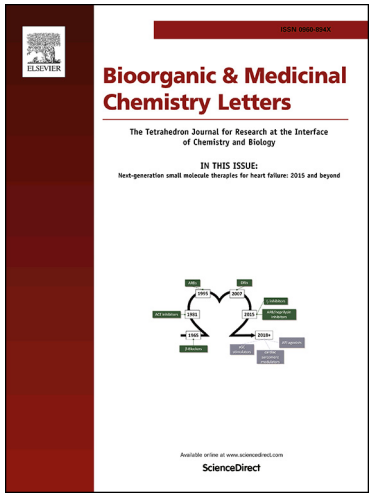
Accepted Manuscript



Design and synthesis of C^{10} modified and ring-truncated canthin-6-one analogues as effective membrane-active antibacterial agents

Jiangkun Dai, Wenjia Dan, Yunyun Zhang, Mengfan He, Junru Wang

PII: S0960-894X(18)30478-5
DOI: https://doi.org/10.1016/j.bmcl.2018.06.001
Reference: BMCL 25882

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 29 March 2018
Revised Date: 25 April 2018
Accepted Date: 1 June 2018

Please cite this article as: Dai, J., Dan, W., Zhang, Y., He, M., Wang, J., Design and synthesis of C^{10} modified and ring-truncated canthin-6-one analogues as effective membrane-active antibacterial agents, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.06.001

Design and synthesis of C^{10} modified and ring-truncated canthin-6-one analogues as effective membrane-active antibacterial agents

Jiangkun Dai[a], Wenjia Dan[a], Yunyun Zhang[a], Mengfan He[a], Junru Wang[a,b,*]

[a]College of Chemistry & Pharmacy, Northwest A&F University, 22 Xinong Road, Yangling 712100, Shaanxi, China.

[b]State key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Zhangjiang Hi-Tech Park, 201203, Shanghai, China.

These two affiliation address are equal.

Corresponding Author

*E-mail: wangjunru@nwafu.edu.cn; wangjr07@163.com.

ABSTRACT: A series of canthin-6-one analogues were designed and synthesized in order to study their antibacterial activity and structure–activity relationships. Compound **22** showed a broad spectrum of antibacterial activity and exhibited better bactericidal effect (8-fold superiority against *Staphylococcus aureus* and 2-fold superiority against *Ralstonia solanacearum*) than fosfomycin sodium and propineb with a minimum inhibitory concentration value of 2 μg/mL. Moreover, it showed low cytotoxicity, stimulation on germination rates and good "drug-like" properties. Membrane-active mechanism was further studied by fluorescent microscopy, scanning electron microscopy, cytoplasmic *β*-galactosidase leakage assay and evaluation of the molecular docking. The results showed that **22** may exert its bactericidal effect by damaging bacterial cell membranes and influencing the membrane formation, both of which could lead to cell death. The *in vivo* antibacterial assay with a protective efficacy of 68% demonstrated the potential of C ring-truncated canthin-6-one **22** as a new bactericide.

Keywords: *canthin-6-one; synthesis; antibacterial activity; structure–activity relationships; mechanism.*

Healthcare systems, farming, and the food production industry are the main sectors driving antibiotic consumption.[1–3] *Staphylococcus aureus* and methicillin-resistant *S. aureus* are the leading causes of bacterial infections in humans with symptoms ranging from simple skin infections to severe necrotizing fasciitis and pneumonia.[4] *Bacillus cereus* could cause food poisoning, such as a diarrheal syndrome and an emetic syndrome, both through the production of distinct toxins.[5] *Bacillus subtilis* and *Ralstonia solanacearum* are major components of plant pathogens.[6] We could see that all of these diseases caused by bacteria constitute a major threat to humans' life and property. The emergence of the antibiotic crisis has further brought great challenges to normal production and life. "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.[7] Therefore, we need to develop new drugs to replace the ones that no longer work.

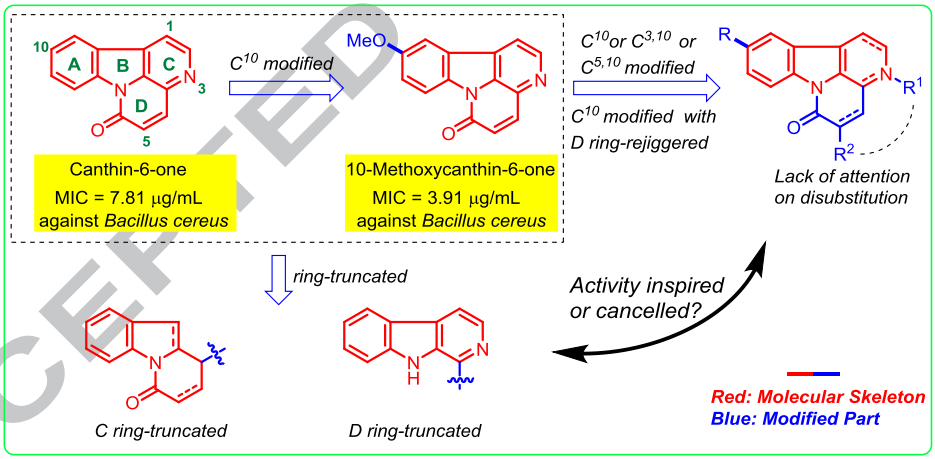


Figure 1. Synthetic tactics of C^{10} modified and ring-truncated canthin-6-one analogues.

Canthin-6-one alkaloids, a subclass of *β*-carboline alkaloids, are reported to have antibacterial activity.[8] Recently, our group also reported the antibacterial activity of canthin-6-one and 10-methoxycanthin-6-one with minimum inhibitory concentration values (MICs) of 7.81 and 3.91 μg/mL against *B. cereus*, respectively (Figure 1).[9,10] The results indicated that C^{10} modified canthin-6-one analogues may be a good option for improving the antibacterial activity. In addition, little attention has been paid to the $C^{3,10}$ or $C^{5,10}$ disubstitution, C^{10} modified with D ring-rejiggered and ring-truncated

(ABC ring and ABD ring) canthin-6-one analogues for seeking highly antibacterial agents. In the present study, such analogues were synthesized and evaluated for their *in vitro* antibacterial activity. In addition, the structure–activity relationships, preliminary antibacterial mechanism, drug feasibility and the *in vivo* protective efficacy were also studied.

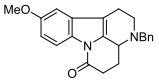
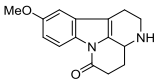
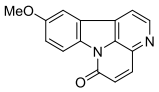
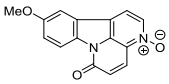
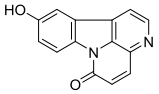
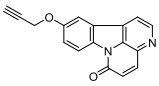
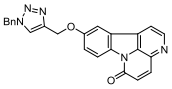
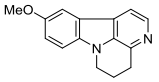
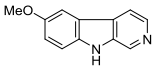
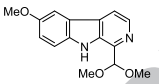
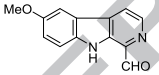
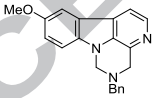
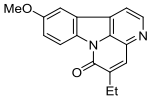
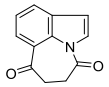
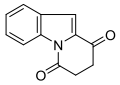
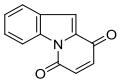
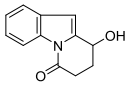
The synthesis of the target compounds **3**–**11**, **13**–**14**, **16**–**17** and **20**–**26** is illustrated in Figures S1–S4. Compound **27** has been early prepared and reported by us.[11] All spectral and analytical data were consistent with the assigned structures. Specifically, the successful synthesis of the canthin-6-one analogues was further confirmed by the crystal structure of compound **21** (CCDC:1820037; Figure S5).

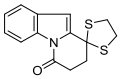
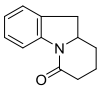
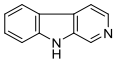
In the ^{13}C nuclear magnetic resonance (NMR) spectra of compound **21**, the signal of the ketone group was detected around δ = 187.68 ppm and the signal of the lactam group was detected around δ = 167.23 ppm. The signals of the methylene hydrogen atoms were observed at δ = 3.17 ppm and δ = 3.10 ppm in the ^{1}H NMR spectra, respectively. Moreover, the signals of the phenyl hydrogen atoms appeared in the aromatic region. After oxidation, the signals of the methylene group were disappeared in the NMR spectra of compound **22**, which indicated that the oxidation reaction was successful. In addition, the signal of $[M+H]^+$ could be found at 198.0556 Da in the high-resolution mass spectroscopy (HRMS) of compound **22** (error = 3.53 ppm), which conformed to the theoretical value within the allowable error range (error < 5 ppm).

Compounds (**3**–**11**, **13**–**14**, **16**–**17** and **20**–**27**) were evaluated for their *in vitro* antibacterial activity against four Gram-positive bacteria and one Gram-negative bacterium, with fosfomycin sodium, cefotaxime sodium and propineb as the positive controls (Table 1). The partial antibacterial activity of compounds **4**, **5** and **7** has been reported by us.[10] However, the activity against *S. aureus* and MRSA of these three compounds has not been reported. Compared with the positive control fosfomycin

sodium (MIC = 16 μg/mL) against *S. aureus*, three analogues (**21**, **22** and **23**) exhibited superior activity with a peak MIC lower than 2 μg/mL. It was worth mentioning that compound **22** has showed about 16-fold superiority than canthin-6-one (MIC = 31.25 μg/mL) against *S. aureus*.[9] Two compounds (**21** and **22**) displayed better activity against MRSA than the positive controls. Specifically, compound **22** was presented as potent antibacterial lead against MRSA (2 μg/mL), 4-fold and 8-fold more promising than the commercial drugs fosfomycin sodium and cefotaxime sodium, respectively. Six compounds (**5**, **7**, **8**, **21**, **22** and **23**) displayed equal or superior activity against *B. cereus* compared with the positive controls cefotaxime sodium and propineb (MIC = 16 μg/mL). For *B. subtilis*, compounds **21** and **22** displayed equal or superior activity compared with the agrochemical fungicide propineb. Compared with the positive control propineb (MIC = 8 μg/mL) against *R. solanacearum*, four analogues (**8**, **10**, **21** and **22**) exhibited equal or better activity with a peak MIC lower than 4 μg/mL. Interestingly, 10-methoxycanthine (**10**), 2-fold more promising than propineb against *R. solanacearum*, exhibited some selectivity for the bacterial. Generally, compound **22** was considered to be the preferred canthin-6-one analogue which showed a broad spectrum of antibacterial activity and exhibited better bactericidal effect than fosfomycin sodium and propineb.

Table 1. Antibacterial activity (MIC in μg/mL) of canthin-6-one analogues.

No.	Structure	Gram-positive bacteria				Gram-negative bacteria
		S. aureus	MRSA	*B. cereus*	*B. subtilis*	*R. solanacearum*
3		64	64	>64	>64	>64
4		>64	>64	>64	>64	>64
5		>64	64	4	8	16
6		>64	>64	>64	>64	>64
7		>64	>64	16	>64	>64
8		64	>64	8	>64	8
9		>64	>64	>64	>64	>64
10		>64	32	32	>64	4
11		>64	>64	>64	>64	>64
13		>64	>64	>64	>64	>64
14		>64	>64	32	>64	>64
16		>64	>64	64	64	>64
17		>64	>64	>64	>64	>64
20		>64	>64	>64	>64	>64
21		8	4	16	4	8
22		2	2	4	2	4
23		8	32	16	16	32

24		32	>64	>64	>64	>64
25		>64	>64	>64	>64	>64
26		>64	>64	>64	>64	>64
27		>64	>64	>64	>64	>64
F.S.[a]	-	16	8	4	2	8
C.S.[a]	-	1	16	16	2	2
P.[a]	-	16	16	16	4	8

[a]Positive controls, F.S. = Fosfomycin sodium, C.S. = Cefotaxime sodium, P. = Propineb.

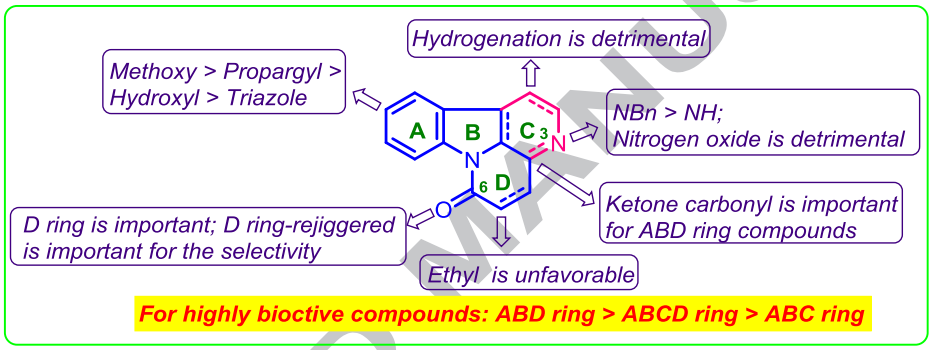


Figure 2. Structure–activity relationships of canthin-6-one analogues against bacteria.

Based on the antibacterial activity data, the structure–activity relationships were carefully investigated (Figure 2). Hydrogenation is detrimental. For hydrogenated canthin-6-ones, the activity of *N*-benzyl compound **3** is better than that of the debenzyl compound **4**. Compared with the MIC of compound **4** against *S. aureus* and MRSA (> 64 μg/mL), compound **3** exhibited a better MIC (64 μg/mL). Nitrogen oxide at N^3 and ethyl substituent at C^5 are unfavorable. Compounds **6** and **17** are inactive under the tested range (64–1 μg/mL). The rejiggered D ring could change the selectivity against bacteria. For example, 10-methoxycanthine (**10**) exhibited a significant MIC (4 μg/mL) against *R. solanacearum*. Compound **16**, D ring-rejiggered analogue with an *N*-benzyl group at position 5, showed weak antibacterial activity. For substituents of fully aromatic canthin-6-ones at position 10, the activity against *B. cereus* trend is: Methoxy > Propargyl > Hydroxyl > Triazole. For C ring-truncated canthin-6-one analogues, *α,β*-unsaturated ketone fragment is favorable for improving

the antibacterial activity. For example, compound **22** showed 4-fold superiority than compound **21** against *S. aureus* and *B. cereus*. De-ketone products (**25** and **26**) were inactive under the tested range which also indicated that the ketone carbonyl group was important. The weak or inactive activity of D ring-truncated canthin-6-one analogues highlighted the importance of D ring. It is obviously to find that the activity trend of the highly bioactive compounds: ABD ring > ABCD ring > ABC ring.

Many natural and synthetic agents often have relatively low application because of their toxicity.[12] The highly bioactive C ring-truncated canthin-6-one analogue **22** was evaluated for their cytotoxicity against Hela (cervical cancer), SGC-7901 gastric adenocarcinoma), A549 (lung cancer) and L02 (normal human hepatocyte) cells (Table S1). The cell viabilities were more than 89% under an effective antibacterial dose (4 μg/mL) and the selectivity indices were greater than 4.0 for the four cells. The results suggested that the compound **22** was low cytotoxicity.

Compound **22** was also evaluated for allelopathic activity against turnip (*Raphanus sativus*) and wheat (*Triticum aestivum* L.) seeds by determining the germination rates with respect to the positive control, glyphosate, a broad-spectrum systemic herbicide.[13] As shown in Table S2, compound **22** showed weak stimulation effect on germination rates under an effective antibacterial dose (4 μg/mL), which indicated that compound **22** will not inhibit plant growth when it acts as bactericides.

In 1997, Lipinski *et al.* published what is widely regarded as the key paper defining physicochemical and structural properties profiles for the optimal oral availability of drugs.[14] In 2003, Clarke and Delaney reported that most herbicides and fungicides also adhere to the Lipinski rule.[15] As shown in Table S3, the log*P* value (2.03) is lower than 5, the molecule weight (197.19) is lower than 500, the number of hydrogen bond acceptors (3) is lower than 10 and the number of hydrogen bond donors (0) is lower than 5. The physicochemical properties therefore conform to the Lipinski rule, which demonstrates that compound **22** has good "drug-like" properties.

The antimicrobial mechanisms of canthin-6-one analogues are complex and elusive. Membrane-active mechanism may be a good breakthrough. The most potent compound **22**, was thus examined for its ability to compromise bacterial membranes of the representative bacterium *S. aureus*.

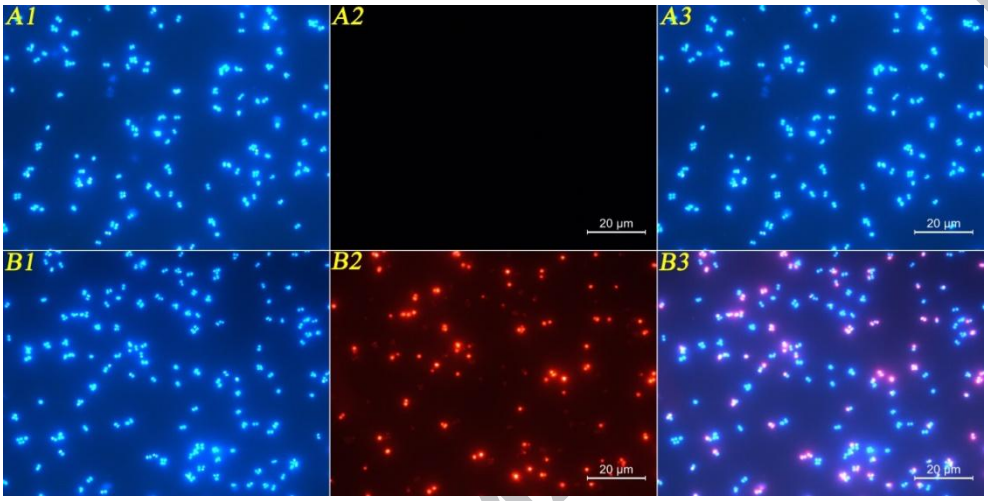


Figure 3. Fluorescence micrographs of *S. aureus* treated or not treated with 1 μg/mL **22** for 1 h. (A1) no treatment, Hoechst stained; (A2) no treatment, PI stained; (A3) no treatment, merge graph; (B1) *S. aureus* treatment with **22**, Hoechst stained; (B2) *S. aureus* treatment with **22**, PI stained; (B3) *S. aureus* treatment with **22**, merge graph.

Two dyes, Hoechst (2'-(4-ethoxyphenyl)-5-(4-methyl-1-pipe-razinyl)-2,5'-bi-1H--benzimidazoletrihydrochl-oride) and PI (propidium iodide), were used to differentiate between cells with either an intact or a damaged membrane (Figure 3).[16] 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 Figure 3A, *S. aureus* exhibited blue fluorescence in the absence of compound **22**, whereas no fluorescence was showed in the PI channel, indicating the membranes of *S. aureus* were intact. However, after *S. aureus* was incubated with **22** for 1 h, they were stained by both Hoechst and PI, suggesting that the membranes of *S. aureus* were damaged (Figure 3B).

SEM (scanning electron microscopy) of *S. aureus* revealed morphological

changes in the bacterial cell surface (Figure 4). The surfaces of cells in the untreated group (Figure 4A) was relatively smooth and regular, whereas when treated with compound **22** (Figure 4B) there was shrinkage. Increased permeabilization of the membrane may explain the leakage of cytoplasmic material.[17]

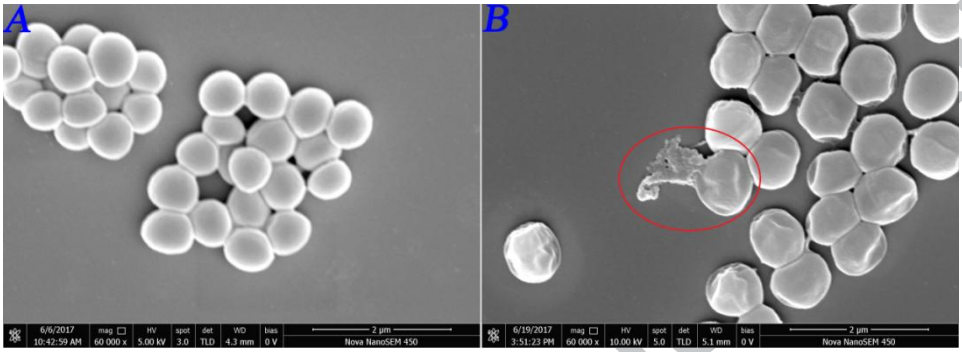


Figure 4. SEM of *S. aureus* cells: (A) blank group; (B) treated group.

In normal conditions, cytoplasmic *β*-galactosidase cannot pass through the cell membrane of bacteria. However, if the cell membrane is damaged, cytoplasmic *β*-galactosidase could be detected due to its leakage through the damaged membrane.[18] In the present study, M9 lactose medium was used to induce the production of cytoplasmic *β*-galactosidase in *S. aureus*. Compound **22** was added to the culture medium and the enzymatic activity of *β*-galactosidase measured. As shown in Figure S6, *β*-galactosidase activity in the culture medium was detected at 10 min after treatment with **22**, and this activity kept increasing during the test period. No *β*-galactosidase activity was detected in the culture medium of the control bacterial cells (not treated with **22**). These results indicate that compound **22** increases the cell membrane permeability of *S. aureus*, which leads to the leakage of *β*-galactosidase.

The Surflex-Dock scoring function is a weighted sum of non-linear functions based on the binding affinities of protein-ligand complexes coupled with their crystallographically determined structures. The function's primary terms involve hydrophobic and polar complementarity, with additional terms for entropic and solvation effects. Surflex-Dock scores are expressed in $-\log_{10}(K_d)$ units to represent binding affinities.[19]

Fatty acids are an important component of the bacterial cell membrane. The bacterial fatty acid biosynthesis pathway has recently generated much interest for the development of novel classes of antibacterial agents.[20] Biotin carboxylase (BC), one important portion of the acetyl-CoA carboxylase which catalyzes the first enzymatic step of fatty acid biosynthesis, is an effective target for antibacterial activity.[21,22] In addition, the compound **22** is similar to the skeleton of the BC inhibitor imidazo[4,5-*b*]pyridine to some extent.[23] So we further explored the inhibition activity of compound **22** against BC through molecular docking evaluation, which might influence the formation of bacterial membranes.

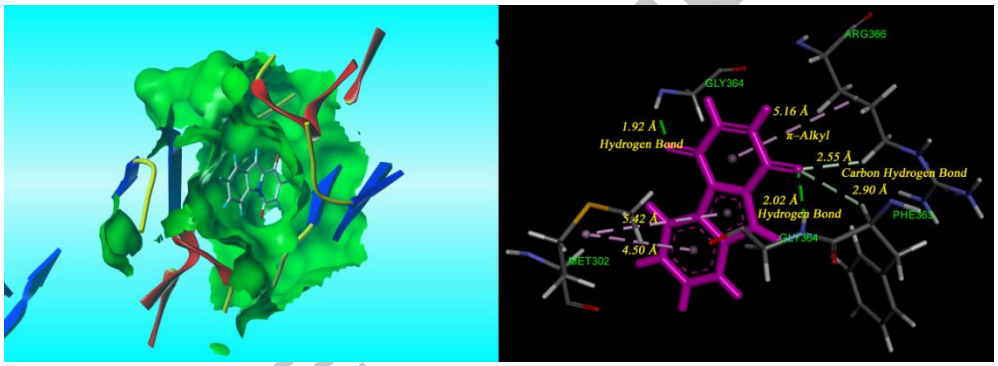


Figure 5. Three-dimensional conformations of compound **22** docked in biotin carboxylase.

A crystal of bacterial biotin carboxylase (PDB ID: 2V58) was selected as a possible target. The docking evaluation gave a good total score (5.5146) for compound **22**. And the calculated dissociation constant (K_d) value was 3.05×10^{-6}. These results might rationalize the antibacterial mechanism that the intercalation of **22** disrupts the function of biotin carboxylase, inhibiting the biosynthesis of fatty acids, further influencing the formation of membrane and ultimately leading to cell death. As shown in Figure 5, there are two π–alkyl interactions between the planar ABD ring and the MET302 residue of the target (4.50 and 5.42 Å). Simultaneously, two hydrogen bonds (1.92 and 2.02 Å) are formed between the carbonyl of D ring and the GLY364 residue. The ketone carbonyl is adjacent to the ARG366 and PHE363 residues, forming two carbon–hydrogen bonds (2.55 and 2.90 Å). These interactions also explained the importance of ketone carbonyl group which is beneficial for

improving antibacterial activity, conforming to the proposed structure–activity relationships.

Fluorescent microscopy, scanning electron microscopy and cytoplasmic *β*-galactosidase leakage assay indicated that **22** may exert its bactericidal effect by damaging bacterial cell membranes. The docking evaluation illustrated that **22** might disrupt the function of biotin carboxylase which could influence the formation of membrane. These findings suggested dual antibacterial mechanism of membrane-active compound **22**, namely membrane damage and membrane formation.

The *in vivo* antibacterial activity (protective effect) of the highly bioactive compound **22** was tested against *R. solanacearum* on eggplant leaf (Figure S7).[24,25] Compound **22** was found to have a good preventative effect against *R. solanacearum*, with a protective efficacy of 68%. This demonstrated the potential of the C ring-truncated canthin-6-one analogue **22** as a new candidate for crop protection.

In summary, we have reported a class of canthin-6-one derivatives as potential antibiotic agents, and compound **22** was considered to be the highly bioactive canthin-6-one analogue which exhibited better bactericidal effect than fosfomycin sodium and propineb. Simultaneously, this study provided a feasible approach, ring-truncated structural modification, for developing new bactericides based on polycyclic functional structure. Moreover, the dual membrane-active antibacterial mechanism, membrane damage and membrane formation, was proposed as one of the possible targets. To our delight, the *in vivo* antibacterial assay demonstrated the potential of the C ring-truncated canthin-6-one **22** as a new candidate for crop protection.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (Grant No. 81773603), the Open Foundation of Key Laboratory of Synthetic and Natural Functional Molecule Chemistry of Ministry of Education (Northwest

University, China), and the State Key Laboratory of Drug Research (Grant No. SIMM1705KF-09).

Supplementary data

Supplementary data associated with this article can be found.

References

[1] Laxminarayan R, Duse A, Wattal C, et al. *Lancet. Infect. Dis.* **2013**, *13*, 1057–1098.

[2] Li DD, Zhang SS, Song ZH, et al. *Eur. J. Med. Chem.* **2018**, *143*, 558–567.

[3] Zhou Y, Zhang SS, He HW, et al. *Bioorg. Med. Chem.* **2018**, *26*, 84–95.

[4] Benoit AR, Schiaffo C, Salomon CE, et al. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 3014–3017.

[5] Griffiths MW, Schraft H. *Foodborne Diseases (Third edition)* **2017**, 395–405.

[6] Salanoubat M, Genin S, Artiguenave F, et al. *Nature* **2002**, *415*, 497–502.

[7] O'Neill J. Government of the UK. http://apo.org.au/node/63983.

[8] Dai JK, Li N, Wang JR, et al. *Molecules* **2016**, *21*, 493.

[9] Dai JK, Dan WJ, Li N, et al. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 580–583.

[10] Zhao F, Dai JK, Liu D, et al. *Molecules* **2016**, *21*, 390.

[11] Li N, Dai JK, Du HT, et al. *Spectrochim. Acta. A Mol. Biomol. Spectrosc.* **2015**, *136*, 900–905.

[12] Xu QB, Chen XF, Feng J, et al. *Sci. Rep.* **2016**, *6*, 36238.

[13] Zhang Q, Wang SQ, Tang HY, et al. *J. Agric. Food. Chem.* **2013**, *61*, 11447–11452.

[14] Lipinski CA, Lombardo F, Dominy BW, et al. *Adv. Drug. Deliver. Rev.* **1997**, *23*, 3–25.

[15] Clarke ED, Delaney JS. *Chimia* **2003**, *57*, 731–734.

[16] Su M, Xia DL, Teng P, et al. *J. Med. Chem.* **2017**, *60*, 8456–8465.

[17] Sharma R, Patel S, Abboud C, et al. *Int. J. Antimicrob. Ag.* **2017**, *49*, 224–232.

[18] Miao J, Zhou J, Liu G, et al. *Food Control* **2016**, *59*, 609–613.

[19] Pham TA, Jain AN. *J. Med. Chem.* **2006**, *49*, 5856–5868.

[20] Wright HT, Reynolds KA. *Curr. Opin. Microbiol.* **2007**, *10*, 447–453.

[21] Cronan Jr. JE, Waldrop GL. *Prog. Lipid. Res.* **2002**, *41*, 407–435.

[22] Miller Richard J, Dunham S, Mochalkin L, et al. *P. Natl. Acad. Sci. USA* **2009**, *106*, 1737–1742.

[23] Cheng CC, ShippsJr. GW, Yang ZW, et al. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6507-6514.

[24] Li P, Shi L, Yang X, et al. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 1677–1680.

[25] Li DD, Zhang SS, Zong ZH, et al. *Eur. J. Med. Chem.* **2017**, *136*, 114–121.

Table Caption

Table 1. Antibacterial activity (MIC in μg/mL) of canthin-6-one analogues.

Figure Captions

Figure 1. Synthetic tactics of C^{10} modified and ring-truncated canthin-6-one analogues.

Figure 2. Structure–activity relationships of canthin-6-one analogues against bacteria.

Figure 3. Fluorescence micrographs of *S. aureus* treated or not treated with 1 μg/mL **22** for 1 h. (A1) no treatment, Hoechst stained; (A2) no treatment, PI stained; (A3) no treatment, merge graph; (B1) *S. aureus* treatment with **22**, Hoechst stained; (B2) *S. aureus* treatment with **22**, PI stained; (B3) *S. aureus* treatment with **22**, merge graph.

Figure 4. SEM of *S. aureus* cells: (A) blank group; (B) treated group.

Figure 5. Three-dimensional conformations of compound **22** docked in biotin carboxylase.

Highlights

- The peak MIC of the synthesized canthin-6-one analogues is 2 μg/mL.
- The structure–activity relationships are summarized.
- The highly bioactive compound **22** has good drug feasibility.
- The compound **22** might damage bacterial cell membranes and influence the membrane formation.