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Influence of α-methylation in constructing stapled peptides with olefin metathesis

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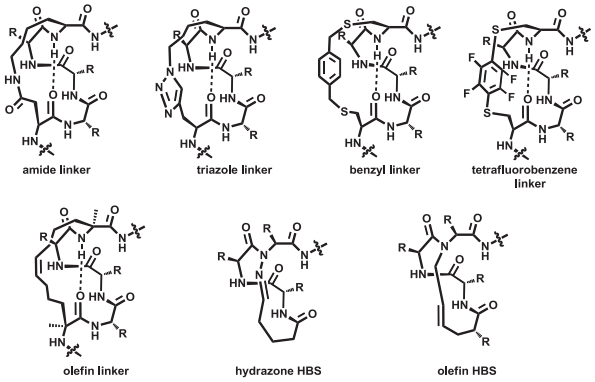
ABSTRACT

Ring-closing metathesis is commonly utilized in peptide macro-cyclization. The influence of α-methylation of the amino acids bearing the olefin moieties has never been systematically studied. In this report, controlled reactions unambiguously indicate that α-methylation at the N-terminus of the metathesis sites is crucial for this reaction to occur. Also, we first elucidated that the *E*-isomers of stapled peptides are significantly more helical than the *Z*-isomers.

1. Introduction

Recent statistical analysis reveals that α-helical interfaces are presented in more than 60% protein–protein interactions (PPIs) based on the Protein Data Bank (PDB), approximately 30%t of these interactions are accomplished by helices composed of 15 amino acids or less.[1] Due to the shallow and large nature of the PPI surface, small molecule PPI ligands are rare; meanwhile, poor bioavailability and enzymatic labiality largely limit the application of peptides.[2–4] How to interrupt PPIs remains a formidable task for the research community. Starting from a few decades ago, researchers noticed that a short peptide with well-defined helical conformation is more resistant to protease. Numerous methods have been developed to construct constraint peptides with a fixed secondary conformation, as shown in Scheme 1.[5–11] In general, most of these methods fix the peptides into cyclic molecules with constraint conformation by adding a tether to the peptide backbone. Salt bridges,[12] disulfide bonds,[13] amide bonds,[14] thiol–ether bonds, photo-clicked heterocycle[15] formation, olefin metathesis, etc. have been employed as efficient linking strategy.

Among them, olefin metathesis is the most commonly used method to construct constraint peptides. Grubbs et al. in 1998 reported that olefin metathesis could be applied in peptide stabilization by adding a tether to the peptides by inserting two *O*-allyl serines at the *i* and *i*+4 positions, respectively.[16] Shortly after, Verdine et al. in 2000 reported an all-hydrocarbon linker constructed by substituting the amino acids at *i*, *i*+4 positions or *i*, *i*+7 positions with unnatural amino acids bearing an alkenyl side chain.[9] These peptides are named as 'stapled peptides' and show higher helix content, better protease resistance, and cell permeability than their linear analogues. In the past decade, stapled peptides are utilized to mimic protein binding surfaces in various biological researches, including Mcl-1,[17] hDM2,[18] ERα,[19] etc. (Scheme 2a). In 2004, Arora et al. constructed a hydrogen bond



Scheme 1. Short summary of α-helix stabilization methods.

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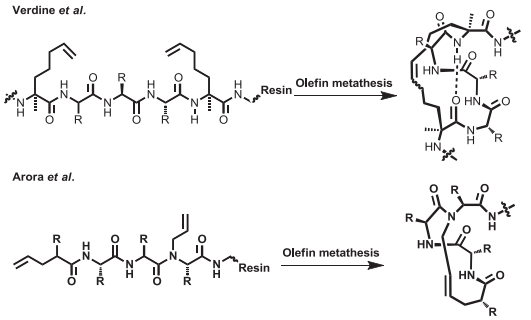
surrogate (HBS) system, which incorporated a 4-pentenoic acid and *N*-allyl residue at *i*, *i*+4 positions, respectively (Scheme 2b).[11] Their HBS peptides are also broadly utilized as PPI inhibitors for hDM2,[20] Ras-Sos,[21] HIF-1,[22] etc.



Scheme 2. Verdine and Arora utilized olefin metathesis in constructing macro-cyclic peptides.

Although olefin metathesis has been proved as an efficient method to construct constraint peptides, a detailed study of its scope and limitation is absent. Due to the limit switching sites of Arora's HBS peptides, we focus on stapled peptides and systematically elucidate the correlation between the linker length, double bond position, α-substitution on the amino acids at the metathesis sites and the products' secondary conformations.

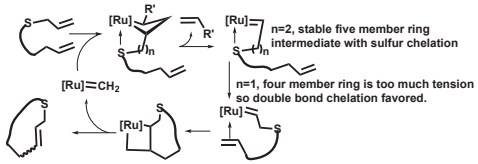
2. Result and discussion

For this study, we try to elucidate the efficiency of metathesis under the standard solid-phase peptide synthesis condition. Pentapeptides with three alanines as the internal residues and the unnatural amino acids bearing an olefin substitution at the N- and C-terminus, respectively, were chosen as the model peptides to avoid any possible sequence perturbation. All metathesis reactions were carried out in dichloroethane (DCE) on resin at room temperature for 2 h without heating or microwave radiation.

*S*5 is a common utilized α-methylated unnatural amino acid for stapled peptides, *S* indicates the absolute configuration and 5 means there are five atoms in the olefin substitution. Reported by Verdine et al., if two *S*5s at *i* and *i*+4 were switched to *S*6 and *S*3, respectively, metathesis efficiency significantly decreased.[9] However, further examination of the tether length was absent. We synthesized *S*5 analogues with and without α-methyl group and named them as *Sn* or mono-*Sn* (m*Sn* for short), then systematically tested the tether length and α-methylation influences on the metathesis efficiency.

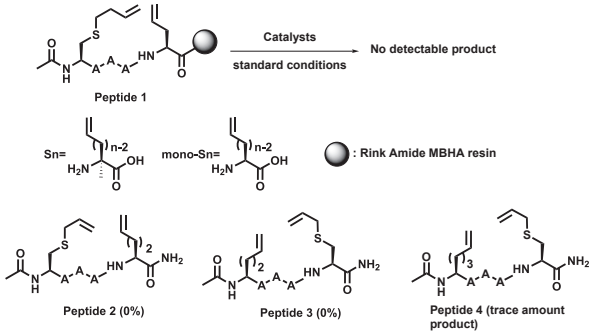
To mimic stapled peptides' tether length, Ac-*S*-homoallyl-C-AAA-m*S*3-NH_2 (**1**) was first synthesized, which would form a seven-atom tether. It was comparable with stapled peptides' eight-atom all-carbon tether since C–S bond length is significantly longer than C–C bond (1.8 Å vs 1.5 Å). Interestingly, this reaction wouldn't occur with all metathesis catalysts tested. As reported by Davis et al. in 2008, homoallyl substitution on sulfur may form an ideal chelating environment for catalyst and the sulfur atom will compete with the other alkene.[23] In contrast, *S*-allyl-cysteine is too tight to form sulfur–olefin chelating of metal catalysts (Scheme 3).

Then Ac-*S*-allyl-C-AAA-m*S*4-NH_2 (**2**) was tested, however, no product was detectable with LC–MS as well. Then the amino acids at the N- and C-terminus were switched, Ac-m*S*4-AAA-*S*-allyl-C-NH_2 (**3**) didn't provide any products as well. Longer C–S bonds may not be enough to compensate one atom less in the tether. Then Ac-m*S*5-AAA-*S*-allyl-C-NH_2 (**4**) was constructed, which would also



Scheme 3. *S*-Homoally-cysteine chelates metal catalyst.

form an eight-atom tether, however, only less than 5% of product was detected (Scheme 4).



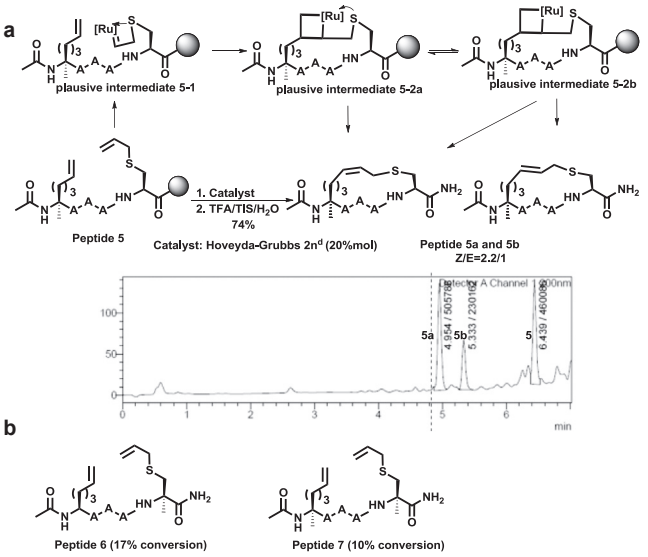
Scheme 4. Linker length has minimal influence on metathesis efficiency. Catalysts tested: Grubbs first generation, Grubbs second generation, Hoveyda–Grubbs second generation; standard conditions: 20% catalyst agitated for 2 h in DCE.

α-Methylation was always present in Verdine's stapled peptides; however, Grubbs et al. utilized two *O*-allyl serines instead of Verdine's unnatural amino acid *S*5, which could undergo metathesis efficiently without α-methylation requirement. In 2013, Yeo et al. successfully used m*S*5 synthesized stapled BID BH_3 peptide, but there was an internal glycine between the *i* and *i*+4 position.[24] Meanwhile, Nomura et al. used m*S*5 and *O*-allyl serine synthesized HIV-1 integrase inhibitors.[25] In their study the metathesis reaction must be heated. The previous reports hinted the importance of α-methylation, however, there was no systematical elucidation.

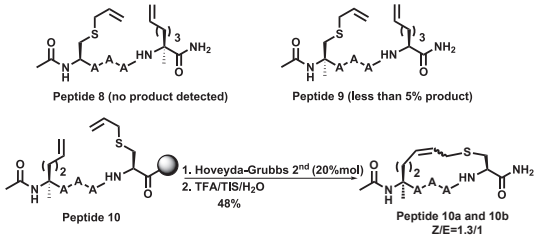
Peptide Ac-*S*5-AAA-*S*-allyl-C-NH_2 (**5**) was synthesized, the metathesis reaction went smoothly to provide a 74% conversion, which highlighted the importance of the α-methylation for the metathesis reactions. The two isomers had a ratio of 2.2:1. This higher ratio may be caused by the formation of plausible intermediate **5-2a**, which preferred isomer **5a** (Scheme 5a). If these two unnatural amino acids were switched, peptide Ac-m*S*5-AAA-*S*-allyl-α-methyl-C-NH_2 (**6**) provided only 17% conversion. If N- and C-terminus were both α-methylated, peptide Ac-*S*5-AAA-*S*-allyl-α-methyl-C-NH_2 (**7**) gave only 10% conversion (Scheme 5b). All these controlled studies showed the importance of incorporating an α-methyl substitution on *Sn* unnatural amino acid while α-methylation of *S*-allyl-cysteine decreased the reaction efficiency (Scheme 5b).

Further study revealed that peptide Ac-*S*-allyl-C-AAA-*S*5-NH_2 (**8**) and Ac-*S*-allyl-α-methyl-C-AAA-m*S*5-NH_2 (**9**) were synthesized and none of them could react. These results indicated that *Sn* amino acids must be positioned at the N-terminus to facilitate this reaction. This finding was further supported by peptide Ac-*S*4-AAA-*S*-allyl-C-NH_2 (**10**), which reacts smoothly to provide 48% conversion to form a seven-atom tether (Scheme 6).

Then the finding was further tested in the stapled peptide system for constructing all-hydrocarbon tether. Peptides Ac-m*S*5-AAA-m*S*4-NH_2 (**11**), Ac-m*S*4-AAA-m*S*5-NH_2 (**12**), Ac-m*S*5-AAA-

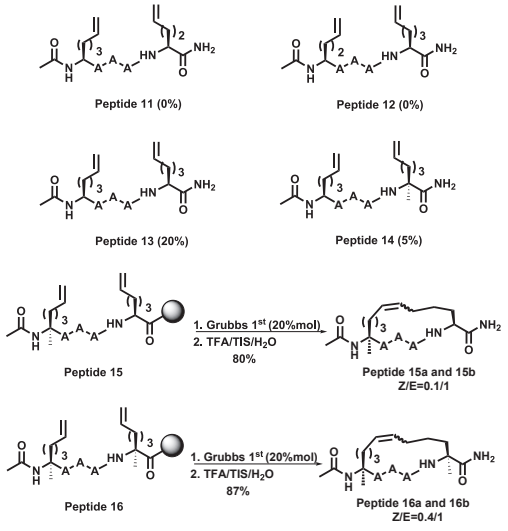


Scheme 5. (a) HPLC analysis of the reaction of peptide **5**. (b) Peptides **7** and **8** have lower yield to peptide **5** for their different α-methyl pattern.



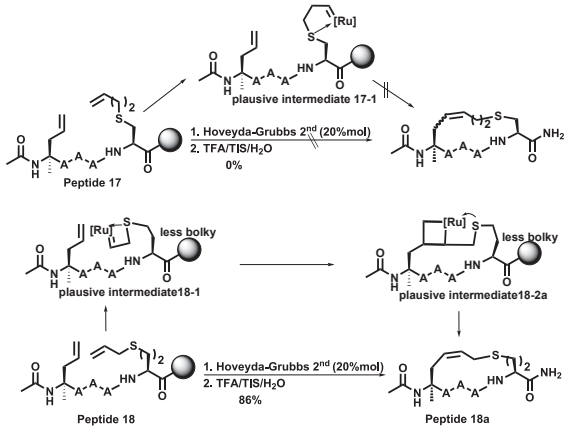
Scheme 6. Influence of α-methyl on metathesis efficiency.

m*S*5-NH_2 (**13**), and Ac-m*S*5-AAA-*S*5-NH_2 (**14**) could not undergo metathesis reactions smoothly since they were missing α-methyl substitutions. On the contrast, peptide Ac-*S*5-AAA-m*S*5-NH_2 (**15**) and Ac-*S*5-AAA-*S*5-NH_2 (**16**) both reacted well with high conversions (Scheme 7).



Scheme 7. Effects of α-methylation on all-hydrocarbon tether construction.

Peptide Ac-*S*3-AAA-*S*-homoallyl-C-NH_2 (**17**) could not undergo metathesis while peptide Ac-*S*3-AAA-*S*-allyl-homoC-NH_2 (**18**) reacted smoothly to give 86% conversion. These two controlled reactions further proved that *S*-homoallyl-cysteine indeed chelated the metal catalyst to inhibit the reaction as shown in Scheme 2 (Scheme 8). Interestingly, peptide **18** gave only one isomer from the metathesis, which suggested plausible intermediate **18-2a**, which provided more space around the sulfur (Scheme 8).



Scheme 8. Influence of sulfur position.

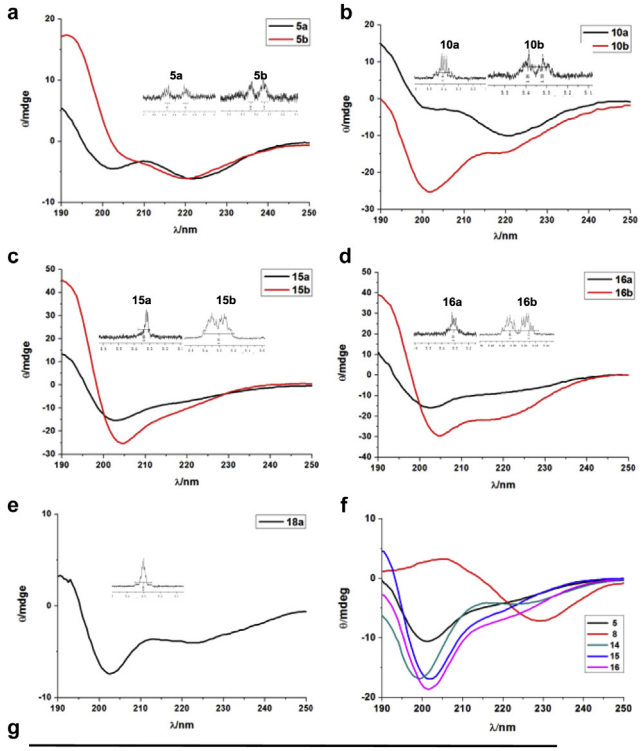
When metathesis is utilized for macro-cyclic peptide construction, the resulting peptides may exist as *E*- and *Z*-mixtures, however, the conformational differences between the *E*- and *Z*-isomers of stapled peptides have never been explored. We indeed separated the *E*- and *Z*-isomers by HPLC and the isomers with the longer retention time showed higher α-helical contents by circular dichroism (CD) measurement (Scheme 9). ^{1}H NMR experiments showed the isomer with longer retention time was *E* isomer and CD measurements showed that the *E*-isomers had significantly higher α-helicity contents (Scheme 9f). This result suggested that for stapled peptides, *E*-isomers gave better α-helicity.

Interestingly, α-methyl of the linear peptide could also enhance its α-helicity, which could then facilitate the metathesis reactions. Peptide **14** Ac-m*S*5-AAA-*S*5-NH_2 is less helical than peptide **16** Ac-*S*5-AAA-*S*5-NH_2, peptide **16** had an 87% conversion in metathesis reaction versus 5% conversion for peptide **14** (Scheme 9f).

3. Conclusion

The systematic study of the α-methylation influence on the stapled peptides unambiguously indicated that *Sn* must be placed at the N-terminus to facilitate the metathesis reactions at mild conditions. Otherwise, harsher reaction conditions were required, which was generally not favorable in peptide synthesis. Interestingly, α-methylation on the *S*-allyl-cysteine casted damaging effects on metathesis reactions, no matter it was placed at N- or C-terminus. One possible explanation is that the α-methylation of *Sn* may help to fix the olefin tether into a preferable torsion angle for the metathesis reaction to occur, while the bond angle difference between sp^3 C and sp^3 S induced the reactivity differences between *S*5 and α-methyl-*S*-allyl-cysteine. Our findings will help the researchers design their peptide sequences with higher successful rate.

Interestingly, for most reported stapled peptides, they were drawn in their *Z*-form; however, our study showed that *E*-stapled peptides show significantly higher α-helical contents than its *Z*-



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Peptide NO.	Relative alpha helicity[a]	Peptide NO.	Relative alpha helicity[a]
5	0.23	10a	0.35
8	ND	10b	0.67
14	0.19	15a	0.38
15	0.31	15b	0.62
16	0.36	16a	0.44
5a	0.20	16b	1.00
5b	0.23	18a	0.17

[a]Relative alpha helicity was calculated by $[\theta_{215}]x/[\theta_{215}]16b$.

Scheme 9. (a) CD spectra of **5a**/**5b**. (b) CD spectra of **10a**/**10b**. (c) CD spectra of **15a**/**15b**. (d) CD spectra of **16a**/**16b**. (e) CD spectra of **18a**. (f) CD spectra of linear peptide **5**, **8**, **14**, **15**, **16**. (g) Calculated helix contents of peptides. The concentration of peptides is normalized by HPLC. All CDs are measured with peptides in acetonitrile/H_2O (1:3).

isomers. This finding gives a hint we may improve the overall helix enhancing effect of stapled peptides by developing a more *E*-selective metathesis catalyst.

4. Experimental

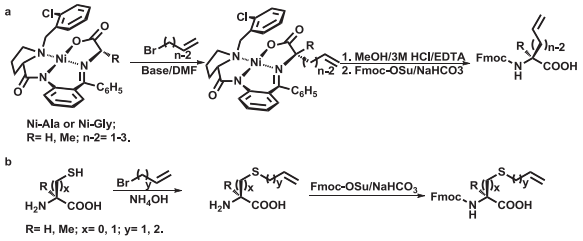
4.1. General method

The Fmoc-protected natural amino acids were purchased from GL Biochem (Shanghai). *S*5 was purchased from Suli Pharmaceutical Technology Jiangyin Co., Ltd. Homocysteine was purchased from Xinxiang Zhiyuan Co., Ltd. Peptides were synthesized manually following standard Fmoc-protected solid phase peptide synthesis procedure. Peptides were purified with Reverse Phase HPLC (Waters 600 and Shimadzu Prominence LC-20AT, column: Agilent Technologies XDB-C18 250×9.4 nm, 5 μm). All compounds were characterized with LC–MS (Shimadzu LCMS2020 using an UHPLC column, Agilent Technologies 'Poroshell 120 SB-C18' 75×3.0 mm, 2.7 μm) and/or nuclear magnetic resonance (NMR) spectroscopy (AVANCE-III 300M, 400, 500M). HRMS was taken on ABI Elite.

4.2. Unnatural amino acid synthesis

4.2.1. Synthesis of mSn and Sn (Scheme 10a).[26]

4.2.1.1. mSn. Alkylation of Ni-Gly: KOH powder (11.2 g, 200 mmol) was added to a stirred solution of Ni–Gly (10.6 g, 20 mmol) in DMF (100 mL) and the mixture was stirred at room



Scheme 10. (a) Synthesis of amino acids m*Sn* and *Sn*. (b) Synthesis of *S*-alkenyl cysteines.

temperature for 1 h. Then 1.05 equiv of alkene bromide was added under ice bath and the mixture was stirred for 2 h.

Work up: After complete consumption of starting material on TLC, the reaction mixture was acidified with 5% acetic acid to pH=5 and left for precipitation overnight. Then, the reaction mixture was filtered and the solid was dried and used for the nest step without purification.

Hydrolysis of Ni-complex procedure: 3 M HCl/DCM/MeOH (2:2:1) cocktail was added to the alkylated Ni–Gly (5 mL cocktail to 1 mmol Ni-complex) and the reaction mixture was heated to reflux until no Ni-complex was detectable on TLC. DCM and MeOH were removed by rotvap. The aqueous solution was extracted with $CHCl_3$ for three times. Then the amino acid aqueous solution was directly used for next step.

Fmoc protection: EDTA (1 equiv) was added to the amino acid aqueous solution and the reaction pH was adjusted to 7–8 with $NaHCO_3$. Then equal volume of acetonitrile and 1.1 equiv of Fmoc–OSu were added. The mixture was stirred for 3 h, concentrated to remove acetonitrile, and acidified with 1 M HCl to pH 2–3, extracted with ethyl ether, washed with brine, dried over sodium sulfate, and purified via column chromatograph with elute DCM/MeOH (50:1).

These procedures have been repeated multiple times for various amino acids, the yields varied between 30% and 50% from Ni-complexes.

4.2.1.1.1. mS3. δ_H (300 MHz, $CDCl_3$) 7.76 (2H, d, *J* 7.5), 7.59 (2H, d, *J* 7.1), 7.40 (2H, t, *J* 7.4), 7.31 (2H, t, *J*7.4), 5.71 (1H, dd, *J* 16.7, 7.5), 5.30 (1H, d, *J* 7.7), 5.18 (2H, d, *J* 12.8), 4.46 (3H, dd, *J* 26.5, 6.3), 4.23 (1H, t, *J* 6.8), 2.72–2.34 (2H, m).[27]

4.2.1.1.2. mS4. δ_H (300 MHz, $CDCl_3$) 7.75 (2H, d, *J* 7.4), 7.58 (2H, d, *J* 6.3), 7.38 (2H, t, *J* 7.4), 7.30 (2H, t, *J* 7.3), 5.85–5.69 (1H, m), 5.24 (1H, d, *J* 7.9), 5.04 (2H, t, *J* 13.3), 4.52–4.35 (3H, m), 4.21 (1H, t, *J* 6.6), 2.20–1.93 (3H, m), 1.87–1.73 (1H, m).[28]

4.2.1.1.3. mS5. δ_H (400 MHz, $CDCl_3$) 7.76 (2H, d, *J* 7.4), 7.60 (2H, d, *J* 5.2), 7.40 (2H, t, *J* 7.3), 7.31 (2H, t, *J* 7.2), 5.84–5.68 (1H, m), 5.24 (1H, d, *J* 8.1), 5.08–4.93 (2H, m), 4.41 (3H, dd, *J* 13.4, 7.0), 4.23 (1H, t, *J* 6.8), 2.14–1.86 (3H, m), 1.72 (1H, d, *J* 9.0), 1.54–1.35 (2H, m).

4.2.1.2. Sn. Alkylation of Ni–Ala: To a stirred solution of Ni–Ala (10.9 g, 20 mmol) in THF (100 mL) was added NaH 60% in mineral oil (3.0 g, 100 mmol) and the mixture was stirred at room temperature for 1 h. Then 5 equiv of alkene bromide was added and the mixture was stirred for overnight. Then the mixture was filtrated carefully and the liquid was concentrated with rotvap. The product was used for the nest step without purification.

The following steps are similar to the synthesis of m*Sn*.

4.2.1.2.1. S3. Yield 1.5 g, 11% overall yield from 20 g Ni-complex. δ_H (300 MHz, $CDCl_3$) 7.76 (2H, d, *J* 7.4), 7.59 (2H, d, *J* 7.3), 7.40 (2H, t, *J* 7.2), 7.31 (2H, td, *J* 7.4, 1.0), 5.67 (1H, s), 5.52 (1H, s), 5.14 (2H, d, *J* 11.5), 4.49–4.29 (2H, m), 4.22 (1H, t, *J* 6.6), 2.81 (1H, s), 2.68 (1H, s), 1.61 (3H, s).

4.2.1.2.2. S4. Yield 0.5 g, 3.9% overall yield from 20 g Ni-complex. δ_H (400 MHz, $CDCl_3$) 7.77 (2H, d, *J* 7.5), 7.59 (2H, d, *J* 7.4), 7.40 (2H, t, *J* 7.4), 7.32 (2H, td, *J* 7.4, 0.9), 5.76 (1H, s), 5.57 (1H, s), 4.99

(2H, dd, *J* 27.6, 13.4), 4.40 (2H, s), 4.22 (1H, t, *J* 6.5), 2.32–2.17 (1H, m), 2.00 (3H, d, *J* 35.3), 1.62 (3H, s).

4.2.2. Synthesis of S-allyl-cysteine and relative derivatives (Scheme 10b).[29] L-Cysteine hydrochloride (2.90 g, 16 mmol) was mixed with allyl bromide (3.0 g, 2.2 mL, 25 mmol) in 50 mL 2 M NH_4OH/EtOH (5:4) and stirred at room temperature for 20 h. The reaction mixture was concentrated and *S*-allyl-C precipitated out as white solid. The solid was filtered, washed with ethanol (50 mL twice), dried under reduced pressure, and used without further purification. Then Fmoc protection was taken in H_2O/acetonitrile with 1 equiv of Fmoc–OSu and 3 equiv of $NaHCO_3$. The product was purified with column with DCM, DCM/MeOH (20:1) to get 4.30 g Fmoc-protected amino acid, 70% overall yield from L-cysteine hydrochloride.

This protocol was used for other cysteine derivative synthesis.

4.2.2.1. Fmoc-S-allyl-C. Yield 4.3 g, 70%. δ_H (400 MHz, $CDCl_3$) 7.76 (2H, d, *J* 7.5), 7.61 (2H, d, *J* 4.2), 7.40 (2H, t, *J* 7.4), 7.31 (2H, t, *J* 7.4), 5.82–5.65 (2H, m), 5.16–5.05 (2H, m), 4.40 (2H, d, *J* 7.1), 4.24 (1H, t, *J* 7.1), 3.16 (2H, d, *J* 7.1), 2.98 (2H, ddd, *J* 34.3, 14.1, 5.0).

4.2.2.2. Fmoc-α-methyl S-allyl-C. α-Methyl cysteine was synthesized as previous reported.[30] Yield 0.3 g, 45% from α-methyl cysteine hydrochloride. δ_H (400 MHz, $CDCl_3$) 7.77 (2H, d, *J* 7.5), 7.63–7.56 (2H, m), 7.40 (2H, t, *J* 7.3), 7.31 (2H, td, *J* 7.4, 1.1), 5.90–5.78 (1H, m), 5.22–5.09 (2H, m), 4.84 (1H, s), 4.43 (2H, d, *J* 6.9), 4.23 (1H, t, *J* 6.7), 3.83 (2H, dd, *J* 9.3, 3.8). δ_C (101 MHz, $CDCl_3$) 177.69, 154.82, 143.75, 143.71, 141.27, 133.93, 127.70, 127.09, 125.06, 119.96, 117.68, 66.84, 60.29, 47.07, 35.86, 30.90, 23.38. ESI HRMS *m*/*z* found 420.1242 $(M+Na)^+$, calcd for $C_{22}H_{23}NNaO_4S$ exact mass: 420.1245.

4.2.2.3. Fmoc-S-allyl-homoC. Yield 0.15 g, 23% from homocysteine hydrochloride. δ_H (400 MHz, $CDCl_3$) 7.76 (2H, d, *J* 7.5), 7.58 (2H, d, *J* 3.3), 7.40 (2H, t, *J* 7.4), 7.31 (2H, t, *J* 7.4), 5.76 (1H, dt, *J* 16.5, 7.1), 5.38 (1H, d, *J* 8.1), 5.08 (2H, dd, *J* 10.9, 4.5), 4.47 (2H, dd, *J* 35.5, 5.7), 4.22 (1H, dd, *J* 8.6, 5.0), 3.13 (2H, d, *J* 7.1), 2.52 (2H, t, *J* 7.3), 2.23–2.13 (1H, m), 1.98 (1H, dd, *J* 14.2, 7.2). δ_C (75 MHz, $CDCl_3$) 176.59, 156.25, 143.82, 143.62, 141.34, 134.04, 127.82, 127.15, 125.11, 120.07, 117.44, 67.21, 53.07, 47.11, 34.48, 31.70, 26.13. ESI HRMS *m*/*z* found 398.1421 $(M+H)^+$, calcd for $C_{22}H_{24}NO_4S$ exact mass: 398.1426.

4.2.2.4. Fmoc-S-homoallyl-C. Yield 0.53 g, 81% from cysteine hydrochloride. δ_H (300 MHz, $CDCl_3$) 7.76 (2H, d, *J* 7.4), 7.60 (1H, d, *J* 7.0), 7.40 (1H, t, *J* 7.4), 7.31 (1H, t, *J* 7.3), 5.75 (1H, ddd, *J* 20.7, 17.1, 9.0), 5.05 (1H, t, *J* 12.6), 4.71–4.52 (1H, m), 4.41 (1H, d, *J* 7.0), 4.23 (1H, t, *J* 6.9), 3.05 (1H, d, *J* 4.7), 2.68–2.53 (1H, m), 2.32 (1H, d, *J* 7.0). δ_C (75 MHz, $CDCl_3$) 175.32, 155.97, 143.67, 143.62, 141.32, 136.19, 127.80, 127.13, 125.12, 120.05, 116.47, 77.48, 77.06, 76.64, 67.42, 53.48, 47.06, 34.12, 33.71, 32.16. ESI HRMS *m*/*z* found 420.1241 $(M+Na)^+$, calcd for $C_{22}H_{23}NNaO_4S$ exact mass: 420.1245.

4.3. Olefin metathesis procedure

Ac-X-A-A-A-Y-Resin (50 mg) was swelled in 1 mL DCM for 5 min then washed with DCE (1 mL) twice. Catalyst of 20% was dissolved in 1 mL DCE and added to the resin. The mixture was agitated with N_2 for 2 h. The resin was then cleaved with cocktail of TFA/TIS/H_2O (95:2.5:2.5) for 30 min, dried with nitrogen, precipitated with cold ether for three times, and the precipitate was collected and dissolved in 0.5 mL acetonitrile/H_2O (1:1) for purification after filtration.

*4.3.1. cyclo-[S5-A-A-A-S-allyl-C]-NH_2 (**5a**/**5b**)*

*4.3.1.1. Compound **5a**.* δ_H (400 MHz, DMSO) 7.97 (1H, d, *J* 8.1), 7.88 (1H, s), 7.84 (1H, s), 7.74 (2H, s), 7.39 (1H, s), 7.16 (1H, s), 5.45 (1H, s), 5.30 (1H, s), 4.34 (1H, s), 4.22 (1H, s), 4.09 (2H, s), 3.08 (2H, d, *J* 6.6), 1.91 (3H, s), 1.82 (3H, s), 1.79–1.73 (2H, m), 1.55 (3H, s), 1.35 (3H, s), 1.28–1.15 (12H, m).

*4.3.1.2. Compound **5b**.* δ_H (500 MHz, DMSO) 8.46 (1H, s), 8.15 (1H, s), 7.85 (1H, s), 7.80 (1H, s), 7.54 (1H, s), 7.14 (1H, s), 7.08 (1H, s), 5.43 (1H, s), 5.35 (1H, s), 4.24 (1H, dd, *J* 9.7, 8.1), 4.12 (1H, s), 4.03–3.96 (2H, m), 2.93 (2H, s), 2.81 (1H, s), 2.73 (1H, s), 2.00 (3H, d, *J* 6.8), 1.89 (4H, s), 1.78–1.69 (2H, m), 1.58 (2H, s), 1.40 (2H, s), 1.38–1.19 (13H, m).

*4.3.2. cyclo-[S4-A-A-A-S-allyl-C]-NH_2 (**10a**/**10b**)*

*4.3.2.1. Compound **10a**.* δ_H (400 MHz, DMSO) 8.12 (1H, d, *J* 6.1), 7.93 (1H, s), 7.80 (1H, d, *J* 7.1), 7.75–7.68 (2H, m), 7.29 (1H, s), 7.17 (1H, s), 5.45–5.36 (2H, m), 4.26 (1H, d, *J* 6.0), 4.20–4.15 (1H, m), 4.12–4.08 (1H, m), 4.03 (1H, d, *J* 7.1), 3.06 (2H, dd, *J* 11.5, 6.2), 2.67–2.65 (1H, m), 2.61 (1H, s), 1.96 (3H, s), 1.86 (3H, s), 1.81–1.74 (1H, m), 1.37 (3H, s), 1.32–1.15 (9H, m).

*4.3.2.2. Compound **10b**.* δ_H (400 MHz, DMSO) 8.30 (1H, s), 8.04 (1H, s), 8.00 (1H, d, *J* 5.6), 7.82 (1H, d, *J* 7.8), 7.69 (1H, d, *J* 7.7), 7.23 (1H, s), 7.09 (1H, s), 5.39 (1H, s), 5.32 (1H, s), 4.17 (2H, d, *J* 7.2), 4.03 (1H, s), 3.97 (1H, d, *J* 7.5), 3.07 (2H, s), 2.87–2.82 (1H, m), 2.72 (1H, dd, *J* 12.7, 7.7), 2.05 (3H, d, *J* 22.6), 1.89 (3H, d, *J* 7.2), 1.79 (1H, s), 1.42–1.15 (12H, m).

*4.3.3. cyclo-[S5-A-A-A-mS5]-NH_2 (**15a**/**15b**)*

*4.3.3.1. Compound **15a**.* δ_H (500 MHz, DMSO) 7.98 (1H, s), 7.91 (1H, s), 7.86–7.83 (1H, m), 7.64 (2H, s), 7.14 (1H, s), 7.03 (1H, s), 5.32 (2H, d, *J* 4.7), 4.27 (1H, s), 4.20–4.14 (1H, m), 4.06 (2H, d, *J* 6.5), 2.02–1.95 (2H, m), 1.86 (7H, s), 1.62 (4H, s), 1.52–1.43 (2H, m), 1.42–1.17 (12H, m).

*4.3.3.2. Compound **15b**.* δ_H (500 MHz, DMSO) 8.84 (1H, d, *J* 3.3), 8.40 (1H, s), 7.97 (1H, d, *J* 6.3), 7.43 (1H, d, *J* 8.6), 7.10 (1H, s), 6.98 (1H, d, *J* 8.2), 6.61 (1H, s), 5.39–5.32 (1H, m), 5.27 (1H, dd, *J* 10.0, 4.2), 4.23–4.16 (1H, m), 3.98–3.88 (2H, m), 3.82 (1H, dd, *J* 7.4, 3.4), 2.00–1.83 (6H, m), 1.76–1.48 (7H, m), 1.44–1.20 (15H, m), 1.16 (2H, d, *J* 12.4).

*4.3.4. cyclo-[S5-A-A-A-S5]-NH_2 (**16a**/**16b**)*

*4.3.4.1. Compound **16a**.* δ_H (500 MHz, DMSO) 8.10 (1H, s), 7.79 (2H, d, *J* 7.7), 7.57 (1H, s), 7.25 (1H, d, *J* 3.6), 7.18 (1H, s), 7.06 (1H, s), 5.32 (2H, s), 4.19–4.08 (3H, m), 1.87 (7H, s), 1.63 (2H, s), 1.27 (15H, ddd, *J* 37.8, 23.5, 11.7).

*4.3.4.2. Compound **16b**.* δ_H (500 MHz, DMSO) 8.86 (1H, s), 8.41 (1H, s), 7.84 (1H, d, *J* 6.6), 7.20 (1H, d, *J* 8.5), 7.07 (1H, s), 6.88 (1H, s), 6.55 (1H, s), 5.42–5.34 (1H, m), 5.34–5.24 (1H, m), 4.10–4.01 (1H, m), 3.96–3.85 (2H, m), 1.93 (4H, s), 1.90–1.83 (1H, m), 1.70 (3H, dd, *J* 15.5, 9.1), 1.55 (1H, d, *J* 7.5), 1.39–1.20 (15H, m).

*4.3.5. cyclo-[S3-A-A-A-S-allyl-homoC]-NH_2 (**18a**).* δ_H (500 MHz, DMSO) 8.81 (1H, s), 8.61 (1H, s), 7.67 (1H, d, *J* 6.4), 7.42 (1H, d, *J* 9.3), 7.21 (1H, d, *J* 7.9), 7.09 (1H, s), 6.64(1H, s), 5.30 (2H, s), 4.30–4.23 (1H, m), 4.06–4.01 (1H, m), 3.97–3.91 (1H, m), 3.77 (1H, d, *J* 10.1), 3.02 (1H, d, *J* 13.7), 2.93 (1H, d, *J* 5.7), 2.80 (1H, d, *J* 7.7), 2.42 (1H, d, *J* 8.9), 2.37 (1H, d, *J* 12.7), 2.23 (1H, d, *J* 5.9), 1.94 (3H, s), 1.76 (2H, d, *J* 9.7), 1.37–1.24 (12H, m).

4.4. CD measurement procedure

Peptides were dissolved in 25% acetonitrile. CD spectra were obtained on a Chirascan Circular Dichroism Spectrometer at 25 °C using the standard measurement parameters: wavelength,

190–250 nm; step resolution, 0.5 nm; speed, 20 nm/s; accumulations, 10; response, 1 s; bandwidth, 1 nm; path length, 0.1 cm. Every sample was scanned twice and the final CD spectra were smoothed to reduce noise.

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Supplementary data

H NMR and C NMR of amino acids and cyclic peptides, LC–MS spectra of peptides. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2014.08.004.

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