Chinese Journal of Chemistry
中国化学 - An International Journal

Accepted Article

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This manuscript has been accepted and appears as an Accepted Article online.

This work may now be cited as: *Chin. J. Chem.* **2020**, *38,* 10.1002/cjoc.202000133.

The final Version of Record (VoR) of it with formal page numbers will soon be published online in Early View: http://dx.doi.org/10.1002/cjoc.202000133.

WILEY-VCH SIOC CCS

ISSN 1001-604X • CN 31-1547/O6
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Design, Synthesis and Biological Evaluation of Bengamide Analogues as ClpP activators

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Summary of main observation and conclusion To combat multidrug-resistant Gram-positive bacteria, new antimicrobials particularly those with novel mechanism of action are badly needed. Different with conventional antibiotics those are typical inhibitors, small-molecule activators of bacterial ClpP represent a new class of antibiotics. No ClpP activators have been developed for clinical trial. Herein, we conducted a screening on our library of bengamide-like ring-opened analogues and found that **L472-2** possesses a low minimum Inhibitory Concentration (MIC) against *S.aureus* and shows no activity for ClpP activation in the *in vitro* study but displayed reduced antibacterial activity against *S. aureus* with *clpP* deletion. In order to obtain bengamide analogues that activate ClpP *in vitro* as well as possess antibacterial activity, we perform further structural modifications starting from **L472-2**. Compound **37** remains the antimicrobial activity and activation of ClpP protein *in vitro*, which could be viewed as a new chemical scaffold for ClpP activators and worthy of further investigation.

Background and Originality Content

The large-scale use of antibiotics and even abuse in clinical and non-clinical settings have led to the rapid emergence of antibiotic resistance[1]. Multidrug-resistant strains of Gram-positive bacteria, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), remain a threat to public health in many parts of the world[2]. In addition, many determinant factors have contributed to the decline of new antibiotics[3]. Thus, we are facing severe antimicrobial

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/cjoc.202000133

resistance and approaching the post-antibiotic era. Therefore, the research of new antimicrobial agents, particularly those with new scaffolds and novel mechanisms is urgent to tackle the antibiotic crisis.

Bacterial Clp protease is an energy-dependent serine protease which plays a pivotal role in the bacterial pathogenesis[4]. The ClpP protease itself is able to degrade short peptides, while it requires the assistance of the chaperone proteins such as ClpX for the proteolysis of polypeptides and proteins. The *S. aureus* ClpP (*Sa*ClpP) has been established to regulate bacterial virulence and pathogenesis, which represents a novel target for antibacterial drug discovery[5].

We are interested in the structural and mechanistic studies on *Sa*ClpP[6], as well as chemical intervention on *Sa*ClpP activity[7]. Both inhibitors and activators for ClpP were developed in the past decade[8], and particularly activators were extensively studies. The first activator was ADEPs (**Figure 1A**), which contended with the ClpX ATPase in complex with ClpP, thus bypassing ClpX's regulation. ACPs displayed a similar mechanism of action to ADEPs, which were identified in high-throughput screening and named after activators of self-compartmentalizing proteases (**Figure 1B)**. In a fluorescence-based assay, a non-peptide natural product called sclerotiamide could activate *E. coli* ClpP, which possesses a distinct three-dimensional bicyclo-[2.2.2]-diazoctane motif (**Figure 1C**).

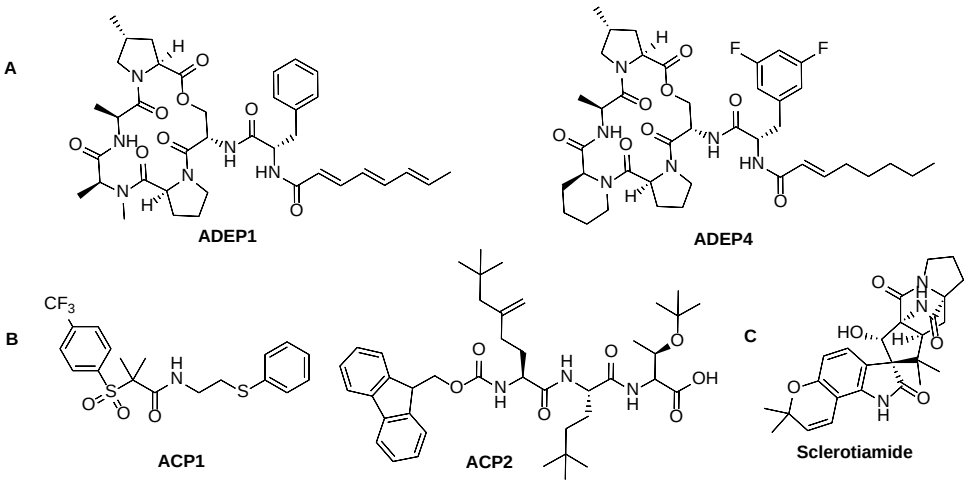


Figure 1. Chemical structures of ClpP activators.

The natural products bengamides and their derivatives exhibited remarkable biological properties, including antitumor, anthelmintic and antibacterial activities[9]. Previously, we carried out systematic structural optimization and simplification of bengamides with regards to the antitumor activities[10], thus establishing a bengamide-like ring-opened focused library. In this study, we have screened our library and performed structural optimization with the aim to obtain antibacterial bengamide-like ring-opened analogues as new activators for *Sa*ClpP.

Results and Discussion

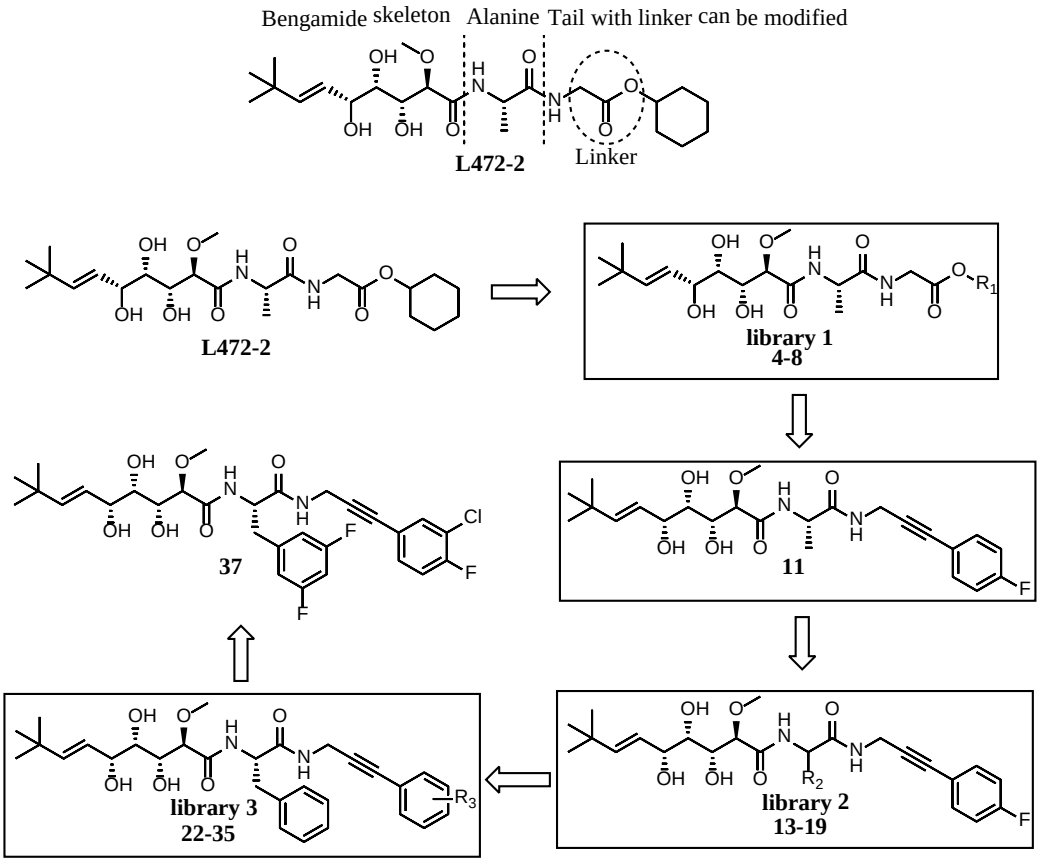


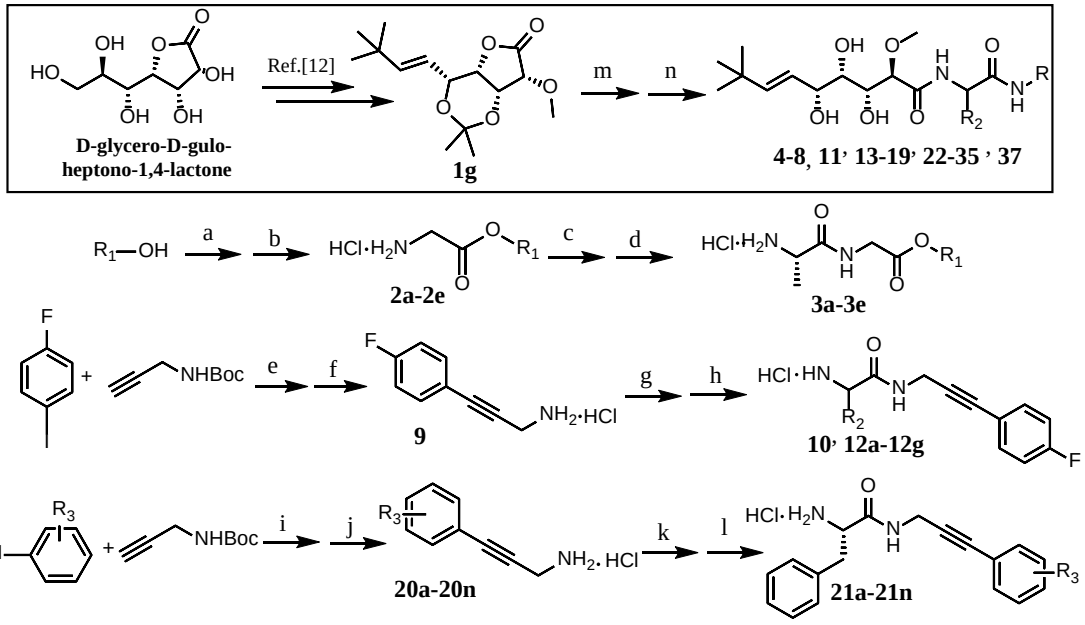
Figure 2. Design of bengamide derivatives as ClpP activators.

In the initial screening of our bengamide-like derivatives, **L472-2** displayed antibacterial activity towards *S. aureus* (**Figure 2**). To evaluate whether the antibacterial activity of **L472-2** is associated with *Sa*ClpP activation, we determined the MIC values of **L472-2** against different strains, including the wild type *S. aureus* (8325-4), the *S. aureus* clpP mutant strain (Δ*clpP*), the *clpP* complementary strain (Δ*clpP*::*clpP*), and the 8325-4 strain overexpressing *clpP* (OE*clpP*)[11]. As shown in **Table 1**, compound **L472-2** displayed a lower MIC of 0.78-1.56 μg/ml and 1.56-3.13 μg/ml for against 8325-4 and Δ*clpP*::*clpP* respectively. Interestingly, this compound showed weak activity against the strain Δ*clpP* with a MIC of 25.0-50.0 μg/ml, while it still showed potent activity against OE*clpP* strain with a MIC of 0.78-1.56 μg/ml. These results indicate that **L472-2** is active against the growth of *S. aureus*, which is related to ClpP.

To confirm whether **L472-2** directly activates ClpP, we performed a α-casein degradation assay. **L472-2** failed to activate ClpP in α-casein degradation assay *in vitro*. **L472-2** showed no activity for ClpP activation in the *in vitro* study but displayed reduced antimicrobial activity against *S. aureus* with *clpP* deletion, just like the positive control ADEP4. There could be some underlying mechanisms affecting the ClpP pathway in an indirect manner. The possibility of alternative target and/or off-target effect needs to be addressed in future study.

Next, we performed further structural modifications beginning with **L472-2**(**Figure 2**). By remaining the skeleton of bengamide-like ring-opened and modifying the R_1, R_2 and R_3, a series of compounds were designed. The types of rings and the linker on **L472-2** was firstly screened in the **library 1**. After the introduction of substituted benzene into **L472-2**, the compound **8** was acquired. Then the ester group replaced with alkynyl group for avoiding potential metabolic problems yielded the compound **11**. Replacing the methyl in the compound **11** with different types of substituents with various amino acids, provided **library 2** for further optimization. The emergence of *Sa*ClpP activation *in vitro* had attracted our great interest among the compound of **library 2**. Then, **library 3** was gained by systematic structural modification of the substituted benzene ring. Given the activation of ClpP protein and the antibacterial activity, the alignment of compound **19** and **30** produced **37** with further improved activity.

In general, the synthesis of the bengamide skeleton was started from a commercially available lactone that was converted to the key intermediate **1g** (**Scheme 1**) [12], which reacted with various amines (**3a**-**3e**, **10**, **12a**-**12g**, **21a**-**21n**) respectively by a coupling reaction and then followed by a acid-catalyzed acetonide deprotection[13]. Throughout various reaction routes, the reaction of amines with lactone **1g** was a versatile process for the preparation of target compounds.



Scheme 1. Synthesis of bengamide derivatives **4-8**, **11**, **13-19**, **22-35 and 37**.

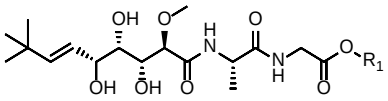
(a) L-glycine, EDCI, DMAP, DCM; (b) HCl/EA; (c) L-alanine, EDCI, DMAP, DCM; (d) HCl/EA; (e) $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, rt; (f) HCl/EA; (g) various amino acids, EDCI, DMAP, DIPEA, DCM; (h) HCl/EA; (i) $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, rt; (j) HCl/EA; (k) L-phenylalanine, EDCI, DMAP, DIPEA, DCM; (l) HCl/EA; (m) **3a-3e**, **10**, **12a-12g**, **21a-21n**, **36**, sodium 2-ethylhexanoate, THF, rt; (n) 1 N HCl, THF.

The alcohols were condensed with L-glycine to give esters **2a-2e** in high yields, which was further condensed with L-alanine providing diverse amines (**3a-3e)**. Intermediate **9** was prepared from *p*-iodofluorobenzene using copper-palladium catalyzed Sonogashira coupling[14], thenceforth reacting with varieties of amino acids commercially available to yield varieties of amines (**10**, **12a-12g**). In order to get intermediates **20a-20n**, the coupling of the derivatives of iodobenzene with N-Boc-propargylamine was applied, which then reacted with L-phenylalanine to afford a range of amines (**21a-21n**). Then the bengamide analogues **4-8**, **11**, **13-19**, **22-35** were steadily synthesized from **1g** and diverse amines (**3a-3e**, **10**, **12a-12g**, **21a-21n**) correspondingly. Inspired by earlier screening for R_2 and R_3, the suitable moieties of

19 and **30** were kept to form bengamide derivative **37**.

These bengamide derivatives **4-8**, **11**, **13-19**, **22-35** and **37** were evaluated for their antibacterial activity and activation of ClpP through *in vitro* assays. The ClpP activator ADEP4 was assayed as the positive control and vancomycin as the negative control. Compounds **4-8** remained the antibacterial activity through modification of the cyclohexane in **L472-2** (**Table 1**). Unfortunately, these derivatives are still unable to promote *Sa*ClpP to degrade α-casein substrate *in vitro*. These data suggest that R_1 are not crucial for the antibacterial effect. Carney reported that N-acyldifluorophenylalanine fragments of ADEPs function via the identical mechanism of action, as evidenced by its requisite and sufficiency for the performance of ClpP activation and antibacterial activity [15]. Inspired by the benzene ring with halogen substitutions in N-acyldifluorophenylalanine fragments, we try to further exploration from compound **8**.

Table 1. MICs of **library 1** against *S. aurues* strains



R_1	ID	MIC(μg/ml)			
		8325-4	Δ*clpP*	Δ*clpP*::*clpP*	OE*clpP*
	**L472-2**	**0.78-1.56**	**25.0-50.0**	**1.56-3.13**	**0.78-1.56**
	**4**	1.56-3.13	>50.0	3.13-6.25	1.56-3.13
	**5**	0.78-1.56	25.0-50.0	0.39-0.78	0.78-1.56
	**6**	0.39-0.78	6.25-12.5	<0.39	0.39-0.78
	**7**	0.39-0.78	12.5-25.0	3.13-6.25	<0.39
	**8**	0.78-1.56	25.0-50.0	0.39-0.78	<0.39
ADEP4		0.39-0.78	>50.0	0.39-0.78	0.39-0.78
Vancomycin		0.78-1.56	0.78-1.56	0.78-1.56	0.78-1.56

By considering the instability of esters and the sensitivity of the linker in metabolism, we replaced the flexible ester in compound **8** with linear alkynyl motif, which gave compound **11** for further evaluation of the biological activity (**Figure 3**). It

is good to find that compound **11** largely kept the antibacterial activity. So we kept the alkyne group and substituted benzene in compound **11** for further improvement.

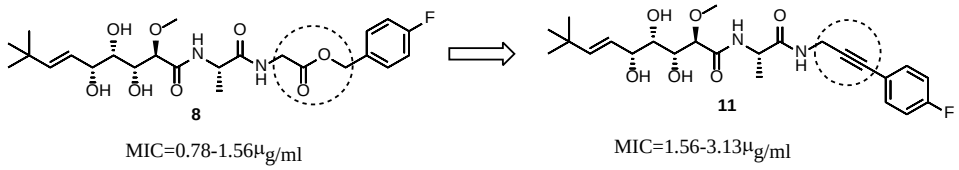
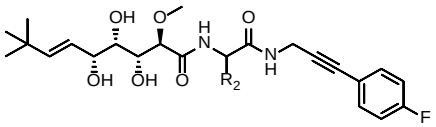


Figure 3. Modification of the linker based on **8**.

Next, we investigated the effect of R_2 substituents on the antibacterial activity (**Table 2**). The comparison of antibacterial activity between compound **13** and **14** clearly revealed that the configuration of L-amino acid is necessary for remaining antibacterial activity. To our delight, compound **13** with moderate antibacterial activity was able to activate ClpP to degrade α-casein with an EC_{50} of 50 μM (**Table 3**). Compounds **15**, **16**, **17** displayed comparable antibacterial activities with compound **11** with low MICs and no appearance of *Sa*ClpP activation in degrading α-casein substrate *in vitro*. Compound **18** with a MIC of 12.5-25.0μg/ml showed very weak activity for *Sa*ClpP activation *in vitro* consistently. Compound **19** with 3, 5-F substituted benzyl exhibited comparable antibacterial activity with compound **13**. Interestingly, compound **19** displayed much stronger activity than **13** for *Sa*ClpP activation *in vitro* (**Table 3**).

Table 2. MICs of **library 2** against *S. aureus* strains



R_2	ID	MIC (μg/ml)			
		8325-4	Δ*clpP*	Δ*clpP*::*clpP*	OE*clpP*
	**11**	**1.56-3.13**	**1.56-3.13**	**1.56-3.13**	**0.78-1.56**
	**13**	6.25-12.5	25-50	3.13-6.25	6.25-12.5

	**14**	>50	>50	>50	25-50
	**15**	0.78-1.56	6.25-12.5	0.78-1.56	1.56-3.13
	**16**	3.13-6.25	6.25-12.5	3.13-6.25	3.13-6.25
	**17**	0.78-1.56	3.13-6.25	0.78-1.56	0.78-1.56
	**18**	12.5-25.0	25.0-50.0	12.5-25.0	6.25-12.5
	**19**	6.25-12.5	12.5-25.0	6.25-12.5	6.25-12.5
ADEP4		0.39-0.78	>50.0	0.39-0.78	0.39-0.78
Vancomycin		0.78-1.56	0.78-1.56	0.78-1.56	0.78-1.56

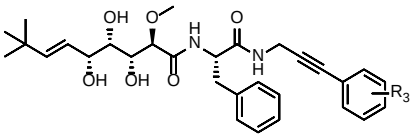
Table 3. EC_{50} values of derivatives activating *Sa*ClpP

Compound	EC_{50} (μM)
ADEP4	3.13-6.25
L472-2	>100
23	>100
27	50-100
13	50-100
24	50-100
30	25-50
29	12.5-25
19	6.25-12.5
37	3.13-6.25

The derivatives in **Table 4** were prepared by changing the *para*-fluorine group in compound **13** to varying substituents at different sites on the phenyl ring. Compound **28** with the *ortho*-fluorine substitution and compound **32** bearing *ortho*-isopropyl

substitution completely lost the antibacterial activity. The MICs of the compounds (**13**, **23-27**, **29-30**) with halogen substitutions at other sites rather than the *ortho*-position were moderate. The properties of electrons in R_3 group minimally affected the antibacterial activity in compounds **31-35**. Only those compounds with halogen substitution display agonistic activity on ClpP enzyme. The halogen substituents in R_3 had different effects on activation of *Sa*ClpP degrading protein substrate. The activity for *Sa*ClpP activation also depends on both the halogen atoms and the substituted positions. Compound **29** and **30** bearing both fluorine and chlorine groups displayed moderate activities for *Sa*ClpP activation *in vitro* (**Table 3**).

Table 4. MICs of **library 3** against *S. aureus* strains



R_3	ID	MIC (μg/ml)			
		8325-4	Δ*clpP*	Δ*clpP*::*clpP*	OE*clpP*
p-F	**13**	12.5-25.0	25.0-50.0	12.5-25.0	6.25-12.5
-	**22**	12.5-25.0	25.0-50.0	12.5-25.0	12.5-25.0
m-Br	**23**	12.5-25.0	12.5-25.0	6.25-12.5	25.0-50.0
m-Cl	**24**	6.25-12.5	12.5-25.0	6.25-12.5	6.25-12.5
p-Br	**25**	6.25-12.5	12.5-25.0	3.13-6.25	6.25-12.5
p-Cl	**26**	6.25-12.5	12.5-25.0	6.25-12.5	6.25-12.5
m-F	**27**	25.0-50.0	25.0-50.0	12.5-25.0	12.5-25.0
o-F	**28**	>50.0	>50.0	>50.0	25.0-50.0
m-F, *p*-F	**29**	12.5-25.0	25.0-50.0	6.25-12.5	6.25-12.5
m-Cl, *p*-F	**30**	6.25-12.5	25.0-50.0	6.25-12.5	6.25-12.5
o-OCF_3	**31**	25.0-50.0	25.0-50.0	12.5-25.0	12.5-25.0
o-$CH(CH_3)_2$	**32**	>50.0	>50.0	>50.0	>50.0
p-CN	**33**	12.5-25.0	25.0-50.0	12.5-25.0	12.5-25.0
o-CH_3	**34**	25.0-50.0	25.0-50.0	25.0-50.0	25.0-50.0
m-NO_2, *p*-F	**35**	12.5-25.0	25.0-50.0	12.5-25.0	6.25-12.5
ADEP4		0.39-0.78	>50.0	0.39-0.78	0.39-0.78
Vancomycin		0.78-1.56	0.78-1.56	0.78-1.56	0.78-1.56

Finally, compound **37** was prepared on the basis of the balanced enzyme activation(**Table 3**) and antibacterial activity(**Table 5**) as observed in compounds **19** and **30**. To our delight, **37** exhibited significantly improved activation on the degradation of protein substrate by *Sa*ClpP with the *in vitro* EC_{50} of 3.13-6.25 μM (**Table 3**), while this compound is still active against *S. aureus* in a ClpP-dependent way (**Table 3**). Therefore, the bengamide derivatives could function as a new class of antibiotics by actively targeting the *S. aureus* ClpP protease.

Table 5. MICs of the compound **37** against *S. aureus* strains

ID	MIC (μg/ml)			
	8325-4	Δ*clpP*	Δ*clpP*::*clpP*	OE*clpP*
37	6.25-12.5	>50	6.25-12.5	6.25-12.5
ADEP4	0.39-0.78	>50.0	0.39-0.78	0.39-0.78
Vancomycin	0.78-1.56	0.78-1.56	0.78-1.56	0.78-1.56

Conclusion

Confronted with antimicrobial resistance, the strategy for the study of new antibiotics with novel mechanisms of action is imperative. ClpP has drawn our attention for its superiority as a new target for the development of novel antibiotics. After screening on the bengamide-like focused compound library, we found bengamide analogues **L472-2** could be viewed as a hit for further optimizations. Three different sets of new derivatives of **L472-2** were synthesized. The introduction of the alkynyl group into compounds **8** yielded compound **11**, which might be much metabolite-tolerant and remains antibacterial activity. Further optimization of the L-alanine with L-phenylalanine provided compound **13**, leading to the occurrence of *Sa*ClpP activation *in vitro*. Furthermore, compound **19** is more active than **13** for *Sa*ClpP activation. In view of the activation of ClpP protein and the antibacterial activity of compound **19** and **30**, the arrangement of them generated **37** with further improved activity. Compound **37** possesses activation of *Sa*ClpP with EC_{50} of 3.13-6.25 μM and

antibacterial activity with a MIC of 6.25-12.5 μg/ml. These data suggest that the bengamide-like ring-opened compounds, especially **37**, is a potent activator of *Sa*ClpP. Collectively, we have identified that unreported bengamide scaffolds as ClpP activators for further investigation.

Experimental section

Experimental procedures are available in the Supporting Information.

Acknowledgements

We would like to thank Hanne Ingmer for providing the strains of 8325-4, Δ*clpP* and Δ*clpP*::*clpP*. This work was supported by Science and Technology Commission Shanghai Municipality (16ZR1407000 and 17XD1404400), and the National Natural Science Foundation of China (81861138046).

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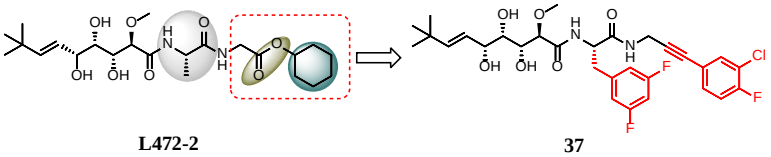

L472-2 **37**

Xue-Qing Kong,Bing-Yan Wei, Chen-Xi Yu, Xiang-Na Guan, Wei-Ping Ma, Gang Liu, Cai-Guang Yang*and Fa-Jun Nan*

8325-4 MIC(μg/ml):0.78-1.56
Δ clpP MIC(μg/ml):25.0-50.0
Δ clpP::clpP MIC(μg/ml):1.56-3.13
OE*clpP* MIC(μg/ml):0.78-1.56
in vitro no activation of SaClpP

8325-4 MIC(μg/ml): 6.25-12.5
ΔclpP MIC(μg/ml)>50.00
ΔclpP::clpP MIC(μg/ml): 6.25-12.5
OEclpP MIC(μg/ml): 6.25-12.5
in vitro activation of SaClpP EC_{50}(μM):3.13-6.25