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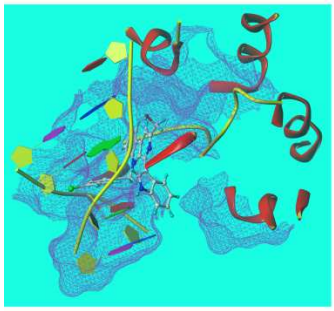
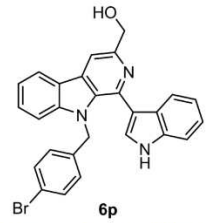
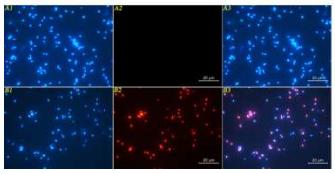
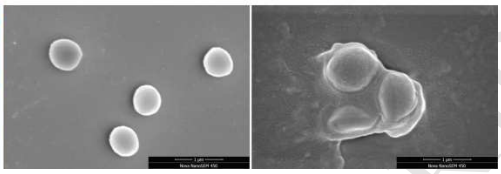




6p

MIC = 3.125 μmol/L against MRSA
(4-fold superiority than ciprofloxacin)
MIC = 1.5625 μmol/L against *R. solanacearum*
(16-fold superiority than propineb)





Computer-aided drug discovery: novel 3,9-disubstituted eudistomin U derivatives as potent antibacterial agents

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ABSTRACT

Thirty-two new 3,9-disubstituted eudistomin U derivatives were designed and synthesized based on computer-aided drug discovery (CADD). Sixteen 3,9-disubstituted eudistomin U derivatives (**6a**–**6p**) have exhibited potent antibacterial activity. Specially, the most active compound **6p** displayed better activity than commercial drugs fosfomycin sodium, ciprofloxacin and propineb, with a peak minimum inhibitory concentration (MIC) of 1.5625 μmol/L. The antibacterial mechanism indicated that these compounds could exert bactericidal effect by damaging bacterial cell membrane and disrupting the function of DNA gyrase.

Keywords: eudistomin U; design; synthesis; antibacterial; molecular docking; mechanism.

1. Introduction

Infections caused by bacterial pathogens are a major cause of morbidity and mortality worldwide.[1] Although the successful treatment of such infections by antibiotic drugs is widely regarded as a major medical breakthrough of the 20th century, this achievement may not be sustainable in the future, as bacteria have counteracted antibiotic pressure and developed or acquired resistances that render formerly efficacious drugs inactive.[2] Moreover, the intensive antibacterial discovery effort has seen a dramatic decline in the large pharmacy industry in the last two decades.[3] "The cost in terms of lost global production between now and 2050 would be an enormous 100 trillion USD if we do not take action," as a UK Government report states.[4] Therefore, a new antibacterial drug is urgently needed.

DNA gyrase is a type II topoisomerase which is independently essential for bacterial DNA replication. Specifically, it is primarily responsible for introducing negative supercoils into conformationally constrained DNA.[5] As DNA gyrase is absent in humans it is an appealing antibacterial target with quinolones developed as a successful class of antibiotics.[5, 6] Mechanism of DNA gyrase inhibition is known to occur in two ways: these inhibitors may bind DNA gyrase directly or they may bind to DNA and alter its structure, so that it cannot be recognized by DNA gyrase.[7]

Natural products are an important source of antibacterial agents.[8] Eudistomin U **L1** (Fig. 1), isolated from several species of marine ascidians, exhibited good antibacterial activity (IC_{50} = 22.61 μmol/L against *Staphylococcus aureus*).[9] Interestingly, it *per se* has two planar structure molecules, *β*-carboline and indole, which is similar to the commercial quinolones to some extent. Recently, Mulcahy's group reported that eudistomin U could bind to DNA.[10] Moreover, many studies of quinolones' antibacterial mechanisms have dismissed these enzymes *per se* as target and point to DNA as the direct binding species.[6, 11] So we speculate that the action mode of eudistomin U is binding to DNA area, resulting to the altering DNA structure could not be recognized by DNA gyrase.

Despite eudistomin U **L1** exhibiting good antibacterial potency, its activity is not sufficient to compete with commercial antibiotics, hindering its potential as a new

bactericide. Here in an effort to enhance the antibacterial activity we designed some eudistomin U derivatives (Fig. 1) based on computer-aided drug discovery (CADD), molecular docking evaluation. Moreover, we studied their antibacterial mechanism based on the most active compound.

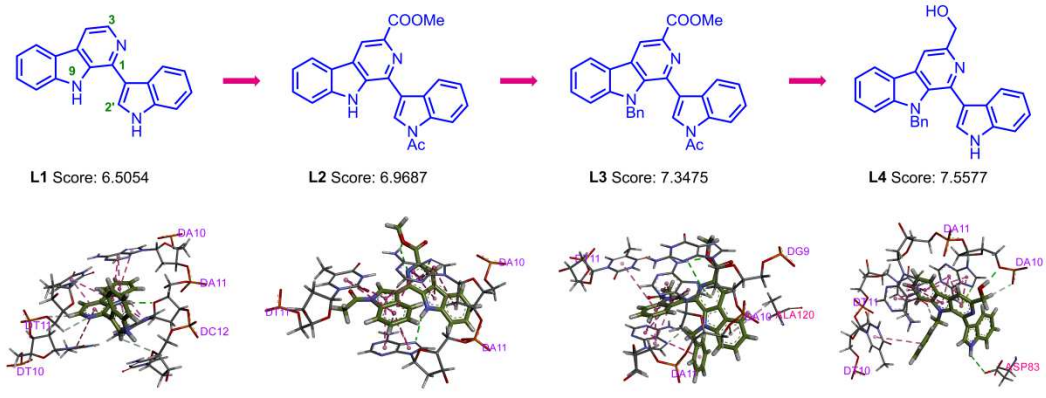

Fig. 1. Design of antibacterial eudistomin U analogues based on computer-aided drug discovery.

2. Results and Discussion

2.1. Design based on CADD

CADD has emerged as a highly successful way to find quality leads for subsequent optimization into drug candidates and approved new medicines.[12] Based on our speculation, the DNA gyrase-DNA complex (PDB ID: 5IWM) was selected as a template target and eudistomin U scaffold was retained as the ligand core, but was elaborated at the C-3, N-9 and N'-1 positions with various chemical moieties. The Surflex-Dock scoring function is a weighted sum of non-linear functions, mainly involving hydrophobic and polar complementarity, with additional terms for entropic and solvation effects.[13] The scores are expressed in -log10(K_d) units to represent binding affinities. As shown in Fig. 1, the score values gradually increased with changes in modifying groups, indicating the enhanced activity. Compounds **L1** and **L2** completely bind to DNA area and compounds **L3** and **L4** mainly interact with DNA, which confirms our proposed inference.

2.2. Chemistry

To realize a synthetic pathway for preparing analogues bearing the eudistomin U core we felt that the 1*H*-indole-3-carbaldehyde **1** would serve as a useful raw material (Fig. 2). Compound **2** was easily prepared by the acetylation reaction using acetic

anhydride with 98% yield. Application of the known synthetic procedures of Waters *et al.*,[14] Pictet-Spengler condensation of **2** and tryptophan methyl ester provided tetrahydro-*β*-carboline **3** as diastereomers (86% yield). Dehydrogenation of **3** under the oxygen with Pd/C as catalyst proceeded smoothly, affording **4** (**L2**) in 74% yield. Further this scaffold **4** was confirmed by x-ray single crystal diffraction (CCDC 1848195). With this compound **4** in hand, attention was focused on modifying the N-9 position. Benzyl group in this position significantly increased the scores (Fig. 1). So we synthesized a series of eudistomin U derivatives **5a**–**5p** (81–96% yields) with diverse substituted benzyl group on the N-9 position which varied in electron-inducing ability and substitution position. Subsequently, eudistomin U analogues **6a**–**6p** (78–95% yields) with hydrophilic hydroxyl and secondary amine groups were converted under $NaBH_4$ and $CaCl_2$.

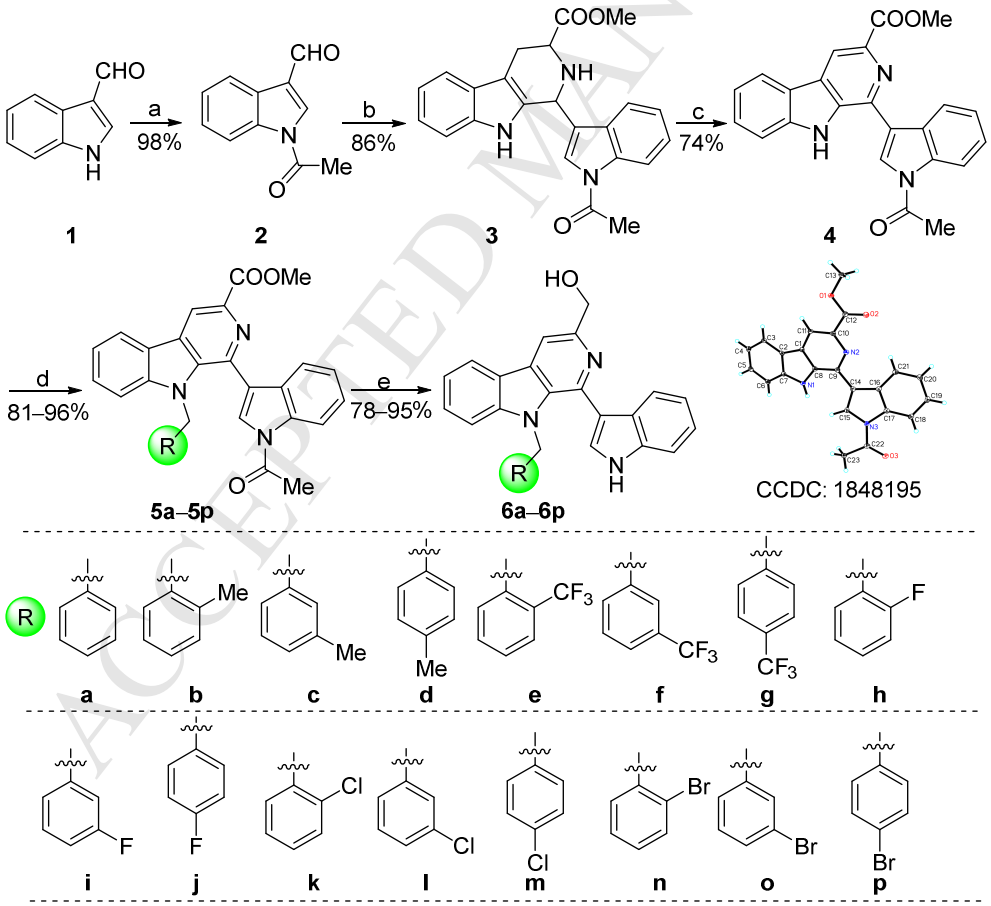

Fig. 2. Synthesis of the eudistomin U derivatives. a) Ac_2O, Et_3N, DMAP, DCM, 0 ^oC ~ r.t., 3 h; b) (i) Trp-COOMe, methylbenzene, reflux, 2 h; (ii) DCM:TFA (2:1), 0 ^oC ~ r.t., 23 h; c) Pd/C, O_2, xylene, 150 ^oC, 48 h; d) K_2CO_3, CH_3CN, benzyl bromide, 80 ^oC, 3 h; e) $NaBH_4$, $CaCl_2$, EtOH, r.t., 0.5 h.

All the structures of the target compounds (**4**, **5a**–**5p** and **6a**–**6p**) were confirmed by ^{1}H NMR, ^{13}C NMR and HRMS spectra. In the ^{1}H NMR spectra of compound **6p** (Supporting Information), the signals of the secondary amine and the hydroxyl group were detected around δ = 9.05 ppm and δ = 3.58 ppm, respectively. Moreover, the signals of the methylene carbon atoms were detected around 64.87 and 47.03 ppm in ^{13}C NMR, respectively. In addition, the signal of $[M+H]^+$ could be found at 482.0853 Da in HRMS of compound **6p** (error = 1.86 ppm), which conformed to the theoretical value 482.0862 Da within the allowable error range (error < 5 ppm).

2.3. Antibacterial activity and structure–activity relationship (SAR)

S. aureus and methicillin-resistant *S. aureus* (MRSA) are the leading causes of bacterial infections in humans with symptoms ranging from simple skin infections to severe necrotizing fasciitis and pneumonia.[15] *Bacillus cereus* could cause food poisoning through the production of distinct toxins.[16] *Ralstonia solanacearum* is a major component of plant pathogens.[17] So three Gram-positive bacteria (*S. aureus*, MRSA and *B. cereus*) and one Gram-negative bacteria (*R. solanacearum*) was selected as the tested bacteria in this work. Thirty-three compounds (**4**, **5a**–**5p** and **6a**–**6p**) were evaluated for their *in vitro* antibacterial activity through double dilution method, with fosfomycin sodium, ciprofloxacin and propineb as the positive controls (Table 1).

Sixteen compounds (**6a**–**6p**) displayed better activity against *S. aureus* compared with the commercial drug fosfomycin sodium (MIC = 100 μmol/L). Specifically, the MIC of compounds **6c**, **6f** and **6p** (MIC = 3.125 μmol/L) was equal to the commercial drug ciprofloxacin. Six compounds (**6d**, **6f**, **6g**, **6j**, **6m** and **6p**) displayed equal or superior activity against MRSA compared with fosfomycin sodium (MIC = 50 μmol/L). It was worth mentioning that compounds **6j** and **6p** have showed about 4-fold superiority than ciprofloxacin (MIC = 12.5 μmol/L) against MRSA. Eleven compounds (**6a**, **6c**, **6d**, **6f**, **6g**, **6j**, **6l**, **6m**, **6n**, **6o** and **6p**) displayed equal or superior activity against *B. cereus* compared with fosfomycin sodium (MIC = 25 μmol/L). Specially, four compounds (**6c**, **6f**, **6j** and **6p**) have exhibited about 2-fold superiority than ciprofloxacin. Compared with the commercial agrochemical bactericide propineb

Table 1. Antibacterial activity and TPSA values of eudistomin U analogues (MIC, μmol/L).

Compd.	*S. aureus*	MRSA	*B. cereus*	*R. solanacearum*	TPSA[b]
4	>100	>100	>100	>100	76.99
5a	>100	>100	>100	>100	66.14
6a	12.5	100	12.5	12.5	53.84
5b	>100	>100	100	>100	66.14
6b	25	>100	50	12.5	53.84
5c	>100	>100	>100	>100	66.14
6c	3.125	100	3.125	3.125	53.84
5d	>100	>100	>100	>100	66.14
6d	25	25	25	12.5	53.84
5e	>100	>100	>100	>100	66.14
6e	50	100	50	50	53.84
5f	>100	>100	>100	>100	66.14
6f	3.125	12.5	3.125	3.125	53.84
5g	>100	>100	>100	>100	66.14
6g	25	25	25	25	53.84
5h	>100	>100	>100	>100	66.14
6h	25	>100	50	25	53.84
5i	>100	>100	>100	>100	66.14
6i	25	>100	50	50	53.84
5j	>100	>100	>100	>100	66.14
6j	6.25	3.125	3.125	6.25	53.84
5k	100	>100	>100	>100	66.14
6k	25	>100	50	25	53.84
5l	>100	>100	>100	>100	66.14
6l	25	>100	25	50	53.84
5m	>100	>100	>100	>100	66.14
6m	25	50	12.5	12.5	53.84
5n	>100	>100	>100	>100	66.14
6n	12.5	>100	25	50	53.84
5o	>100	>100	>100	>100	66.14
6o	25	>100	25	25	53.84
5p	>100	>100	>100	>100	66.14
6p	3.125	3.125	3.125	1.5625	53.84
F.S.[a]	100	50	25	50	NC[c]
C.[a]	3.125	12.5	6.25	3.125	NC[c]
P.[a]	50	25	50	25	NC[c]

[a]F.S. = Fosfomycin sodium, C. = Ciprofloxacin, P. = Propineb.

[b]TPSA = Topological polar surface area.

[c]NC = No calculation.

(MIC = 25 μmol/L) against *R. solanacearum*, twelve compounds (**6a**, **6b**, **6c**, **6d**, **6f**, **6g**, **6h**, **6j**, **6k**, **6m**, **6o** and **6p**) exhibited equal or better activity with a peak MIC

lower than 1.5625 μmol/L, 16-fold superiority. Compound **6p** was considered to be the highly active eudistomin U derivative which exhibited better activity than these three commercial drugs.

In general, the activity data of compounds **6a**–**6p** conformed to our design. To explore the loss in activity of compounds **4** and **5a**–**5p**, we calculated the topological polar surface area (TPSA) values (Table 1), which was used extensively in medicinal chemistry to predict absorption and optimize a compound's membrane permeability.[18, 19] The larger the value, the poorer the absorption and membrane permeability. Obviously, the TPSA values of compounds **4** and **5a**–**5p** were greater than compounds **6a**–**6p**, which indicated that the loss in activity might be due to these compounds could not completely penetrate the cell membrane to active on the target. SAR studies for diverse substituted benzyl group on the N-9 position could provide some guidance for future design of eudistomin U-based antibacterial agents. Electron-donating methyl and electron-withdrawing trifluoromethyl groups incorporated into the *meta*-position of the benzyl backbone were better than that on *ortho*- and *para*-positions. Electron-withdrawing fluorine, chlorine and bromine substituents on *para*-position displayed higher activity than that on *ortho*- and *meta*-positions.

2.4. Preliminary antibacterial mechanism

2.4.1. Fluorescence microscopy analysis

The highly active compound **6p** and *S. aureus* were selected to explore the antibacterial mechanism. Two dyes, Hoechst (2'-(4-ethoxyphenyl)-5-(4-methyl -1-pipe-razinyl)-2,5'-bi-1*H*-benzimidazoletrihydrochl-oride) and PI (propidium iodide), were used to differentiate between cells with either an intact or a damaged membrane (Fig. 3).[20] Hoechst can easily permeate the membrane of intact cells and show blue fluorescence regardless of cell viability. In contrast, PI is a DNA intercalator but lacks cell permeability which fluoresces in red only when cell membranes are disrupted. As shown in Fig. 3A, *S. aureus* exhibited blue fluorescence in the absence of compound **6p**, whereas no fluorescence was showed in the PI channel, indicating the membranes of *S. aureus* were intact. However, after *S. aureus*

was incubated with **6p** for 1 h, they were stained by both Hoechst and PI, suggesting that the membranes of *S. aureus* were damaged (Fig. 3B).

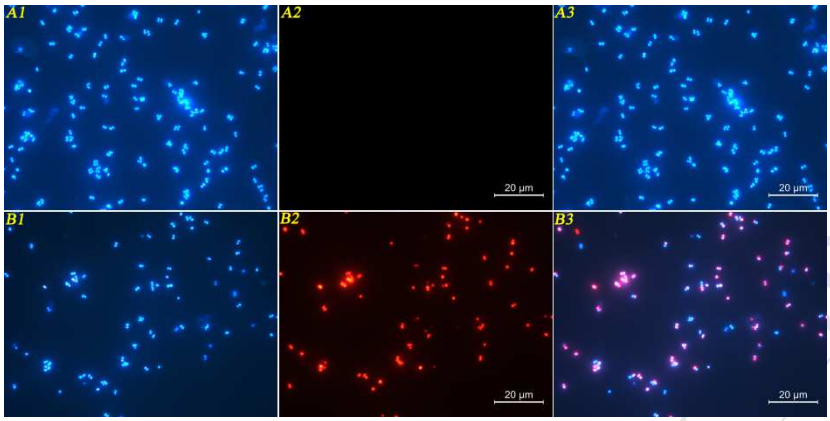

Fig. 3. Fluorescence micrographs of *S. aureus* treated or not treated with **6p** for 1 h. (A1) no treatment, Hoechst stained; (A2) no treatment, PI stained; (A3) no treatment, merge graph; (B1) *S. aureus* treatment with **6p**, Hoechst stained; (B2) *S. aureus* treatment with **6p**, PI stained; (B3) *S. aureus* treatment with **6p**, merge graph.

2.4.2. Scanning electron microscopy analysis

SEM of *S. aureus* revealed morphological changes in the bacterial cell surface (Fig. 4). The surfaces of cells in the untreated group (Fig. 4A) was relatively smooth and regular, whereas when treated with compound **6p** (Fig. 4B) there was rough and irregular. Increased permeabilization of the membrane may explain the leakage of cytoplasmic material.[21]

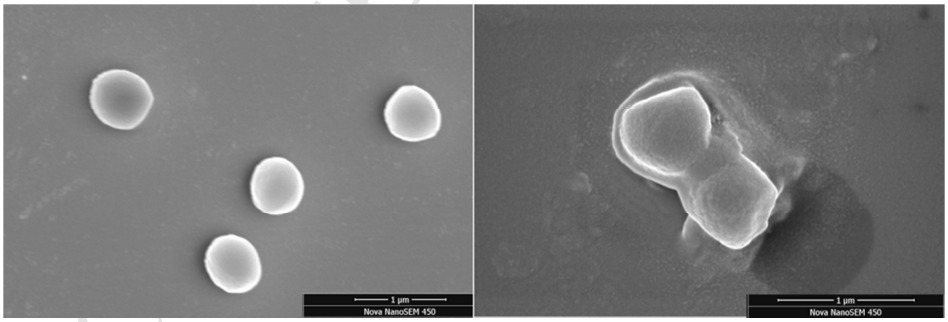

Fig. 4. SEM of *S. aureus* cells: (A) blank group, left; (B) treated group (**6p**), right.

2.4.3. Transmission electron microscope analysis

To further characterize the bactericidal effects of compound **6p**, TEM was also used to visualize the morphological changes of *S. aureus* cells (Fig. 5). In the absence of compound **6p**, the *S. aureus* cells showed a well-defined cell membrane. After *S. aureus* cells were treated with compound **6p** for 1h, the cells lose or began to lose the

clear boundary of cell membrane.[22] Overall, the compound **6p** could increase permeabilization and disrupt integrity of the cell membrane.

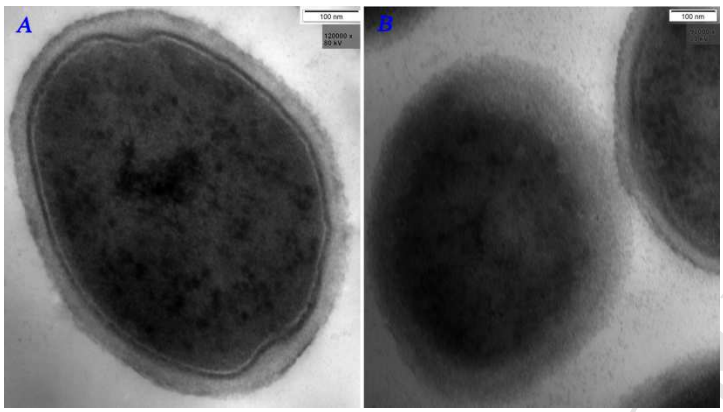

Fig. 5. TEM of *S. aureus* cells: (A) blank group, left; (B) treated group (**6p**), right.

2.4.4. Molecular docking study

Molecular docking studies allow us to visualize the molecular interactions between compound **6p** and DNA gyrase-DNA complex. The docking evaluation gave a good total score (7.4079) for compound **6p**. As shown in Fig. 6, compound **6p** completely bound to DNA area. DNA binding agents tend to interact noncovalently with the host molecule through two general modes: in a groove-bound fashion and intercalative association.[23] Obviously, the mainly binding mode was groove-bound fashion in Fig. 6. Moreover, the eudistomin U core completely bound to DNA in a groove-bound fashion stabilized by a mixture of carbon–hydrogen bond, π–π stacked, π–donor hydrogen bond and hydrogen bonds. Specially, the hydroxyl and secondary amine fragments of compound **6p** were adjacent to DA10 and DA11, forming two strong hydrogen bonds (1.96 and 2.13 Å), respectively. The benzyl group bound to DNA through an intercalative association mode. Interestingly, the bromobenzene ring was locked into the DNA base pairs through π–alkyl bonds between bromine atom and DA10, DA11. The score (7.4079) of **6p** is lower than **L4** (7.5577). However, **6p** exhibited better activity, indicating completely binding to DNA area may be favorable for improving the activity.

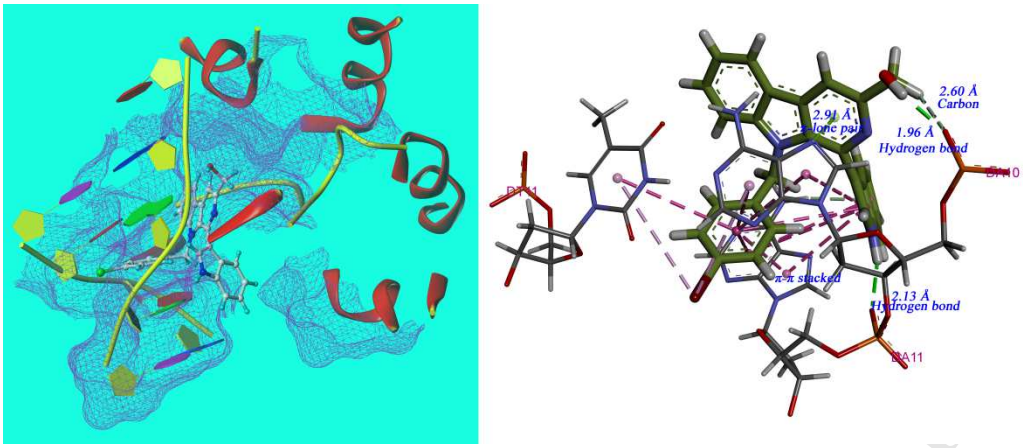

Fig. 6. Three-dimensional conformations of compound **6p** docked in DNA gyrase-DNA complex.

2.4.5. Isothermal titration calorimetry measurements

To investigate the binding studies, calf thymus DNA was selected as DNA model because of its medical importance, low cost and ready availability properties.[6, 24] Isothermal titration calorimetry (ITC),[25] which could give direct measurement of the dissociation constant, the stoichiometry, the heat of reaction, and indirect access to other thermodynamic parameters such as entropic binding contribution or Gibbs free energy, was carried out to study the interactions of **6p** with DNA. As shown in Fig. 7, the pink purple dotted line corresponded to a binding model with a 1:1 stoichiometry and was fitted with the change of enthalpy $\Delta H = -26.73$ kJ/mol, entropy $\Delta S = 30.14$ J/mol·K and free energy $\Delta G = -35.72$ kJ/mol. The data indicated that the binding was enthalpy-driven and entropy-driven spontaneous reaction.[26] The large negative enthalpy change mainly contributed by hydrogen bonds, favored the molecular docking results.[27] The dissociation constant K_d and binding constant K_a values were 5.526×10^{-7} M and 1.810×10^{6} M^{-1}, respectively. According to the calculation formula, Score = $-\log10(K_d)$, the calculated score is 6.2576, which is close to the molecular docking score 7.4079. The difference between these two scores may be mainly caused by differences in DNA, which need further research.

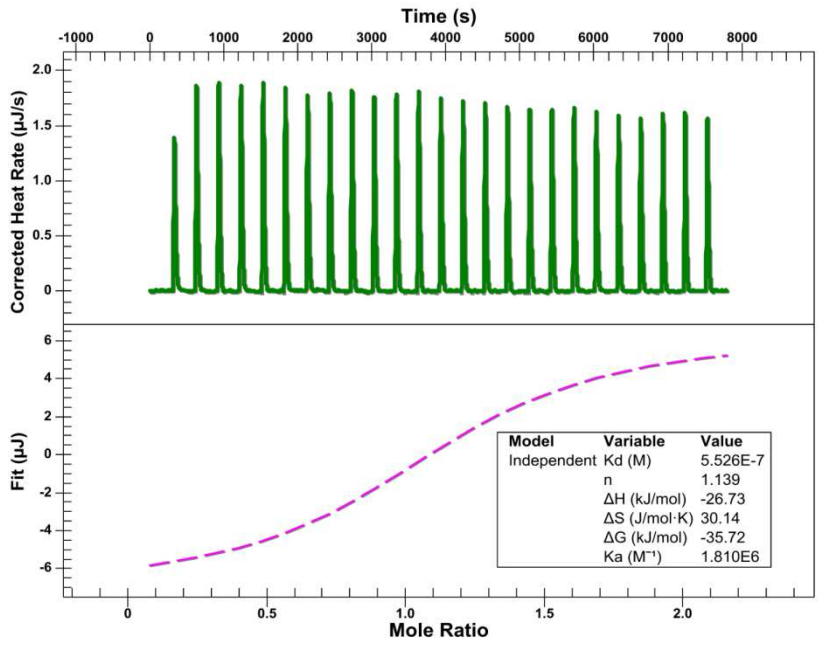

Fig. 7. Calorimetric titration of the DNA with compound **6p** at 298 K. (A) Heat flow as a function of time (green); (B) The pink purple dotted line corresponds to the theoretical independent model. The thermodynamic constants are presented in the pane.

The antibacterial mechanism was deduced that compound **6p** might attack the bacterial cell membrane and cause the membrane damage. The consequent increased membrane permeability will then allow **6p** to enter the cells. The compound **6p** in the cytoplasm will interact further with DNA, which in turn disrupt the function of DNA gyrase and cause cell death. Overall, these compounds could exert bactericidal effect by damaging bacterial cell membrane and disrupting the function of DNA gyrase.

3. Conclusions

In conclusion, 3,9-disubstituted eudistomin U derivatives have exhibited potent antibacterial activity by damaging bacterial cell membrane and disrupting the function of DNA gyrase. The most active compound **6p** displayed better activity than commercial drugs, 4-fold superiority against MRSA than ciprofloxacin and 16-fold superiority against *R. solanacearum* than propineb. Overall, this work demonstrated here the antibacterial potential of eudistomin U scaffold, enriched the types of candidate antibiotics and provided more options for solving the current antibiotic crisis.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data related to this article can be found.

Conflict of interest

The authors state no conflict of interest.

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Highlights

- Thirty-two new eudistomin U derivatives were designed and synthesized based on CADD.
- These compounds exerted bactericidal effect by damaging bacterial cell membrane and disrupting the function of DNA gyrase.
- Compound **6p** displayed better activity than commercial drugs.