterial cultures at 0.5 OD, was treated with serial dilutions of inhibitors and incubated at 37 °C for 48 h. 20

μL of resazurin (stock-0.02%) was added and incubated on a shaking incubator at 37 °C for 1 h. The lowest concentration at which the color of resazurin was completely retained as blue was read as the MIC (pink=growth, blue=no growth). The MBC defines that blue color retains over 4h. If necessary, viable bacteria in each well (96-well plate) were measured via colony-forming unit (CFU) on the agar plate. The absorbance measurements were also performed using a Biotek Synergy XT (Winooski, VT, USA), 96-well plate reader at 570 nm and 600 nm.

Synergistic Effect of Doxycycline with **1** or Valnemulin

The synergistic or antagonistic activities of doxycycline with **1** or valnemulin were assessed *in vitro* via micro dilution broth checkerboard technique. The FIC index was calculated according to the following equation. $\Sigma FIC = FIC_A + FIC_B = C_A/MIC_A + C_B/MIC_B$ where, MIC_A and MIC_B : MIC of drugs A and B, C_A and C_B =concentrations of drugs A and B used in combination. In these interaction studies, ΣFIC of less than 0.5 represents synergistic activity.

Cytotoxicity Assays

Cytotoxicity assays were performed using Vero monkey kidney (ATCC CCL-81) and HepG2 human hepatoblastoma cell (ATCC HB-8065) lines. Vero or HepG2 cells were cultured in 75 cm² flasks and transferred to 96-well cell culture plates using ATCC-formulated Eagle's minimum essential medium containing 10% FBS, and penicillin-streptomycin. Serially diluted aliquots of each test compound at concentrations ranging from 0.78–200 μg/mL were added to the cells. Control compounds with known toxicity such as tunicamycin, colistin or tobramycin were included on each plate. The plates were incubated and cytotoxic effects were determined via the MTT assay.

Microsomal Stability

Pooled Sprague-Dawley rat liver microsomes were purchased from Corning Life Sciences (Oneonta, NY, USA). Microsomes (20 mg/mL) were thawed on ice and diluted using phosphate buffer (100 mM, pH: 7.4), resulting in a protein concentration of 1 mg/mL. Stock solutions (10 mg/L) of **1**, valnemulin and verapamil (positive control) was prepared in DMSO (50%). A final concentration of 500 ng/mL was used for incubation with microsomes. NADPH (final concentration: 1 mM) was used as a co-factor. All the above solutions except NADPH were added to individual wells (12-well) in triplicate and were allowed to equilibrate for 5 min at 37 °C. NADPH was then added. 50 μL aliquots in triplicate were drawn from the incubation mixture at 0, 5, 10, 20, 30, 45 and 60 min and immediately the reaction was quenched by addition of ice-cold methanol (4-volumes). Analysis was performed by LC-MS.

Protein Binding

Plasma protein binding of **1** and verapamil (reference) was determined by equilibrium dialysis. The ready to use red device inserts (MW cutoff 6000–8000 D, RED® device, Thermo Scientific, Rockford, USA) containing plasma and buffer chambers for dialysis were used. The inserts were placed in a base plate. High and low concentrations (5000

ng/mL and 500 ng/mL) of **1** and reference were prepared in rat plasma (Innovative Grade US Origin Sprague-Dawley Rat Plasma (anticoagulant: Lithium Heparin), catalog# IGRT-N)) and an aliquot of 300 μ L was added in the plasma chamber in duplicate. A 500 μ L aliquot of blank isotonic phosphate buffer, pH 7.4 was added to buffer chamber of dialysis device. The base plate was covered with sealing tape and incubated at 37°C at approximately 100 r.p.m on an orbital shaker for 4h to achieve equilibrium. At the end of incubation, 50 μ L aliquots were withdrawn from plasma and buffer chambers. Four volumes of internal standard spiked methanol were vortexed for 30 seconds and centrifuged at 1000 rpm for 5 min. The supernatant was analyzed by LC-MS. The free fraction of the drug was calculated as ratio of the concentrations in the buffer and in plasma. The results were expressed in terms of % bound to plasma proteins.

***In vivo* Efficacy using a Mouse Model of Infection**

16–18 gram female C57BL/6 mice (n=5) were used for the animal studies. The animal study protocol (protocol number: 1410–31903A) was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota on May 5, 2016. *A. baumannii* (ATCC19606) cultured at 37 °C were collected by centrifugation and infected into C57BL/6 mice to create a mouse septicemia model. One hour after the infection (at a dose that lead to 75% of death), the molecules (**1**, **1**-Dox 35/1, or tobramycin) were administered intraperitoneally (IP) at single dose (from 2 to 60 mg/kg). Mice were monitored for 5 days and death was defined as the end point.

Gene Analyses of Resistant Strain of *A. baumannii* against **1**

Spontaneous resistant mutants of *A. baumannii* (ATCC 19606) with decreased sensitivity towards **1** were isolated by sub-culturing bacteria on agar plates with concentrations of **1** at MIC and above. Resistant mutants were isolated at $16 \times$ MIC of **1**. Spontaneous resistant mutants with decreased sensitivity against rifampicin were isolated at $32 \times$ MIC of rifampicin (control). The chromosomal DNAs from the resistant mutant (**1**^R) and wild-type *Acinetobacter baumannii* ATCC19606 were isolated. The *rplC* gene fragment was amplified using *A. baumannii* *rplC* specific primers (AbrplCupPrimer 5' CTTTGGGTTA AGGCTTTCGG 3' and Abrplcdn 5'CAACAGCAGAGCCG GAAACAG 3'), purified, and DNA sequenced. The DNA sequencing result was used to blast against *rplC* DNA sequence of *A. baumannii* in NIH Genome database.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

THF	tetrahydrofuran
CH₂Cl₂	methylene chloride
DMSO	Dimethyl sulfoxide
DMF	<i>N,N</i> -dimethylformamide
MeOH	methanol
EtOAc	ethyl acetate
CHCl₃	chloroform
HRMS	high resolution mass spectrometry
HPLC	high performance liquid chromatography
TLC	thin layer chromatography
Bu	<i>n</i> -butyl
Ts	<i>p</i> -toluenesulfonyl
DMAP	<i>N,N</i> -dimethyl-4-aminopyridine
Boc	<i>tert</i> -butoxycarbonyl
EDCI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
Val	Valine
Orn	ornithine
Gly	glycine
rt	room temperatures
ATCC	American type culture collection
MIC	Minimum inhibitory concentration
FIC	Fractional inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
FBS	Fetal bovine serum
NADPH	Nico-tinamide adenine dinucleotide phosphate

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57. Growth rate of *A. baumannii* strains in tryptic soy broth is similar to that in Mueller-Hinton broth (CLSI conditions), and the MIC and MBC values obtained with both media are identical. Because tryptic soy broth is available from several vendors at much lower-cost than Mueller-Hinton broth, we utilized tryptic soy broth or tryptic soy agar plate in this study.

Figure 1.
Pleuromutilin and Representative Pleuromutilin Analogues.

General structures of a 50-membered library

Hit compounds (< MIC 12.5 mg/mL)

Figure 2.
General Structures of Pleuromutilin Derivatives and Anti-*Acinetobacter* Molecules.

Figure 3.
SAR of Valnemulin Core Structure for Anti-*Acinetobacter* Agents.

Figure 4.

Half-life and Cytotoxicity (**1** vs. Valnemulin).

A. Half-life ($t_{1/2}$) in the rat liver microsomes^a

B. Cytotoxicity against Vero cells^a

IC₅₀ 45.3 µg/mL (for **1**), 14.9 µg/mL (for valnemulin), 0.20 µg/mL (tunicamycin, SI), > 200 µg/mL (colistin, SI), > 200 µg/mL (tobramycin, SI).

^aThe graphs were obtained via graph pad prism 7.0.

Figure 5.

Effect of 1-Dox 35/1 and 1 on Survival Rate in the Mouse Infected with *A. baumannii*.^a

^aThe C57BL/6 mice (n = 5) were infected intraperitoneally with *A. baumannii* (ATCC19606) strain at a dose that lead to 100% of death in 2 day. 4 mice was used for the non-treatment control group. The test molecules were intraperitoneally administered once after 1 h of the infection. Mortality was monitored for 5 days for all groups. $P < 0.05$.

```
Query 367 FGGVKRWNFRTQDATHGNSVSHRVLGSTGO KOTPGRVFKGKKMAGHLGDERVTVOGLEI
          FGGVKRWNFRTQDATHGNSVSHRVLGSTGO QTPGRVFKGKKMAGHLGDERVTVOGLEI
Sbjct 121 FGGVKRWNFRTQDATHGNSVSHRVLGSTGO KOTPGRVFKGKKMAGHLGDERVTVOGLEI
```

Figure 6.

The Amino Acid Alignment of the 50S Ribosomal Protein L3 (RplC) from a **1**-Resistant *A. baumannii* Strain (**1^R**).

1^R (Query) and wild-type control (Sbjct): the red color represents the site mutation in RplC.

Scheme 1.
Syntheses of Pleuromutilin Analogues **1–4**.

Table 1

MICs and MBCs of **1–4**, Representative Antibacterial Agents (Clinically Used), and Combinations of **1**-Doxycycline.

Entry	Species and strain	MIC or MBC ($\mu\text{g/mL}$) ^a									
		1		2		3		4		Valnemulin	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	<i>A. baumannii</i> ATCC 19606	1.75	6.25	3.13	>12.5	3.13	>12.5	3.13	>12.5	3.13	12.5
2	MDR- <i>A. baumannii</i> ATCC BAA-1800	6.25	12.5	3.13	>12.5	3.13	>12.5	3.15	>12.5	6.25	25.0
										>25	>12.5
											25.0

Entry	Compound(s)	MIC or MBC ($\mu\text{g/mL}$) ^a											
		<i>A. baumannii</i> ATCC 19606		MDR- <i>A. baumannii</i> ATCC BAA-1800		<i>K. pneumoniae</i> ATCC 8047		<i>E. coli</i> ATCC 10798		<i>P. aeruginosa</i> ATCC 27853		<i>S. aureus</i> ATCC 25923	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
3	1 -Dox 60/1	0.5	2.0	1.56	6.25	1.56	3.25	0.25	1.56	12.5	25.0	12.5	25.0
4	1 -Dox 35/1	0.4	2.0	1.56	3.13	0.78	1.56	0.25	1.56	12.5	25.0	12.5	25.0
5	1 -Dox 2/1	0.5	2.0	1.56	3.13	0.78	1.56	0.25	1.56	6.25	12.5	12.5	25.0
6	Doxycycline (Dox)	0.3	0.4	>25	>25	0.25	0.78	3.13	6.25	12.5	25.0	3.13	12.5
7	1	1.75	3.13	6.25	12.5	<6.25	6.25	3.13	6.25	>25	>25	12.5	25.0
												–	6.25
												–	6.25
												–	6.25
												–	–
												–	6.25

^aThe broth dilution method was used; MBC 2.00 $\mu\text{g/mL}$ for **1**-Dox 35/1; *A. baumannii*: Acinetobacter baumannii; *K. pneumoniae*: Klebsiella pneumoniae; *E. coli*: Escherichia coli; *P. aeruginosa*: Pseudomonas aeruginosa; *S. aureus*: Staphylococcus aureus; *M. tuberculosis*: Mycobacterium tuberculosis. MIC: the lowest concentration that inhibit bacterial growth; MBC: the lowest concentration required to kill 99.9% of bacteria (in CFU/mL)

Table 2Fractional Inhibitory Concentration of a Combination of Doxycycline and **1** or Valnemulin.^a

Entry	Combination of A and B ^b	MIC (µg/mL) C _A and C _B ^c	ΣFIC ^d
1	A: 1	0.20	0.16
	B: Doxycycline	0.025	
2	A: 1	0.40	0.19
	B: Doxycycline	0.025	
3	A: 1	0.78	0.25
	B: Doxycycline	0.025	
4	A: 1	1.56	0.38
	B: Doxycycline	0.025	
5	A: 1	0.20	0.28
	B: Doxycycline	0.05	
6	A: 1	0.40	0.31
	B: Doxycycline	0.05	
7	A: 1	0.78	0.37
	B: Doxycycline	0.025	
8	A: 1	1.56	0.50
	B: Doxycycline	0.025	
9	A: Valnemulin	0.78	0.375
	B: Doxycycline	0.025	
10	A: Valnemulin	1.56	0.375
	B: Doxycycline	0.05	

^aΣFIC index for the wells at growth–no growth interface.^bThe MIC values of molecule **1**, valnemulin, and doxycycline against *A. baumannii* (ATCC 19606) are 6.25, 12.5, and 0.20 µg/mL, respectively.^cC_A and C_B are concentrations of A and B.^dΣFIC is the sum of fractional inhibitory concentration calculated by the equation $\Sigma FIC = FIC_A + FIC_B = C_A/MIC_A + C_B/MIC_B$.