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Original article

Synthesis and evaluation of antibacterial activities of andrographolide analogues

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ABSTRACT

Andrographolide (Andro), the main active component of the herb *Andrographis paniculata*, has been used for many years to treat a variety of diseases including bacterial and viral infections. Andro was recently reported to act by inhibiting the bacterial quorum sensing system. We have synthesized several Andro analogues and investigated their antibacterial activity and mechanism of action. The new compounds were found to be much more potent than the parent Andro in inhibiting bacterial growth and quorum sensing system. Compounds **5** and **7** significantly reduced virulence factor production. Compound **7** completely inhibited *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilm formation, and exhibited synergistic activity with conventional antibiotics. These findings suggest that compound **7** may be the basis for future drug development to combat the unmet needs of virulence factor production, biofilm formation and antibiotic resistance.

1. Introduction

Bacterial resistance to the currently used antibiotics is a major cause of death in outpatient clinics and in intensive care units of hospitals worldwide [1–3]. Methicillin-resistant *Staphylococcus aureus* (MRSA) now accounts for approximately 20% of serious infections in some parts of the United States [4]. Most bacterial strains are becoming resistant to multiple antibiotics including vancomycin, the current drug of choice for MRSA treatment.

Pseudomonas aeruginosa is a common environmental microorganism that has acquired the ability to take advantage of weaknesses in the host immune system to become an opportunistic pathogen in humans [5]. *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections. According to the US CDC, *P. aeruginosa* is the fourth most commonly isolated nosocomial (hospital-acquired) pathogen accounting for 10.1 percent of all hospital-acquired infections in the US. *P. aeruginosa* infection is a serious problem in immunocompromised patients hospitalized with cancer, cystic fibrosis, and burns. The case fatality rate in these patients approaches 50%. *P. aeruginosa* infections are especially troublesome because the bacterium continues to grow more resistant to many antibiotics. Therefore, drugs with new mechanisms of action are urgently needed to combat the growth of antibiotic-resistant bacteria.

Bacteria can attach to surfaces and form communities enmeshed in a self-produced polymeric matrix, which can consist of protein, polysaccharide, and nucleic acid. These communities comprise a biofilm [6,7]. When *P. aeruginosa* chronically infects patients it adapts to the biofilm mode of growth. For example, the persistence of *P. aeruginosa* in the lungs of cystic fibrosis patients has been linked, in part, to its ability to form biofilms [8,9]. Destruction of established biofilm is one of the most challenging problems in treating chronic *P. aeruginosa* infections [6]. Once this structure forms, the colony is protected from the patient's immune system and is less susceptible to drug treatments. Effective biofilm inhibitors could dramatically improve treatment regimens for many infectious diseases and benefit large patient populations.

P. aeruginosa produces a wide range of virulence factors that are regulated by a cell-to-cell signaling mechanism known as quorum sensing (QS). QS was first discovered as a mechanism to control the luminescence of *Vibrio fischeri*, a bacterium that forms a mutualistic light organ symbiosis with certain marine animals [10,11]. Bacteria that use QS produce and secrete certain signaling compounds (called autoinducers, AI) to signal each other and to coordinate their activities. For example, gram-positive bacteria use small

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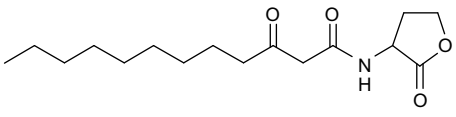
peptides to signal one another [12]. Among gram-negative bacteria perhaps the best-studied signaling system is the N-acylhomoserine lactone (AHL) system [13,14]. AHL signals are generated by the activity of a single enzyme that uses S-adenosylmethionine as a substrate and an intermediate of fatty acid biosynthesis, acyl–acyl carrier protein. [15–17] The enzyme is generally a member of the LuxI family of AHL synthases. Different LuxI homologs generate different AHLs. In *P. aeruginosa* LasI primarily catalyzes the synthesis of N-(3-oxododecanoyl)homoserine lactone (3-oxo-C_{12}-HSL), and RhlI directs synthesis of N-butyrylhomoserine lactone (C_4-HSL) (Fig. 1) [18]. The acyl side-chain length and the substitutions on the side chain provide signal specificity. The specific receptors for AHL signals are the members of the LuxR family of transcriptional regulators.

At low population densities LasI produces a basal level of 3-oxo-C_{12}-HSL. As bacterial cell density increases, 3-oxo-C_{12}-HSL builds to a critical concentration, at which point it interacts with LasR. The LasR-3-oxo-C_{12}-HSL complex then activates transcription of a number of genes, including LasB, ToxA, RhlR, and LasI [19,20]. Activation of LasI by LasR creates a positive autoregulatory loop. The activation of RhlR by LasR results in a quorum-sensing regulatory cascade, in which activation of the Rhl system requires an active Las system. RhlR responds best to the RhlI-generated C_4-HSL. RhlR then activates expression of genes required for production of a variety of secondary metabolites such as hydrogen cyanide and pyocyanin [21].

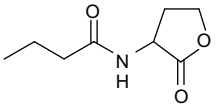
On one hand, bacteria employ QS to regulate their growth and production of various virulence factors, and on the other hand, QS regulates non-essential phenotypes. Thus inhibition of QS is not expected to create the severe selection pressure observed with antibiotics. This should, in theory, avoid development of resistance to QS inhibiting drugs making QS is an ideal target for antibacterial drug design. In fact, during the last 20 years, intensive efforts have been made to find and synthesize compounds that can inhibit the QS [3,22,23].

Andrographis paniculata, commonly known as the "King of Bitters" in China, is a member of the plant family Acanthaceae, which has been widely used in China, India, Japan, Korea and other Asia countries for more than two thousand years for treatment of human diseases including influenza with fever, sore throat, ulcers in the mouth or on the tongue, acute or chronic cough, colitis, upper respiratory and urinary infection, rheumatoid arthritis, laryngitis, diarrhea, and diabetes. Clinical studies of *A.* paniculata extract for treatment of acute upper respiratory tract infections, cold symptoms, and adults with sore throat and fever, demonstrated some benefits [24–29].

Over the last 30 years, the main active ingredient of this herb, Andro, a diterpenoid lactone, and three of its derivatives (Fig. 2) have been used clinically in China. These drugs are effective in the relief of symptoms of inflammation, fever, and pain due to bacterial and viral infections [30–34]. DASM, a derivative of Andro, was found to be an inhibitor of HIV *in vitro* [35]. Andro was found to decrease the HIV-1 RNA level and increase $CD4^+$ lymphocyte count in HIV positive patients [36]. Although the herb, Andro and its derivatives have been used to treat bacterial infections for many years, these drugs have been found to have minimal or no direct inhibition on bacterial growth, especially on *P. aeruginosa* [36,37]. Thus the antibacterial and anti-inflammatory effects have been attributed to stimulation of the immune system. Andro has been recently identified as a potent inhibitor of NF-κB [38,39]. In a significant finding, Li et al. reported that Andro inhibited the QS system of *P. aeruginosa* [37].

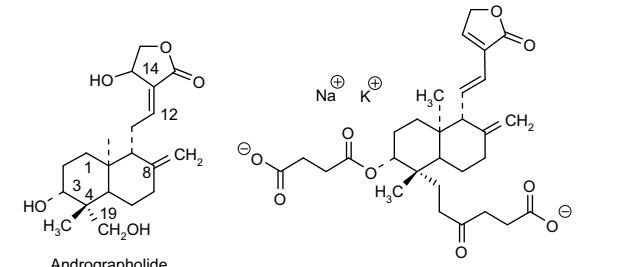


N-(3-oxododecanoyl) homoserine lactone
(3-oxo-C_{12}-HSL)



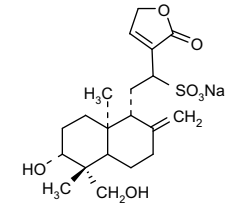
N-butyrylhomoserine lactone (C_4-HSL)

Fig. 1. Chemical structures of autoinducers.

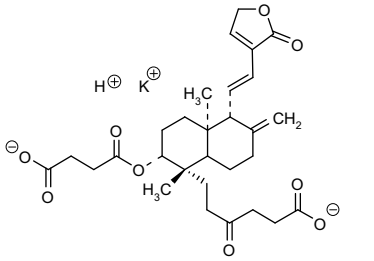


Andrographolide
(Andro)

14-dehydroxy-11,12-didehydroandrographolide-
3,19-bis(succinic acid) potasium sodium salt
10



14-dehydroxyandrographolide-
12-sulfonic acid sodium salt
11



14-dehydroxy-11,12-didehydroandrographolide-
3,19-bis(succinic acid) potasium salt
12

Fig. 2. Structures of andrographolide and related analogues.

Although many Andro analogues have been synthesized over the last 20 years, the antibacterial activities of these compounds are rarely reported. Based on the significant clinical benefits seen with these important drugs, and their newly found mechanism of action, i.e., QS system inhibition, we have designed and synthesized new Andro analogues, and studied their antibacterial activities and investigated their mechanism of action. We herein report the results of these studies.

1.1. Chemistry

1.1.1. Drug design

Nanduri et al. recently reported the synthesis and anticancer activity of a series of Andro analogues [40]. SAR analyses reveal that (a) replacing the C-8 ethylene moiety with an 8,17-epoxy changed neither the potency nor antitumor efficacy; (b) esterification of all three hydroxyl groups seemed to increase the compound's potency but not the antitumor efficacy *in vivo*; (c) an electron-withdrawing group at C-14 resulted in an increase in both the compound's potency and antitumor efficacy *in vivo*. While the C-14 hydroxyl moiety can be modified, its removal resulted in a decrease of activity; (d) an increase in the size of substituent at C-19 resulted in a decrease in the compound's potency. Based on these results we decided to modify the C-14 hydroxyl moiety. A Boc-protected

glycine analogue of Andro conjugated at the C-14 position (compound **4**) has been reported [40], but a free glycine conjugate (compound **5**) was not seen. For SAR studies, we synthesized both compounds **4** and **5**. Compound **4** has a bulky Boc group and is highly lipophilic. In contrast, compound **5** has no bulky group and is hydrophilic since the amino group of the glycinyl part of the molecule is positively charged at physiologic conditions. Alpha-lipoic acid (LA) is a powerful antioxidant, which directly terminates free radicals, chelates transition metal ions (iron and copper), and increases cytosolic glutathione and vitamin C levels [41–43]. These diverse actions suggest that LA acts by multiple mechanisms both physiologically and pharmacologically [42]. Because of the powerful antioxidative effect and other mechanisms of action, LA was conjugated to Andro to make compound **7**. Because an electron-withdrawing group at C-14 resulted in an increase in the compound's potency [40], compound **9** with a strong electron-withdrawing nitro group at the cinnamoyl moiety was synthesized.

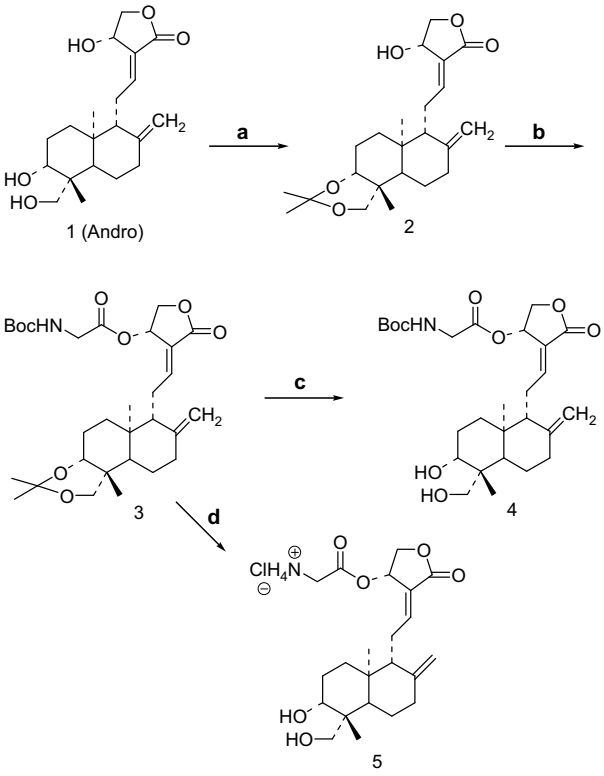
1.1.2. Chemical synthesis

Compounds **2**–**4** were synthesized according to a reported procedure [40]. Compound **3** was treated with anhydrous HCl in ethyl acetate to remove the Boc-protective group, affording compound **5**. Compounds **6** and **8** were prepared by reacting with *R*-(+)-lipoic acid and 4-nitrocinnamic acid, respectively, using a procedure similar to that used for synthesis of compound **4**. Removal of the protective groups from compounds **6** and **8** by treatment with acetic acid afforded target compounds **7** and **9** (Schemes 1 and 2).

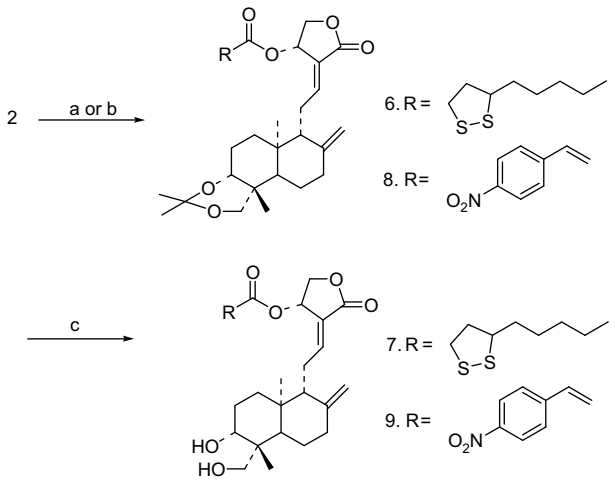
1.2. Biological results and discussion

1.2.1. Inhibition of bacterial growth

Inhibition of bacterial growth of the new compounds was tested against several strains of bacteria including two MRSA strains by a traditional zone of inhibition method, where bacteria are grown in a Luria–Bertani broth (LB) agar medium, and the results are summarized in Table 1. All of the compounds (**4**, **5**, **7** and **9**) and the clinically used Andro and its analogues (**10**–**12**) had no activity against *Escherichia coli*, (*E. coli*), *P. aeruginosa*, *Bacillus subtilis* (*B. subtilis*) and *Candida albicans* (*C. albicans*) at the concentrations tested. Compounds **4**, **5**, **7** and **9** were active against *S. aureus* including the methicillin-resistant MRSA5676 and MRSA5677 strains. In contrast, Andro and its analogues **10**–**12** were not active. These results are in agreement with previous reports indicating that Andro did not directly inhibit bacterial growth [36,37]. These results showed that compounds **4**, **5**, **7** and **9** are more potent than the parent Andro and analogues **10**–**12** against some strains of bacteria. Compounds **4**, **5**, **7** and **9** apparently directly inhibit *S. aureus* growth. Furthermore, the two MRSA strains were susceptible to these new compounds.



Scheme 1. Synthesis of compound **5**. Reagents and conditions: (a) 2, 2-dimethoxypropane/benzene and DMSO/PPTS/8h; (b) for 3, N-Boc-glycine/ClCOOEt/NEt_3/CH_2Cl_2/2 h; (c) AcOH/H_2O/1 h (d) HCl/EtOAc, 15 min.



Scheme 2. Synthesis of compounds **7** and **9**. Reagents and conditions: (a) for **6**, alpha-lipoic acid/ClCOOEt/NEt_3/CH_2Cl_2/2 d; (b) for **8**, 4-nitrocinnamic acid/ClCOOEt/NEt_3/CH_2Cl_2/2 d; (c) AcOH/H_2O/1 h.

1.2.2. Inhibition of virulence factor production

Although Andro and the new compounds were not active against *P. aeruginosa* in the zone of inhibition growth assay, Andro has been shown to inhibit the *P. aeruginosa* QS system [37]. To determine if the new compounds can inhibit the *P. aeruginosa* QS system, wild-type *P. aeruginosa* (PAO1) was grown in LB medium, and bacterial growth, production of virulence factors pyocyanin and protease were measured.

Table 1
Inhibition of bacterial growth[a].

Compd	Zone of inhibition diameter (mm)					
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*		
				Wild type	MRSA5676	MRSA5677
Andro	n	n	n	n	n	n
4	n	n	n	10.2 ± 0.78	8.4 ± 0.92	7.6 ± 0.57
5	n	n	n	9.3 ± 0.78	8.7 ± 0.75	5.2 ± 0.81
7	n	n	n	4.9 ± 0.81	6.9 ± 0.80	4.1 ± 0.77
9	n	n	n	8.3 ± 0.99	8.1 ± 0.64	6.4 ± 0.42
10	n	n	n	n	n	n
11	n	n	n	n	n	n
12	n	n	n	n	n	n

[a] All compounds were tested at 0.05 mg/disc. Results were expressed as mean ± S.D. values of three replicates; n: No activity (diameter of the inhibition zone < 4 mm).

Andro and its analogues **5** and **7** inhibited PAO1 growth albeit at a very high concentration of 1 mM (Fig. 3). Compound **7** was the most potent among the tested compounds. In sharp contrast, the water-soluble analogues **10–12** had no activity at 10 mM, a concentration 10-times higher than that of Andro and its analogues **5** and **7**.

The pigment pyocyanin is a hallmark product of QS system in *P. aeruginosa* [44,45], and proteases, including metalloprotease elastase B, degrade immune components and cause tissue damage [46]. PAO1 bacteria were grown for 3–4 h to mid-log phase (OD_{600} of 0.3–0.5) and then diluted to an OD_{600} of 0.05, and aliquoted to test tubes containing a dilution series of test compounds. Following another 18 h of growth, pyocyanin was extracted and quantified (Fig. 4).

At 0.1 mM, Andro and compounds **4**, **5**, **7** and **9** inhibited pyocyanin production (Fig. 4A), with compound **7** being the most potent. In contrast, compounds **10–12** had no activity. The data suggest that the C-14 hydroxyl moiety is important not only for the anticancer activity as previously reported [40], but also for the antibacterial activity. To demonstrate a dose-response effect, the assay was repeated with higher drug concentrations, and the results are shown in Fig. 4B. Higher drug concentrations increased inhibition of pyocyanin production for all compounds. Again, compound **7** was the most potent. Since compound **4** is a conjugate of Andro and glycine, and **7** is a conjugate of Andro and lipoic acid, we tested the mixtures of Andro and glycine (A + g) and lipoic acid (A + la), respectively, to see if the effect of compounds **4** and **7** was due to a combined effects of Andro and glycine or lipoic acid. Andro was just as potent as the mixtures with glycine and lipoic acid, confirming that compounds **4** and **7** were more potent than the parent Andro even when mixed with free glycine or lipoic acid. The fact that Andro, with a C-14 OH, and compound **5**, with a terminal NH_2 at the C-14 glycinyl, were less potent than **4**, with a lipophilic terminal Boc group at the C-14 glycinyl, and **7** with a lipophilic lipoyl suggests that a lipophilic group at that position increases antibacterial activity. This observation is in agreement with what has been reported on SAR of Andro analogues tested for anticancer activity [40]. However; this hypothesis cannot explain the fact that compound **9**, possessing even a larger lipophilic group than both **4** and **7** was not any more potent than Andro and compound **5**.

A colorimetric method using azocasein as a substrate was used to measure protease activity. At 0.1 mM all compounds suppressed protease production (Fig. 5A). Similar to the pyocyanin assay, compound **7** was the most potent. Higher concentrations further suppressed protease production (Fig. 5B). Compound **7** almost completely suppressed protease production at 1 mM. Compounds **4** and **7** were more potent than the parent Andro, and combinations of glycine or lipoic acid with Andro did not increase activity. Apparently, production of proteases is more sensitive to inhibition than is pyocyanin synthesis. These results confirm the observation

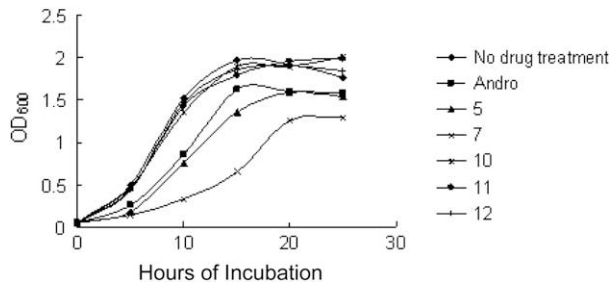


Fig. 3. Inhibition of bacterial (PAO1) growth. The concentration for Andro, **5** and **7** was 1 mM, and for **10–12** was 10 mM, respectively.

reported by Li et al. that Andro is an inhibitor of the *P. aeruginosa* QS system and demonstrate that the new compounds can inhibit the *P. aeruginosa* quorum sensing [37].

Compounds **5** and **7** were selected for further study. Compound **7** is the most potent compound and compound **5** is more water-soluble and actively suppressed protease production. PAO1 cells were grown in the presence and absence of the test compounds for 15 h, and cell population density/community size was visualized by atomic force microscopy (AFM). In this assay Andro did not reduce the PAO1 cell growth (Fig. 6). In contrast, both compounds **5** and **7** significantly inhibited PAO1 growth. The fact that compounds **5** and **7** inhibited PAO1 growth suggested that they can probably inhibit PAO1 biofilm formation.

There is increasing evidence that QS plays a crucial role in the maturation of *P. aeruginosa* biofilms. Therefore, we examined the effect of Andro, compounds **5**, **7** and **10** on PAO1 biofilm formation. PAO1 biofilms were cultivated on a respiratory track suction tube (polythene) surface for 3 and 7 days, respectively, in presence and absence of the test compounds. Biofilm formation was observed using a scanning electron microscope (SEM). As shown in Fig. 7A, PAO1 biofilms began to form after 3 days of incubation. At 1 mM concentration, Andro demonstrated some inhibition of biofilm formation. Surprisingly, compound **5,** which had showed activity in decreasing the production of pyocyanin, protease and reducing the bacterial cell density did not inhibit biofilm formation. However, biofilm formation was almost completely inhibited by compound **7**. Compound **10** did not have activity at 10 mM, a concentration 10-times higher than that of the other compounds.

When PAO1 cells were cultured for 7 days, mature biofilms were formed in the absence of any drug (Fig. 7B). Although Andro and compounds **5** and **10** did not display any obvious inhibition of biofilm formation, compound **7** almost completely inhibited biofilm formation. This result is significant given the difficulty to arrest PAO1 biofilm formation.

Because the Andro compounds are postulated to have a mechanism different from conventional antibiotics and Feng et al. had reported that combining ciprofloxacin and roxithromycin with Andro significantly reduced PAO1 growth compared to either drug used alone [47], Andro and compound **7** were combined with ciprofloxacin and erythromycin, and their effects on PAO1 growth were determined. Results in Table 2 indicated that the best inhibition was achieved by a combination of ciprofloxacin and erythromycin with a survival rate of 35.7% after PAO1 was incubated for 20 h. The combination of compound **7** with ciprofloxacin and erythromycin produced a survival rate of 54.9% and 40.8% respectively, which was much better than when either compound was used alone, confirming the results of Feng et al. [47]. Combination of compound **7** with either ciprofloxacin or erythromycin inhibited bacterial growth better than Andro with either ciprofloxacin or erythromycin.

Analysis of SAR in terms of antibacterial and QS system activity of the compounds reveals some important information. (a) Removal of the C-14 hydroxyl moiety significantly reduces activity. For example, Andro, compounds **5** and **7**, all possessing a C-14 modified hydroxyl moiety, were at least 10-times more potent than compound **10**, which has lost the C-14 hydroxyl moiety, in inhibiting bacterial growth and the production of virulence factor pyocyanin (Figs. 3 and 4); (b) In Fig. 4 (pyocyanin production), Andro, compounds **5** and **9** had the same potency. However, in Fig. 5 (protease production), compounds **5** and **9** were at least 5-times more potent than Andro. In Fig. 6 (AFM Assay of PAO1's cell population density), compound **5** was also much more potent than Andro. The opposite effects in different assays suggest that the compounds act differently at the pathways of pyocyanin and protease production and bacterial cell growth. Further work is

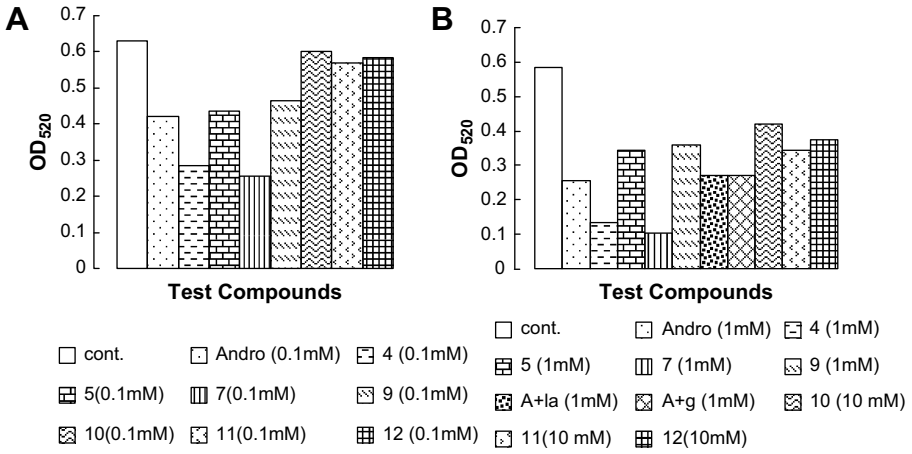


Fig. 4. Inhibition of pyocyanin production. cont.: control (no drug); A + la (Andro + lipoic acid); A + g (Andro + glycine).

required to explain these findings; (c) Compound **7** is the most potent in all assays. It is certainly more lipophilic than both Andro and compound **5**, but probably not more lipophilic than compound **9**. These results suggest that lipophilicity is not the most important determinant for inhibition of *P. aeruginosa* growth and its QS system and the potent effects of compound **7** are due to reasons other than just the increased lipophilicity. The lipoyl moiety may play a key role. Understanding factors accounting for the potent activity of compound **7** will likely lead to the design of better antibacterial agents.

2. Conclusions

Andro and its analogues as well as various herbal preparations are widely used to treat a variety of diseases, especially bacterial and viral infection-related symptoms in many countries. The drug has been reported to have multiple mechanisms of action including inhibition of NF-κB and the QS system of *P. aeruginosa*. We now have synthesized Andro analogues which can inhibit the growth of both gram-positive bacteria such as *S. aureus* and the gram-negative bacteria such as *P. aeruginosa*. We have demonstrated that some of these compounds, such as **7**, have significant effects on the QS system in *P. aeruginosa*, leading to strong inhibition of biofilm formation. More significantly, compound **7** is at least 10-times more potent than its parent Andro, and 100-fold more potent than compounds **10**–**12**, in inhibition of pyocyanin and protease production of *P. aeruginosa*. Further experiments to explore the mechanism(s) of action of these promising antimicrobial agents may lead to new drugs in combating the worldwide problem of antibiotic-resistant bacteria and the difficulty of eradicating *P. aeruginosa* biofilms.

3. Experimental section

3.1. Chemistry

Melting points were measured using a Mel-Temp (X_7L_{20}, Beijing) and are uncorrected. ^{1}H NMR spectra were recorded at ambient temperature on a 400 MHz spectrometer (AV-400, Bruker) in $CDCl_3$ or DMSO-d_6 solution. Electrospray ionization mass spectra (ESI-MS) were obtained in the positive ion detection mode on a Finnigan LCQ Advantage MAX mass spectrometer (AB company, 4000 Q TRAP). Elemental analysis was performed at the Experimental Center, Jinan University, Guangzhou, China, and the results were within ±0.4% of the theoretical values unless otherwise noted. High resolution mass spectra (HRMS) were performed at the Experimental Center of Sun Yat-Sen University, Guangzhou, China. Analytical thin-layer chromatography was performed on silica-coated plastic plates (silica gel 60 F-254, Merck) and visualized under UV light. Preparative separations were performed by flash chromatography on silica gel (Merck, 70–230 mesh).

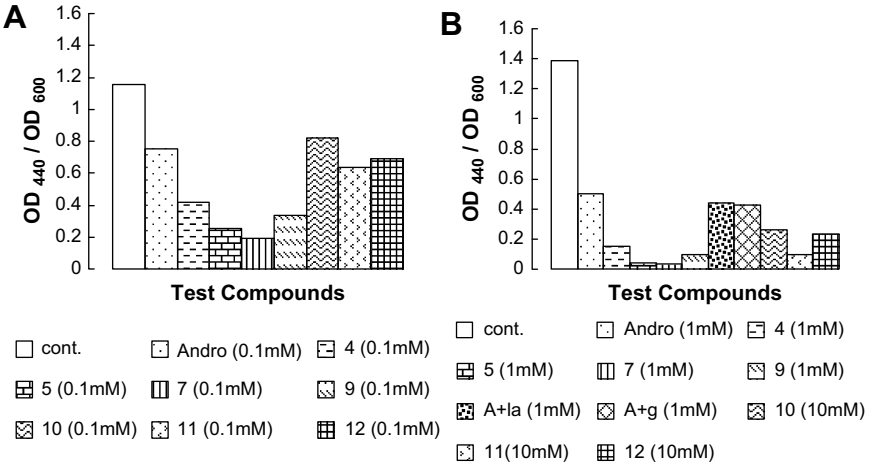


Fig. 5. Inhibition of protease production. cont.: control (no drug); A + la (Andro + lipoic acid); A + g (Andro + glycine).

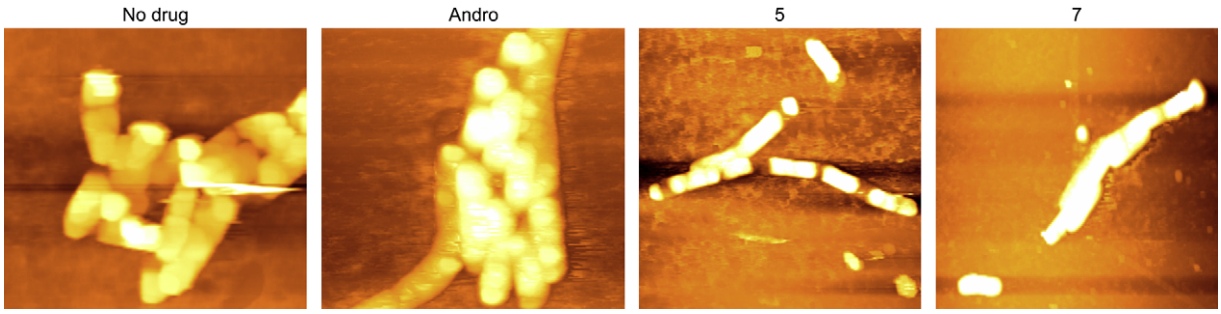


Fig. 6. AFM assay of PAO1's cell population density after 15 h of incubation (concentration: 1 mM).

3.2. 14-Glycinyl andrographolide hydrochloride (**5**)

Ethyl chloroformate (0.15 mL, 1.65 mmol) was added to N-Boc-glycine (200 mg, 1.15 mmol) and Et_3N (0.30 mL, 2.31 mmol) in CH_2Cl_2 (10 mL) at −25 °C and the reaction mixture was stirred for 15 min. Compound **2** [40] (150 mg, 0.39 mmol) was added, and the reaction mixture was stirred at room temperature for another 3 h. After that, CH_2Cl_2 (20 mL) and a saturated solution of $NaHCO_3$ were added. The organic phase was separated, washed with water, dried over $NaSO_4$, and then filtered. The filtrate was concentrated in vacuo and purified by column chromatography, eluting with ethyl acetate/petroleum ether (1/1), affording compound **3**. Then HCl/EtOAc (6 mL) was added, and the reaction mixture was stirred at room temperature for 15 min to remove the protective Boc and C-3, C-19 isopropylidene groups. Solvent was removed in vacuo, affording compound **5** (107 mg, 30% yield) as a colorless solid. ^{1}H NMR (DMSO-d_6, ppm): 7.25 (t, $J = 5.98$ Hz, 1H) 6.90 (t, $J = 5.96$ Hz, 1H), 6.04 (d, $J = 5.75$ Hz, 1H), 4.88 (s, 1H), 4.58 (d, $J = 5.60$ Hz, 2H), 4.24 (d, $J = 1.53$ Hz, 1H), 3.88 (d, $J = 11.64$ Hz, 2H), 3.41 (q, $J = 6.97$ Hz, 2H), 3.12 (d, $J = 11.59$ Hz, 1H), 2.51–2.49 (m, 3H), 1.99–1.94 (m, 3H), 1.68–1.66 (m, 3H), 1.32 (m, 4H), 1.23 (s, 3H), 1.13 (t, $J = 8.63$ Hz, 2H), 0.86 (s, 3H). MS (ESI) $[M + H]^+$ m/z 408.4. Anal. ($C_{22}H_{34}ClNO_6$) C, H, N.

3.3. 14-Alpha-lipoyl andrographolide (**7**)

Ethyl chloroformate (0.23 mL, 2.53 mmol) was added to *R*-alpha-lipoic acid (428 mg, 2.08 mmol) and Et_3N (0.42 mL, 3.22 mmol) in CH_2Cl_2 (15 mL), and the reaction was stirred for 1 h at 0 °C. Compound **2** (400 mg, 1.06 mmol) was added to the resulting mixture which was stirred at room temperature for 48 h. To quench the reaction CH_2Cl_2 (30 mL) and a saturated solution of $NaHCO_3$ were added. The organic phase was separated, and washed with water. The solution was dried over $NaSO_4$, and was then filtered. The filtrate was concentrated in vacuo and purified by column chromatography, eluting with ethyl acetate/petroleum ether (2/1, v/v), affording compound **6** (292 mg, 50% yield) as a yellow powder. ^{1}H NMR ($CDCl_3$, ppm): 7.03 (t, $J = 5.20$ Hz, 1H), 5.90 (d, $J = 6.50$ Hz, 1H), 4.90 (s, 1H), 4.20 (dd, $J = 7.20$ Hz, 1H), 3.90 (d, $J = 11.99$ Hz, 1H), 1.42 (s, 3H), 1.37 (s, 3H), 1.21 (s, 3H), 0.81 (s, 3H). MS (ESI) $[M + H]^+$ m/z 578. Without further purification, compound **6** (235 mg, 0.47 mmol) was added to a diluted acetic acid solution ($AcOH/H_2O = 7/3$, v/v, 10 mL), and the solution was stirred at room temperature for 2 h. Water was added, and the solution was neutralized with $NaHCO_3$. The product was extracted with CH_2Cl_2, and the organic phase was dried over $NaSO_4$, then filtered. The filtrate was concentrated in vacuo. The product was purified by column chromatography, eluting with ethyl acetate/petroleum (1/1), affording compound **7** (150 mg, 64% yield) as a yellow powder. ^{1}H NMR ($CDCl_3$, ppm): 7.00 (t, $J = 5.68$ Hz, 1H), 5.92 (d, $J = 5.84$ Hz, 1H), 4.86 (s, 1H), 4.52 (m, 1H), 4.48 (s, 1H), 4.21 (d, $J = 1.68$ Hz, 1H), 4.16 (d, $J = 11.12$ Hz, 1H), 3.52–3.48 (m, 2H), 3.32 (d, $J = 11.08$ Hz, 1H), 3.11 (m, 2H), 2.76 (br, s, 3H), 2.34–2.43 (m, 6H), 1.84–1.81 (m, 6H), 1.69–1.64 (m, 5H), 1.48 (m, 2H), 1.46–1.34 (m, 1H), 1.23 (br s, 4H), 0.65 (s, 3H). HRMS (positive ion EI), calcd. $C_{28}H_{42}O_6S_2$: $[M]^+$ m/z 538.2408; found: 538.2417.

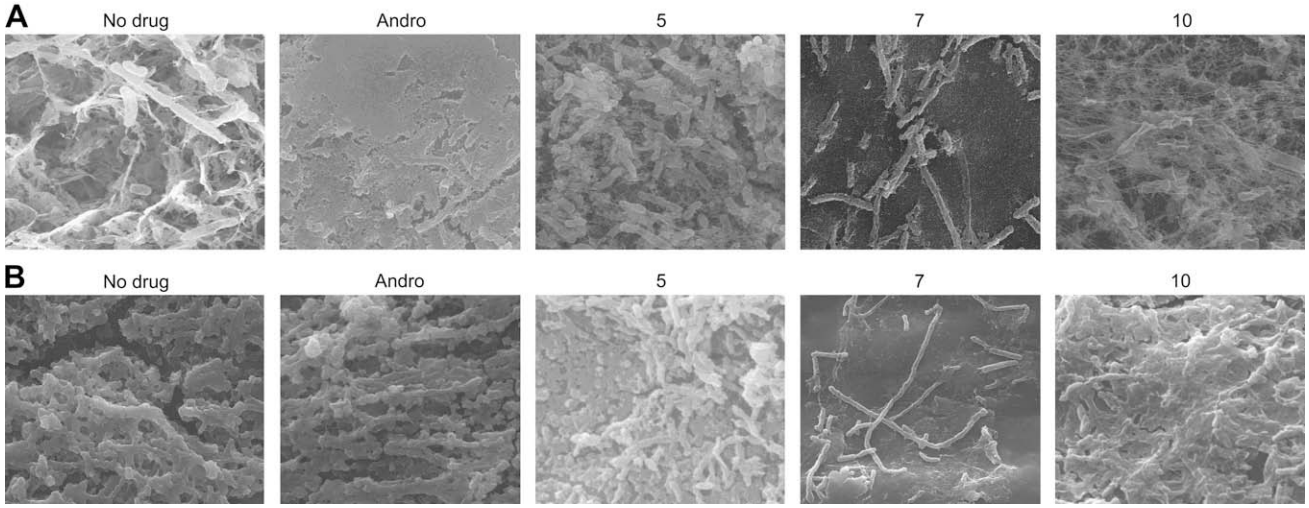


Fig. 7. SEM assay of PAO1's biofilm formation after 3 (A) and 7 (B) days of incubation (Mag 4000×, 5.0 μm). The concentration of Andro, **5** and **7** was 1 mM, and **10** was 10 mM.

Table 2
Survival rate of PAO1 treated with a combination of drugs.

Drug (μg/mL)	Survival rate (%)
Ciprofloxacin (0.017)	85
Erythromycin (3.75)	93.5
Andro (350)	90.1
7 (538)	84
Ciprofloxacin (0.017) + erythromycin (3.75)	35.7
Ciprofloxacin (0.017) + Andro (350)	65.3
Ciprofloxacin (0.017) + **7** (538)	54.9
Erythromycin (3.75) + Andro (350)	63.5
Erythromycin (3.75) + **7** (538)	40.8

The molar concentrations of Andro (350 μg/mL) and **7** (538 μg/mL) are equal (1 mM).

3.4. 14-(4-Nitrocinnamoyl)-3,19-isopropylidene andrographolide (**8**)

Ethyl chloroformate (0.12 mL, 1.32 mmol) was added to 4-nitrocinnamic acid (200 mg, 1.04 mmol) and Et_3N (0.30 mL, 2.31 mmol) in CH_2Cl_2 (10 mL) at 0 °C, and the reaction mixture was stirred for 1 h. Compound **2** (150 mg, 0.39 mmol) was then added, and reaction mixture was stirred at room temperature for another 24 h. Then CH_2Cl_2 (20 mL) and a saturated $NaHCO_3$ solution were added. The organic phase was separated, washed with water, dried over $NaSO_4$, and then filtered. The filtrate was concentrated in vacuo and purified by column chromatography, eluting with ethyl acetate/petroleum ether (2/1), affording compound **8** (230 mg, 79% yield) as a white powder, mp: 81.3 °C. ^{1}H NMR ($CDCl_3$, ppm): 8.28 (dd, $J = 8.56$ Hz, 2H), 7.69 (dd, $J = 8.64$ Hz, 2H), 7.77 (t, $J = 16.08$ Hz, 1H), 6.55 (d, $J = 16.04$ Hz, 1H), 6.10 (d, $J = 5.76$ Hz, 1H), 4.90 (s, 1H), 4.59 (d, $J = 14.36$ Hz, 2H), 4.33 (d, $J = 11.32$ Hz, 1H), 3.94 (d, $J = 11.60$ Hz, 1H), 3.17 (d, $J = 11.52$ Hz, 1H), 2.53–2.40 (m, 3H), 1.72 (m, 5H), 1.52 (s, 6H), 1.38–1.31 (m, 6H), 1.26 (s, 3H), 0.93 (s, 3H). MS (EI) $[M + H]^+$ *m/z*: 566.9. Anal. ($C_{32}H_{39}NO_8 + 0.5H_2O$) C, H, N.

3.5. 14-(4-Nitrocinnamoyl)andrographolide (**9**)

Compound **8** (95 mg) was added to acetic acid (3 mL, AcOH/$H_2O = 7/3$), and the solution was stirred at room temperature for 45 min. Water was added, then neutralized with $NaHCO_3$. The product was extracted with CH_2Cl_2, dried over $NaSO_4$, and then filtered. The filtrate was concentrated in vacuo and purified by column chromatography, eluting with ethyl acetate/petroleum ether (2/1), affording compound **9** (60 mg, 68% yield) as a white powder, mp: 153.0 °C. ^{1}H NMR ($CDCl_3$, ppm): 8.27 (dd, $J = 8.72$ Hz, 2H), 7.68 (dd, $J = 8.72$ Hz, 2H), 7.77 (d, $J = 16.04$ Hz, 1H), 7.08 (t, $J = 1.32$ Hz, 1H), 6.55 (d, $J = 16.08$ Hz, 1H), 6.09 (d, $J = 5.8$ Hz, 1H), 4.88 (d, $J = 1.44$ Hz, 1H), 4.34 (d, $J = 1.48$ Hz, 1H), 4.15 (d, $J = 11.20$ Hz, 1H), 3.72 (br s, 1H), 3.47 (br s, 1H), 3.33 (t, $J = 9.00$ Hz, 1H), 2.66 (d, $J = 7.20$ Hz, 1H), 2.50–2.41 (m, 4H), 1.85–1.80 (m, 2H), 1.54 (s, 3H), 1.28–1.26 (m, 8H), 0.65 (s, 3H). MS (ESI) $[M + H]^+$ *m/z*: 526. Anal. ($C_{29}H_{35}NO_8 + 2.5H_2O$) C, H, N.

3.6. Cell growth assay

P. aeruginosa cells (American Type Culture Collection, ATCC) were grown overnight in LB medium for 8 h, and were then diluted to an OD_{600} of 0.05. The culture (3 mL) was aliquoted to test tubes containing appropriate amount of test compound. Cell growth was tested following 5, 10, 15, 20 and 25 h of incubation, respectively. Growth data were expressed as OD_{600}.

3.7. Virulence factor assays

For assay of pyocyanin, cells were grown overnight in LB medium, washed in fresh media and diluted to an OD_{600} of 0.05. This culture was grown for 3–4 h to mid-log phase (OD_{600} of 0.3–0.5), and was then diluted to an OD_{600} of 0.05, and was aliquoted to test tubes containing appropriate amount of test compound. Following 18 h of growth, pyocyanin was extracted from filtered culture supernatants, and quantified using standard methods [48]. For assay of protease activity, cells were grown overnight in PB media (20% Protease Peptone, 1.4% $MgCl_2$, 10% K_2SO_4) at 37 °C. Cells were washed, and diluted to an OD_{600} of 0.05. After growth to mid-log phase, the cells were washed again, and resuspended to an OD_{600} of 0.05. This culture was then added to test tubes containing test compound and cultured for another 8 h. Protease activity was quantified by incubation of 100 μL of filtered culture supernatant with 5 mg azocasein substrate and 1 mL of 10 mM Tris (pH 7.2), 1 mM $CaCl_2$ for 8 h at 37 °C with agitation. Protease activity was represented by the OD_{440} of the enzyme assay following reaction quenching with EDTA and centrifugation to remove unreacted substrate, divided by the OD_{600} of the cell culture [49].

3.8. Visualization and analysis of P. aeruginosa population density and biofilms

Bacteria were grown overnight in LB medium, washed in fresh media and diluted to an OD_{600} of 0.05. This culture was grown for 3–4 h to mid-log phase (OD_{600} of 0.3–0.5) and then diluted with MH media to an OD_{600} of 0.05. An aliquot (1 mL) was transferred to test tubes containing a dilution series of test compounds, and incubated for 15 h. Bacterial cells were washed with distilled water, and were dried at room temperature. Bacterial population density was visualized with an atomic force microscopy (Autoprobe CP-Research, Thermomicroscopes, USA).

For biofilm assay, cells were grown overnight in LB medium, washed in fresh media and diluted to an OD_{600} of 0.05. This culture was grown for 3–4 h to mid-log phase (OD_{600} of 0.3–0.5), and diluted with MH media to an OD_{600} of 0.05. An aliquot (1 mL) was transferred to a dilution series of the test compounds. Biofilms were grown on respiratory track suction tube (polythene) surfaces. The surface was inoculated with *P. aeruginosa* (PAO1) and cultured for 3 and 7 days, respectively. After incubation, the surfaces were washed with physiological saline to remove the nonadherent cells. The biofilms were fixed with glutaral (2.5%), washed with PBS (pH 7.4) and dried with ethanol (50%, 70%, 80%, 90% and 100%, respectively). Biofilms were visualized with a scanning electron microscope (Quanta 400, Thermal FE Environment Scanning Microscope, FEI Philips, Dutch).

3.9. Drug combination assay

Cells were grown overnight in LB medium, diluted to an OD_{600} of 0.05, and aliquoted to test tubes containing a dilution series of the test compound. The cultures were incubated for 20 h, and cell growth was determined as OD_{600}.

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