ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue



Cite this: *Chem. Commun.*, 2019, 55, 4198

Received 4th February 2019,
Accepted 5th March 2019

DOI: 10.1039/c9cc01027k

rsc.li/chemcomm



# Autophagy inducing cyclic peptides constructed by methionine alkylation†

Xuan Qin,‡ Xiaodong Shi,‡ Licheng Tu, Yue Ma, Ziyuan Zhou, Rongtong Zhao, Meimiao Zhan, Feng Yin * and Zigang Li *

**Peptides that induced autophagy at micromolar concentrations with improved proteolytic resistance properties were generated using the facile methionine bis-alkylation method. Notably, a short bicyclic peptide 7f was proven to be the most potent one among the designed peptides in regards to autophagy inducing activity. This study facilitated the development of a peptide-based autophagy inducer and demonstrated the potential applications of the methionine alkylation-based macrocyclization method for the diversity-oriented generation of peptide-based autophagy inducers.**

Autophagy is a lysosomal-dependent degradation pathway that is highly conserved in eukaryotic cells.[1,2] As an important mechanism of cellular quality control, autophagy plays an important role in maintaining the homeostasis of an organism's tissues and in resisting the invasion of exotic pathogens or hunger stress.[3] Thus, the dysregulation of autophagy is closely associated with neurodegeneration disorders, cancers and other pathological conditions.[4,5] Various chemical molecules have been found to effectively modulate the autophagy process either as inducers or inhibitors.[6–8] Although these molecules have been widely used for exploring the autophagy process, the complicated autophagy mechanism involved multiple interaction role of the autophagy-related (ATG) proteins may give rise to the unspecific results for autophagy modulation.[9] Thus, the search for more selective autophagy modulators is highly required.

Protein and protein interaction modulation has been regarded as an emerging way to develop selective modulators.[10] Particularly, peptide-based modulators have been widely investigated for autophagy modulation.[11] In the phase of phagophore nucleation, Beclin 1 acts as a scaffolding protein for Beclin 1-Vps34 complex formation.[12–15] Based on the C terminal of the Beclin 1 coiled-coil domain, Zhao *et al.* designed stapled peptides that could promote Beclin 1-Atg14L/UVRAG interactions and induce autophagic flux.[16] Levine *et al.* identified a potent peptide candidate named TAT-Beclin 1, which consisted of a peptide derived from Beclin 1 (269–278) and the polycationic TAT sequence. TAT-Beclin 1 could induce autophagy activity both *in vitro* and *in vivo* through binding with the negative regulator GAPR-1.[17] However, this linear peptide has some intrinsic disadvantages such as serum instability and low cell permeability without the polycationic Tat sequence, which limit its further applications. To overcome this problem, a cyclization strategy based on thiol bis-alkylation has been considered as an effective method to increase the cellular uptake and stability of peptides.[18]

Currently, various chemical strategies have been employed for peptide stapling and further peptide inhibitor designs,[19] including side chain crosslinking,[20] N-capped nucleation,[21] and hydrogen bond surrogates.[22] Recently, we developed a novel and facile peptide macrocyclization strategy based on methionine bis-alkylation.[23] This method allowed us to generate a series of constrained peptides with different stapling positions and sequence lengths as well as polycyclic peptides. The addition of these linkers to the peptides resulted in increased proteolysis resistance and negligible cytotoxicity. Notably, the original peptide could be released after a relatively long treatment time with GSH. An effective HBV antigen secretion peptide inhibitor was developed using this strategy with improved cell permeability and binding affinity compared with those of the linear peptide.[24] Herein, we employed this strategy to the design of Beclin 1-derived constrained peptides for generating potential autophagy peptide inducers (Fig. 1).

Based on the sequence reported by Levine and Kritze,[17,18] we prepared a series of short cyclic/bicyclic peptides with a good yield (Fig. 2). First, we utilized an alkylating linker **a** (1,2-dibromomethyl-benzene) to react with methionine-containing peptides for generating the corresponding bis-alkylated cyclic peptides **1a–4a** (Fig. 2B). The N-terminal cap of the peptide was important for the autophagy inducing activity, as reported by Kritzer *et al.*[18] Among the caps screened, 4-pentynoic acid

*State Key laboratory of Chemical Oncogenomics, School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, 518055, P. R. China. E-mail: yinfeng@pkusz.edu.cn, lizg@pkusz.edu.cn*

† Electronic supplementary information (ESI) available: Experimental procedures, supporting tables and figures. See DOI: 10.1039/c9cc01027k

‡ These authors contributed equally to this work.

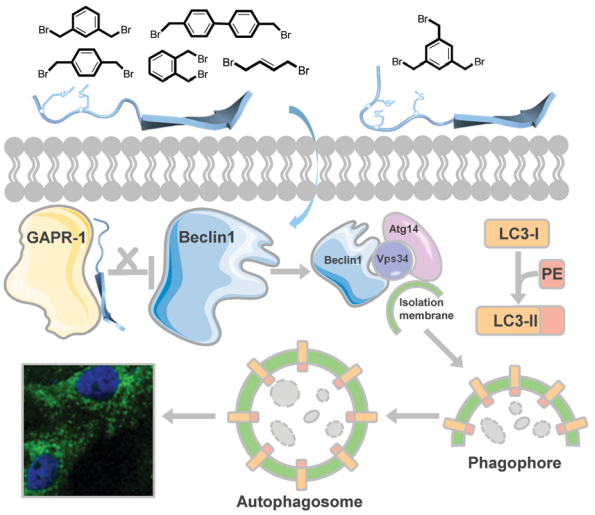
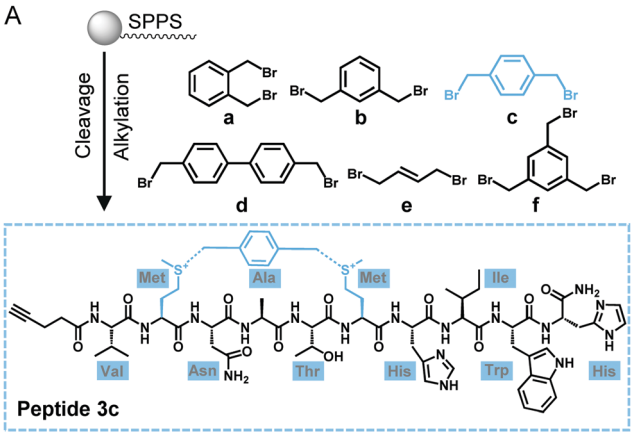



Fig. 1 Schematic presentation of the peptide autophagy inducer. Based on the Beclin 1 (269–278) sequence, the peptide autophagy inducers are synthesized by the methionine bis-alkylation method using different linkers. GAPR-1 is a negative regulator of autophagy and binds with Beclin 1 to inhibit autophagy. When the peptide inducers penetrate the cell membrane and occupy the interaction domain between Beclin 1 and GAPR-1, Beclin 1 is released from GAPR-1 and promotes autophagosome formation.

showed the best autophagy inducing activity.[18] Accordingly, all peptides were N-terminal capped with 4-pentynoic acid. A Western blot analysis of the endogenous LC3B-I conversion to LC3B-II in HeLa cells was used to monitor the autophagy inducing activity.[25]

As shown in Fig. 3A, peptides **1a** and **2a** with two methionine substitutions at *i*, *i* + 3 or *i*, *i* + 4 spacing exhibit negligible increase in the LC3-I conversion to LC3-II under the adopted conditions (50 μM, 12 h). Peptide **4a**, by changing peptide **1a** with two D-methionines, showed mild enhancement. Peptide **3a** significantly improved the conversion levels for LC3-II with a different substituted position. Thus, peptide **3a** was used as a starting point for further optimization. Then, the autophagy inducing activities were compared between the constrained peptides modified with different linkers (**a**–**e**). As shown in Fig. 3B, peptides **3a**, **3b** and **3c** show more potent accumulation in LC3-II than peptide **3d**, **3e** and a scramble control peptide **5c**. Peptide **3c** shows the most improved activity among the monocyclic peptides. The overall comparison of these screening peptides is shown in Fig. S2 (ESI†).

Several studies have revealed that bicyclic peptides exhibit significantly improved proteolysis stability, binding affinity, selectivity and consequently excellent cellular activity.[26–29] This led us to generate the methionine-containing bicyclic peptides for autophagy inducement. Two bicyclic peptides **6f** and **7f** (Fig. 2B) were constructed by alkylating peptides **6** and **7** with the reagent **f** (1,3,5-tris-bromomethyl-benzene). A significant improvement in the autophagy inducing activity was observed when the HeLa cells were treated with **7f** under the same conditions (50 μM, 12 h). Peptide **6f** showed reduced activity compared with **7f**, which was likely due to the absence of the two conserved FF residues (Fig. 3C).[17]




B

| Peptide | Linear Sequence | Linker | Peptide |
|---|---|---|---|
| 1 | pa-VMNAMFHIWH | a | 1a |
| 2 | pa-VWMATFMIWH | a | 2a |
| 3 | pa-VMNATMHIWH | a | 3a |
| | | b | 3b |
| | | c | 3c |
| | | d | 3d |
| | | e | 3e |
| 4 | pa-VmNAmFHIWH | a | 4a |
| 5 | pa-VMHAHMTWIN | c | 5c |
| 6 | pa-VMNATMHIWHMG | f | 6f |
| 7 | pa-VFMATFMIWHMG | f | 7f |

Fig. 2 Schematic presentation of the prepared peptides. (A) The representative synthesis procedures and chemical structure for peptide **3c**. (B) Cyclic peptides synthesized in this paper [Pa: peptide N terminus capped with pentynoic acid; M: L-methionine; m: D-methionine].

To further confirm the efficiency of the autophagy inducement, we chose the most potent peptides **3c** and **7f** to treat the HeLa cells at different concentrations/time intervals. We also examined the expression levels of LC3-II and p62. As shown in Fig. 4A, time-dependent increase in the LC3-I conversion to LC3-II is observed when HeLa cells are treated with peptide **3c**. An obvious increase in the amount of LC3-II was observed at 6 h and it continued to 36 h. When the HeLa cells were treated with peptide **7f**, on prolonging time, the amount of LC3-II reached the maximum level at 12 h and then degraded gradually (Fig. 4D). As shown in Fig. 4B and E, the autophagy activity of the HeLa cells can be induced to the maximum amount by treatment with 50 μM peptides.

Autophagy was also measured with the LC3 turnover assay and mCherry-GFP-LC3 color change assay to confirm that **3c** and **7f** indeed induced autophagic flux rather than the blocking of autophagosome maturation or lysosomal function. HeLa cells were pretreated with the lysosomal fusion inhibitor hydroxychloroquine (HCQ). Autophagy induction would further increase after HCQ, thus preventing the fusion of autophagosomes with lysosomes by inhibiting vacuolar $H^+$ ATPase. As shown in Fig. 4C

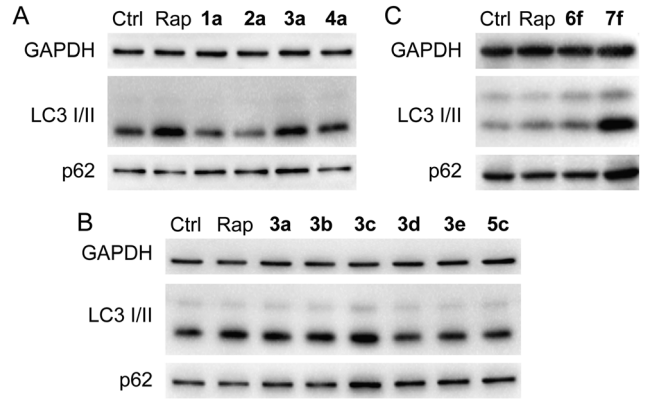



Fig. 3 Cyclic peptides induce autophagy. (A) Peptides **1a–4a** constrained with the same linker **a** with different cyclization sizes or positions. **3a** showed significantly increased amounts of LC3-II. (B) Peptides **3a–3e** cyclized with different linkers **a–e** and a scramble peptide **5c**. Peptide **3c** showed improved autophagy inducing activity among the cyclic peptides with different linkers. (C) Autophagy inducing activity of two bicyclic peptides **6f** and **7f**. **7f** showed the most augmented expression levels of LC3-II. The bar graph is shown in Fig. S1 (ESI†).

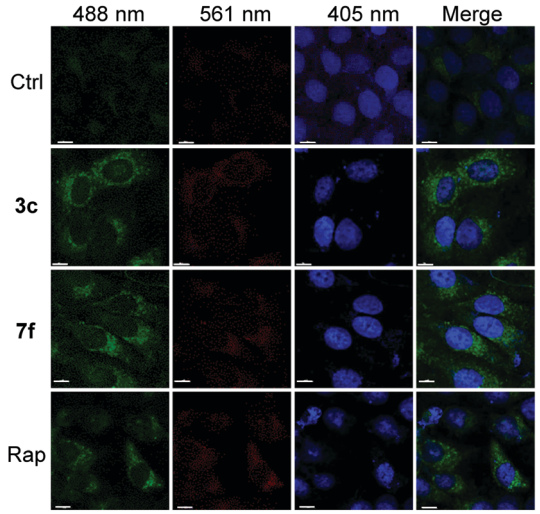



Fig. 5 Peptides **3c** and **7f** truly induce autophagic flux at an early stage of autophagy. HeLa cells transfected with mCherry-GFP-LC3B and cultured in DMEM with 10% FBS for 24 h, followed by treatment with 50 μM peptides **3c**, **7f** or 5 μM rapamycin for 12 h. The cells were imaged using a laser scanning confocal microscope with a 60× oil objective. Scale bar: 10 μm.

and F, the two peptides further generate an increased amount of LC3-II after HCQ treatment, indicating that the autophagic flux increased. Autophagic flux was also measured by detecting the autophagosome formation. HeLa cells were transfected with mCherry-GFP-LC3B and cultured in DMEM with 10% FBS for 36 h, followed by treatment with 50 μM peptide **3c** or **7f** or 5 μM rapamycin for 12 h. In the early stage of autophagy, LC3B localized in autophagosomes and emitted both red (mCherry) and green (GFP) fluorescences. In the later stage of autophagy, when the autophagosome fused with the lysosome, the low pH inside the lysosome could quench the green fluorescence signal rapidly.[30] As shown in Fig. 5, treatment with peptides **3c** and **7f** greatly enhances the green and red signals in HeLa cells compared to the observations for the control group, implying that a large number of autophagosomes are generated and the

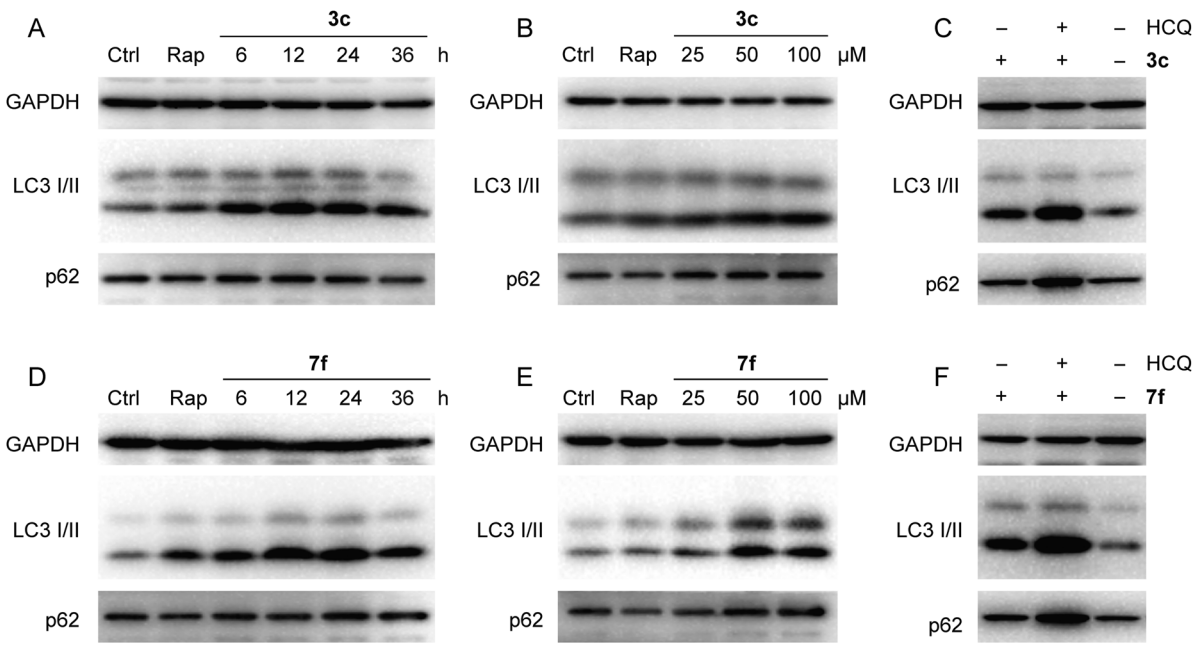



Fig. 4 Peptides **3c** and **7f** increase autophagic flux in HeLa cells. (A and D) HeLa cells were treated with peptides **3c** and **7f** at 50 μM concentrations for 6–36 h. Treatment with 5 μM rapamycin for 12 h is shown as a positive control. (B and E) HeLa cells were treated with different concentrations (25, 50 and 100 μM) of peptide **3c** and **7f** for 12 h. Treatment with 5 μM rapamycin for 12 h is shown as a positive control. (C and F) HeLa cells were pre-treated with 5 μM hydroxychloroquine (HCQ) for 1 h and then incubated with peptides **3c** and **7f** (50 μM) for 12 h. The bar graph is shown in Fig. S3 (ESI†).

two peptides truly induce autophagic flux at the early stage of autophagy.

Besides, the cell penetration of the peptides was also evaluated by the fluorescence activated cell sorting (FACS) assay by using the cell penetrating peptide **FITC-TAT** as a positive control. Peptides **3c-FAM** and **7f-FAM** showed significantly better cell permeabilities than **FITC-TAT** and slightly better cell permeabilities than their linear peptides **3-FAM** and **7-FAM** (Fig. S4, ESI†). In addition, peptides **3c** and **7f** showed significantly enhanced trypsin and cell lysate stabilities compared with the linear peptide **3** (Fig. S5 and S6, ESI†). These properties may be beneficial for the autophagy inducing activity of the peptides.

In summary, using the methionine alkylation method, we generated two cyclic peptides that could induce an autophagic flux in HeLa cells at micromolar concentrations with improved proteolysis resistance. Beclin 1-derived peptide **3** alkylated by linker **c** showed favorable autophagy inducing activity among the mono-cyclic peptides, thus demonstrating the cyclization impact on the peptide activity and the potential of the linker tunable property of the strategy. The bicyclic peptide **7f** showed more potential in LC3-II accumulation and autophagosome formation, as indicated by the autophagic flux detection assay, which may be attributed to the improved cell permeability and stability. This study demonstrated that the Beclin 1-based peptide candidate is a practical template to generate autophagy activators and for the potential applications of the methionine alkylation methods for the diversity-oriented generation of autophagy inducing peptides.

We gratefully acknowledge that this work was supported by the National Natural Science Foundation of China (Grant 21778009, 81701818 and 81572198), the Shenzhen Science and Technology Innovation Committee (JCYJ20170412150609690, KQJSCX20170728101942700 and JCYJ20170807144449135) and the high-performance computing platform of Peking University.

## Conflicts of interest

There are no conflicts of interest to declare.

## Notes and references

1 L. Galluzzi, J. M. Bravo-San Pedro, B. Levine, D. R. Green and G. Kroemer, *Nat. Rev. Drug Discovery*, 2017, **16**, 487.
2 D. C. Rubinsztein, P. Codogno and B. Levine, *Nat. Rev. Drug Discovery*, 2012, **11**, 709.
3 Y. Cheng, X. Ren, W. N. Hait and J. M. Yang, *Pharmacol. Rev.*, 2013, **65**, 1162.
4 J. M. M. Levy, C. G. Towers and A. Thorburn, *Nat. Rev. Cancer*, 2017, **17**, 528.
5 J. Nah, J. Yuan and Y. K. Jung, *Mol. Cells*, 2015, **38**, 381.
6 J. Ha and J. Kim, *Expert Opin. Ther. Pat.*, 2016, **26**, 1273.
7 T. Kimura, Y. Takabatake, A. Takahashi and Y. Isaka, *Cancer Res.*, 2013, **73**, 3.
8 Y. X. Lin, Y. Wang and H. Wang, *Small*, 2017, **13**, 1700996.
9 L. Gao, C. E. Jauregui and Y. Teng, *Future Med. Chem.*, 2017, **9**, 335.
10 G. Muciño, S. Castro-Obregón, R. Hernandez-Pando and G. Del Rio, *IUBMB Life*, 2016, **68**, 259.
11 E. Catalani, C. De Palma, C. Perrotta and D. Cervia, *BioMed Res. Int.*, 2017, 5856071.
12 W. Huang, W. Choi, W. Hu, N. Mi, Q. Guo, M. Ma, M. Liu, Y. Tian, P. Lu, F. L. Wang, H. Deng, L. Liu, N. Gao, L. Yu and Y. Shi, *Cell Res.*, 2012, **22**, 473.
13 X. Li, L. He, K. H. Che, S. F. Funderburk, L. Pan, N. Pan, M. Zhang, Z. Yue and Y. Zhao, *Nat. Commun.*, 2012, **3**, 662.
14 M. J. Ranaghan, M. A. Durney, M. F. Mesleh, P. R. McCarren, C. W. Garvie, D. S. Daniels, K. L. Carey, A. P. Skepner, B. Levine and J. R. Perez, *Biochemistry*, 2017, **56**, 6639.
15 A. B. Birgisdottir, T. Lamark and T. Johansen, *J. Cell Sci.*, 2013, **126**, 3237.
16 S. Wu, Y. He, X. Qiu, W. Yang, W. Liu, X. Li, Y. Li, H. M. Shen, R. Wang, Z. Yue and Y. Zhao, *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**, 5669.
17 S. Shoji-Kawata, R. Sumpter, M. Leveno, G. R. Campbell, Z. Zou, L. Kinch, A. D. Wilkins, Q. Sun, K. Pallauf, D. MacDuff, C. Huerta, H. W. Virgin, J. B. Helms, R. Eerland, S. A. Tooze, R. Xavier, D. J. Lenschow, A. Yamamoto, D. King, O. Lichtarge, N. V. Grishin, S. A. Spector, D. V. Kaloyanova and B. Levine, *Nature*, 2013, **494**, 201.
18 L. Peraro, Z. Zou, K. M. Makwana, A. E. Cummings, H. L. Ball, H. Yu, Y. S. Lin, B. Levine and J. A. Kritzer, *J. Am. Chem. Soc.*, 2017, **139**, 7792.
19 M. Pelay-Gimeno, A. Glas, O. Koch and T. N. Grossmann, *Angew. Chem., Int. Ed.*, 2015, **127**, 9022–9054.
20 C. E. Schafmeister, J. Po and G. L. Verdine, *J. Am. Chem. Soc.*, 2000, **122**, 5891; Y. Tian, J. Li, H. Zhao, X. Zeng, D. Wang, Q. Liu, X. Niu, X. Huang, N. Xu and Z. Li, *Chem. Sci.*, 2016, **7**, 3325–3330; K. Hu, H. Geng, Q. Zhang, Q. Liu, M. Xie, C. Sun, W. Li, H. Lin, F. Jiang, T. Wang, Y. D. Wu and Z. Li, *Angew. Chem., Int. Ed.*, 2016, **55**, 8013.
21 H. Zhao, Q. S. Liu, H. Geng, Y. Tian, M. Cheng, Y. H. Jiang, M. S. Xie, X. G. Niu, F. Jiang, Y. O. Zhang, Y. Z. Lao, Y. D. Wu, N. H. Xu and Z. G. Li, *Angew. Chem., Int. Ed.*, 2016, **55**, 12088.
22 D. Wang, W. Liao and P. S. Arora, *Angew. Chem., Int. Ed.*, 2005, **44**, 6525.
23 X. Shi, R. Zhao, Y. Jiang, H. Zhao, Y. Tian, Y. Jiang, J. Li, W. Qin, F. Yin and Z. Li, *Chem. Sci.*, 2018, **9**, 3227.
24 X. Cai, W. Zheng, X. Shi, L. Chen, Z. Liu and Z. Li, *Mol. Pharmaceutics*, 2018, **15**, 5646.
25 N. Mizushima, T. Yoshimori and B. Levine, *Cell*, 2010, **140**, 313.
26 J. M. Wolfe, C. M. Fadzen, R. L. Holden, M. Yao, G. J. Hanson and B. L. Pentelute, *Angew. Chem., Int. Ed.*, 2018, **57**, 4756.
27 S. Chen, R. Gopalakrishnan, T. Schaer, F. Marger, R. Hovius, D. Bertrand, F. Pojer and C. Heinis, *Nat. Chem.*, 2014, **6**, 1009.
28 P. M. Cromm, S. Schaubach, J. Spiegel, A. Furstner, T. N. Grossmann and H. Waldmann, *Nat. Commun.*, 2016, **7**, 11300.
29 Y. Wang, B. J. Bruno, S. Cornillie, J. M. Nogieira, D. Chen, T. E. Cheatham, C. S. Lim and D. H. Chou, *Chemistry*, 2017, **23**, 7087.
30 G. B. Ding, J. Sun, G. Wu, B. Li, P. Yang, Z. Li and G. Nie, *ACS Appl. Mater. Interfaces*, 2018, **10**, 5227.