Chemoselective Peptide Cyclization and Bicyclization Directly on Unprotected Peptides

Yue Zhang[a], Qing Zhang[a], Clarence T. T. Wong[b], Xuechen Li[a]*

[a]Department of Chemistry, State Key Laboratory of Synthetic Chemistry, The University of Hong Kong, Hong Kong

[b]Department of Chemistry, The Chinese University of Hong Kong, Hong Kong, P. R. China, SAR

ABSTRACT: Cyclic peptides are drawing wide attentions as potential medium-sized modulators of biomolecular interactions with large binding surfaces. Simple but effective peptide cyclization methods are needed to construct cyclic peptide libraries by both peptide and non-peptide chemists. Herein, we report a highly chemoselective and operation-simple method directly cyclizing unprotected peptides, in which *ortho*-phthalaldehyde (OPA) is found to react with the lysine/N-terminus and cysteine within one unprotected peptide sequence effectively to form the isoindole-bridged cyclic peptides. This reaction is carried out in the aqueous buffer and features tolerance of diverse functionalities, rapid and clean transformation and operational simplicity. In addition, OPA peptide cyclization can also be combined with native chemical ligation-mediated cyclization to generate bicyclic peptides. Furthermore, the OPA peptide cyclization product can further react with the N-maleimide moiety in a one-pot manner to introduce additional functional motifs, like fluorophore probe, biomolecules (e.g., glycan, peptide or DNA). This OPA cyclization method extends the toolbox for integrating post-cyclization modification and bioconjugation into peptide cyclization with all-in-one manner strategy.

INTRODUCTION

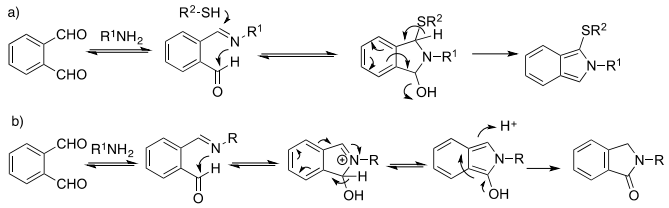
Peptide cyclization confers peptides with more rigid conformation[1-3] and enhanced stability towards enzymatic proteolysis, as compared to the linear peptides.[4, 5] Many cyclic peptides have been discovered from different kingdoms of organisms, which exhibit diverse biological activities, including anti-tumor, antimicrobial and anti-inflammatory activities.[3, 6, 7] The rigidity of cyclic peptides can lower the entropic cost of the Gibbs free energy when engaged in large binding surface. As a result, cyclic peptides are being used to probe the new chemical space and disturb protein-protein interactions (PPIs),[8] which are considered as "undruggable" targets in conventional small-molecule based drug discovery. Based on the structures, cyclic peptides can be classified into head-to-tail, head-to-side chain, side chain-to-tail and side chain-to-side chain cyclization. Various methods and strategies have been developed to construct cyclic peptides.[9] In particular, advances in effective chemoselective reactions have enabled peptide stapling and macrocyclization directly on unprotected peptides. For example, Yudin and co-workers developed multicomponent peptide macrocyclization,[10] Pentelute and co-workers used palladium-mediated lysine or cysteine arylation,[11-14] Dawson and co-workers used dichloroacetone,[15] Chou used thiol-ene reaction[16] and Greenbaum used dibromo-m-xylene[17] to form Cys-Cys dialkylation cyclization. In Dawson's case, the cyclization provided the exocyclic carbonyl functional group for further late-stage conjugation via oxime reactions.[15]

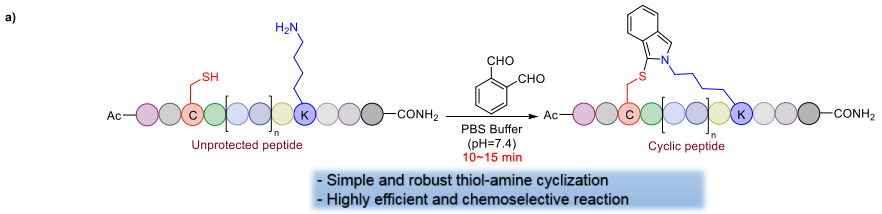
Herein, we report a highly effective chemoselective peptide cyclization and bicyclization directly on unprotected peptides, which generates novel cyclic peptide structural motifs. This method relies on a chemoselective reaction between the amine group (lysine side chain or N-terminus) and the thiol group of cysteine present in the peptide, to form both side chain-to-tail and side chain-to-side chain cyclic peptides. The fast reaction rate and operational simplicity render this method to be highly effective to synthesize cyclic peptides.

RESULTS AND DISCUSSION

We were inspired by the three-component coupling reaction of *ortho*-phthalaldehyde (OPA), a thiol moiety (*i.e.*, 2-mercaptoethanol) and an amine to form the fluorescent 1-substituted-thio-2-substituted-isoindole, which had historically been used to detect the amino group during the peptide Edman degradation process and analytical determination of amino acids. Roth first reported this reaction and its use for fluorometric detection of amino acids in 1971.[18] Benson and co-workers later improved the reaction reproducibility by using a large excess of 2-mercaptoethanol and adding Brij.[19] The established protocol for this three-component reaction required premixing of large excess of both mercaptoethanol and OPA prior to addition of the amino acid in boric acid buffer (pH 9.7) in order to achieve clean and reproducible results.[20]

Scheme 1. a) Proposed three-component reaction mechanism. b) Proposed two-component reaction mechanism.





b)

No.	Sequence	Calculated MS	Obtained MS	Conversion /%
1.	NH_2-AAACF-$CONH_2$	$[M+H]^+$=578.20	579.2	>98
2.	NH_2-AAAACF-$CONH_2$	$[M+H]^+$=649.66	650.3	96
3.	NH_2-AAAAACF-$CONH_2$	$[M+H]^+$=720.31	721.3	>98
4.	Ac-KAAACF-$CONH_2$	$[M+H]^+$=748.30	749.4	98
5.	Ac-KAAAACF-$CONH_2$	$[M+H]^+$=819.30	820.2	93
6.	Ac-KAAAAACF-$CONH_2$	$[M+H]^+$=890.41	891.3	>98
7.	Ac-KAAAAAACF-$CONH_2$	$[M+H]^+$=961.41	962.4	>98
8.	Ac-CHHALTHAK-$CONH_2$	$[M+H]^+$=1155.50	1156.5	>98
9.	Ac-CAHNLTHAK-$CONH_2$	$[M+H]^+$=1132.50	1133.5	>98
10.	Ac-ENPECILDKHVQRVM-NH_2	$[M+2H]^{2+}$=976.02	975.9	>98
11.	Ac-ENCPEILDKHVQRVM-NH_2	$[M+2H]^{2+}$=976.02	976.0	89.5
12.	Ac-CENPEILDKHVQRVM-NH_2	$[M+2H]^{2+}$=976.02	976.1	93
13.	Ac-CENPEILDHVKQRVM-NH_2	$[M+2H]^{2+}$=976.02	976.0	90
14.	Ac-CENPEILDHVQRVMK-NH_2	$[M+2H]^{2+}$=976.02	976.0	94
15.	NH_2-GKAFQESC-NH_2[a]	$[M+H]^+$=966.97	966.44	96

c)

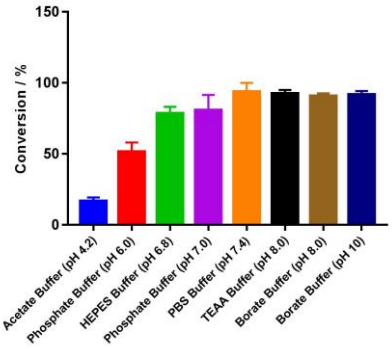


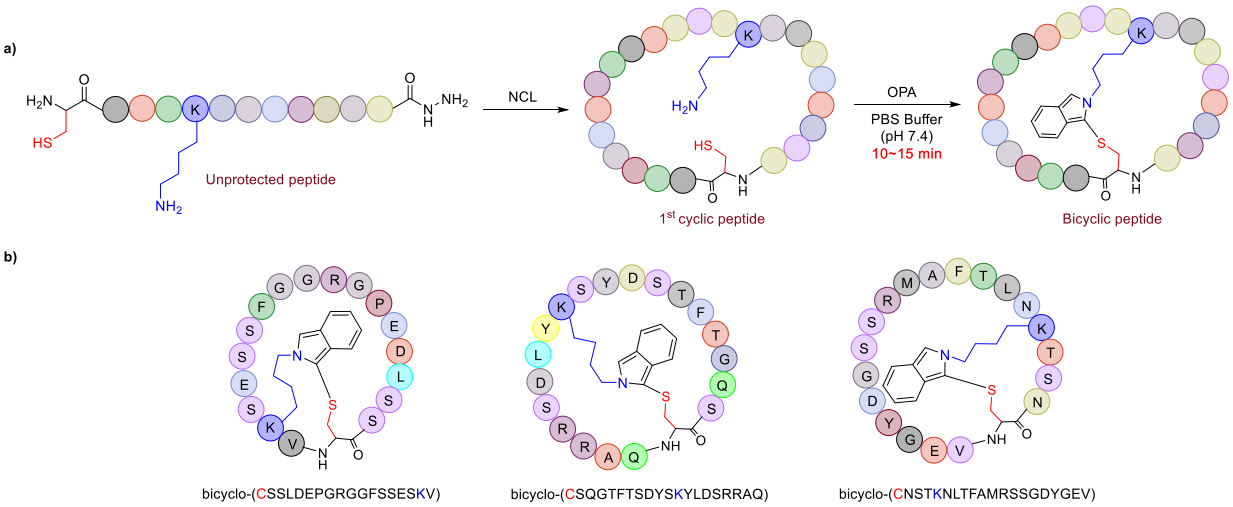
Figure 1. OPA-guided chemoselective cyclization with unprotected peptides. a) Schematic diagram of the OPA-guided chemoselective cyclization. b) The OPA-guided chemoselective cyclization with different peptide sequences. c) The reaction between Ac-KAAACH-$CONH_2$ (0.5 mM) and OPA (1 equiv.) under various buffer conditions for 15 min. [a] The ratio of side-chain-to-side-chain vs. head-to-side-chain cyclization product was roughly 1.7 : 1 based on the NMR and analysis.

Mechanistically, the abovementioned phenomena had not been clearly explained by the proposed mechanism[30] (Scheme 1a). Recently, we found that OPA could react with free amine groups rapidly in aqueous buffers to form phthalimidines (Scheme 1b).[21, 22] This finding suggested that two-component reaction of amine and OPA to form phthalimidines might compete with the three-component reaction of amine, OPA and thiol to form isoindoles, which might account for the necessity of large excess of thiol groups and premixing as in the Benson's protocol.

OPA-guided Chemoselective Cyclization. We were pondering what would happen to a molecule carrying both thiol and amine for the reaction with OPA in aqueous buffers,[23] as two-component reaction or three-component reaction? To answer this question, we prepared the model peptide (AcNH-KAAAAAACF-$CONH_2$), which carried a cysteine residue and a lysine. Upon addition of OPA to this peptide in aqueous PBS buffer (pH 7.4), surprisingly, the reaction turned out to be very smooth and rapid to form the isoindole cyclic peptide, with full conversion within 10 minutes and without any trace of the two-component reaction product (*c.f.*, phthalimidines) or other byproducts, judged by LCMS analysis of the crude reaction mixture (Figure S2.1.7). It is also worth to mention that OPA was used in stoichiometric amount. Further conditions screening showed that buffers with pH 7.4 or above gave very clean reaction, while buffers with pH 7 or below resulted in some minor unidentified byproducts, and pH 3 was not good at all (Figure 1c and S2.2).

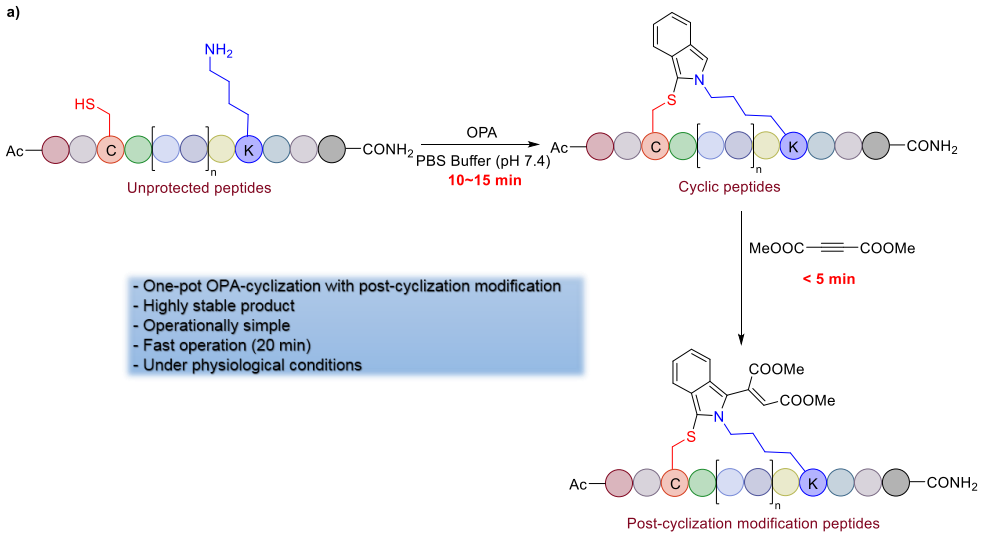
To probe the reaction pathway, we conducted the competition experiment. The peptide (AcNH-AFAQK-$CONH_2$) with only lysine and the peptide (AcNH-ENPECILDKHVQRVM-$CONH_2$) with both lysine and cysteine were mixed in 1:1 ratio and reacted with OPA (1.0 equiv.) at a concentration of 0.02 mM in the PBS buffer. Both the two-component reaction product (6%) and three-component reaction product (94%) were observed from LCMS analysis (Figure S2.3). This result suggested that after the imine formation, the intramolecular thiol attack on the imine was 15 times faster than the hydroxyiminum formation (Scheme 1), affording the three-component reaction product as the major product. It is also possible that the thiol reacted with the OPA to form a thiohemiacetal first, followed by the reaction with the intramolecular amine. In any scenario, the reaction of OPA and intramolecular thiol-amine offers a very effective peptide cyclization.

Next, the scope and the limitation of this chemoselective OPA-cyclization were studied. Fifteen model peptides with different lengths and spacing amino acids of 4 to 13 residues between the N-terminus or lysine side chain and the cysteine residue reacted with OPA in the PBS buffer to afford cyclic peptides of different rings, with 90- >98% conversions judged by LCMS analysis of the crude reaction mixtures (Figure 1b and S2.1). Various side chain functionalities present in the unprotected peptides did not interfere the reaction. Thus, this chemoselective OPA-cyclization provided a simple way to cyclize unprotected native peptides (Figure 1a). It should be pointed out that this reaction couldn't differentiate the side chain amino group and the N-terminal amine, thus capable of producing both side chain-to-tail and side chain-to-side chain cyclic peptides. Orthogonal amine protecting groups need to be used when multiple lysine residues are present in the peptide sequence.



Calculated MS	$[M+2H]^{2+}$=960.97	$[M+2H]^{2+}$=1196.5/ $[M+3H]^{3+}$=797.96	$[M+2H]^{2+}$=1130.0
Obtained MS	961.87	1197.56; 798.99	1131.27
Conversion /%	>98	94	90

Figure 2. Bicyclization via NCL-cyclization followed by OPA-cyclization. a) Schematic diagram of the bicyclization via NCL-cyclization followed by OPA-cyclization. b) Bicyclization with different peptide sequences.



b)

No.	Peptides	Conversion /% [a]		HPLC Yield /%
		Step1	Step2	
1.	Ac-KAAAAACH-$CONH_2$	98.0	80.4	53.2
2.	Ac-ACFALPKG-$CONH_2$	92.5	90.3	48.6
3.	Ac-NYRWRCKN-$CONH_2$	98.0	84.3	28.3
4.	cyclo-(CSSLDEPGRGGFSSESKV)	86.9	89.8	43.7
5.	cyclo-(CNSTKNLTFAMRSSGDYGEV)	89.2	78.1	32.5

[a] conversion percentage was calculated based on LCMS profile

Figure 3. The OPA-guided chemoselective cyclization followed by post-cyclization modification with DMAC. a) Schematic diagram of post-cyclization modification. b) Results of post-cyclization modification with different peptide sequences.

Bicyclization via NCL followed OPA-cyclization. These results of OPA-cyclization were so encouraging that we came up with a peptide bicyclization strategy via native chemical ligation (NCL) followed by the OPA-cyclization (Figure 2a). Three peptide hydrazides with N-terminal cysteine were readily prepared by Fmoc-solid phase peptide synthesis (Fmoc-SPPS). The peptide thioesters obtained from the peptide hydrazides smoothly cyclized via intramolecular native chemical ligation. Subsequently, OPA-cyclization gave rise to the bicyclic products cleanly with >90% conversion analyzed by LCMS (Figure 2b and S3).

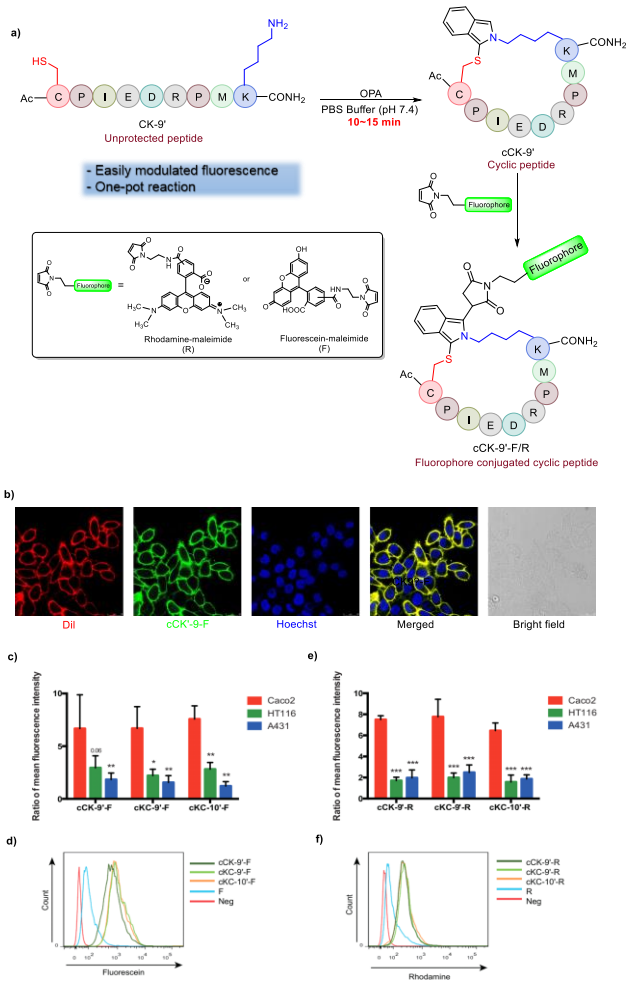
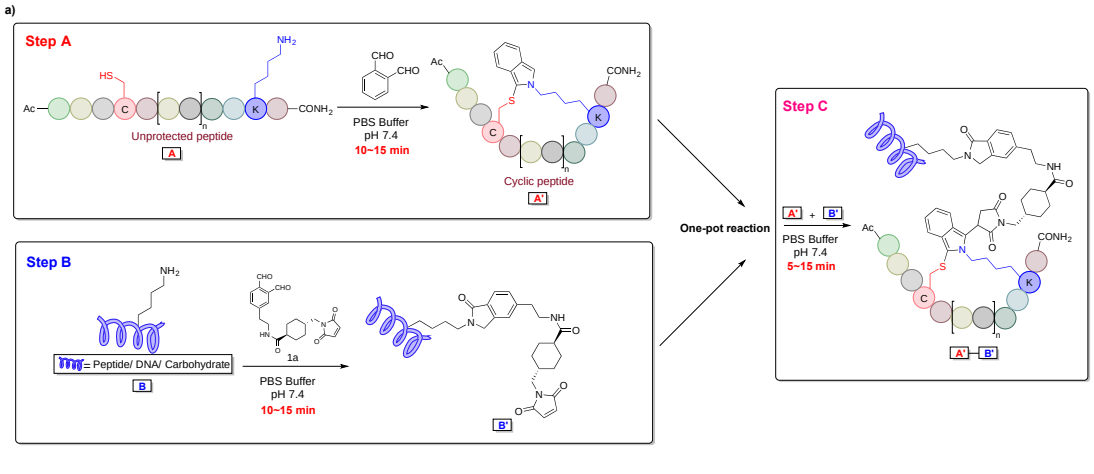


Figure 4. Fluorophore assembled OPA-guided cyclic peptides. a) Schematic diagram of the fluorophore assembled OPA-cyclization by N-maleimide derivatives. b) Fluorescent images of Caco2 by cCK-9′-F. c) The statistics analysis of fluorescein modified peptides (cCK-9′-F, cKC-9′-F, and cKC-10′-F) with different cell lines (Caco2, HT116, and A431). d) The statistics analysis of fluorescein modified peptides (cCK-9′-F, cKC-9′-F, and cKC-10′-F) with Caco2. e) The statistics analysis of rhodamine modified peptides (cCK-9′-R, cKC-9′-R, and cKC-10′-R) with different cell lines (Caco2, HT116, and A431). f) The statistics analysis of rhodamine modified peptides (cCK-9′-R, cKC-9′-R, and cKC-10′-R) with Caco2. (KC-10′: Ac-KTPSPFDSHC-$CONH_2$, KC-9′: Ac-KSDSWHYWC-$CONH_2$, CK-9′: Ac-CPIEDRPMK-$CONH_2$, F: fluorescein, R: rhodamine, Neg: DMSO, Dil: 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate). (*: P value <0.05, **: P value <0.01, ***: P value<0.001.)

One-pot OPA-cyclization with post-cyclization modification. Furthermore, we realized that the isoindole moiety after OPA-cyclization might serve as a useful handle for further derivatization. Towards this end, the obtained cyclic peptides reacted with either dimethyl acetylenedicarboxylate (DMAC) or N-maleimide (NMM) very rapidly with completion within minutes. By modification of the DMAC or N-maleimide, the OPA-cyclization guided modification could be a module-assembled approach for constructing functional peptide architectures. It is worth to note that the OPA-cyclization and post-cyclization modification could be performed in one-pot, by sequentially adding OPA and DMAC/or NMM into the reaction mixture (Figure 3a). The resultant product was found to be the addition product (Figure 3a and S4.1).[23-26] Apparently, this post-cyclization modification can further diversify the structural complexity of the cyclic peptides (Figure 3b). In addition, the resultant moieties showed much enhanced stability as compared with the isoindoles that were prone to oxidation over time (Figure S4.2 and S4.2.1).[27-30]

In addition, we synthesized two modules of N-maleimide-conjugated fluorophores (fluorescein and rhodamine), which could be easily conjugated with OPA cyclic peptides (Figure 4a). Cyclic peptide CK-9 cyclo(CPIEDRPMC) was previously reported to specifically target poorly differentiated colon carcinoma cells.[31] To apply OPA cyclization, CK-9 derivative peptide CK-9′ (CPIEDRPMK) was first synthesized, KC-9′ (KSDSWHYWC) and KC-10′ (KTPSPFDSHC) were synthesized as analogs.[32, 33] OPA-mediated cyclization followed by one-pot N-maleimide conjugation smoothly afforded the cCK-9′-fluorophore conjugate (Figure 4a) and other fluorophore conjugate analogs. Next, fluorescence confocal imaging was employed to prove the specificity of the fluorophore conjugated cyclic peptide cCK-9′. As shown in Figure 4b, the images of fluorescein conjugated cyclic peptide cCK-9′ was overlapped very well with that of cell membrane dye (DiI). Flow cytometry was also used to test the targeting specificity of fluorophores conjugated cyclic peptides (Figure 4c-f and S4.3.2). Both fluorescein and rhodamine conjugated cyclic peptides showed specific binding to Caco2 cells, as compared with HT116 and A431 cells, which is in accordance with previous reports.[30-32] These studies indicated that the OPA-mediated cyclization could effectively mimic the disulfide cyclic linkage, maintaining the cell targeting ability. In all, we have demonstrated that the cyclic peptides from the OPA-mediated cyclization could be further modified with functional groups, such as fluorophores. Via easily changing the functionalized groups and recognition groups, various functional cyclic peptide biomolecules can be synthesized by this robust OPA cyclization-guided modification.

One-pot Cyclization and Bioconjugation. Next, we further derivatized N-maleimide with various functional molecules including glycans, peptides, and amine modified DNA, which could be subsequently introduced onto the cyclic peptides, providing useful bioconjugates (Figure 5a). Towards this end, we designed an N-maleimide-OPA bifunctional linker, which could readily react with an amine group present on the biomolecule **B** (e.g., glycan, peptide or DNA) via phthalimidine chemistry as we previously developed to afford conjugates **B′** (Figure 5a). The resultant conjugate **B′** was subsequently reacted with OPA cyclic peptides **A′** in one-pot manner to generate various cyclic peptide-peptide, cyclic peptide-glycan and cyclic peptide-DNA hybrids. All examples tested could be completed cleanly in around 30 minutes in one-pot manner without any purification step (Figure 5 and S5).



b)

No.	Step A: A + OPA		Step B: B + 1a		Step C: A' + B'
	A' [a]	Time A	B'	Time B	Time for A' + B' [c]
1.	Ac-KAAAAACH-$CONH_2$	15 min	Ac-AFAQK-$CONH_2$ [a]	15 min	10 min
2.	Ac-ENPECILDKHVQRVM-$CONH_2$	15 min	Ac-AFAQK-$CONH_2$	15 min	5 min
3.	cyclo-(CNSTKNLTFAMRSSGDYGEV)	15 min	Ac-AFAQK-$CONH_2$	15 min	15 min
4.	Ac-ENPECILDKHVQRVM-$CONH_2$	15 min	Glucosamine [a]	15 min	15 min
5.	Ac-ENPECILDKHVQRVM-$CONH_2$	15 min	5'-NH_2-C_6-ATCGATCGATCG [b]	15 min	15 min

[a] Reaction concentration is 0.5 mM. [b] Reaction concentration is 0.2 mM. [c] A:B=1:1 mol/mol

Figure 5. OPA-derived one-pot cyclization and bioconjugation. a) Schematic diagram of the OPA-mediated one-pot cyclization and bioconjugation. b) Examples of the OPA-mediated one-pot cyclization and bioconjugation.

CONCLUSION

In summary, we have demonstrated that OPA-amine-thiol three-component reaction could be effectively used for the synthesis of novel cyclic peptide motifs, directly using unprotected peptides as the starting material. The OPA cyclization proves to be highly chemoselective under physiological conditions. Furthermore, by integrating NCL and OPA-mediated chemoselective peptide cyclization, bi-cyclic peptides were easily prepared. More interestingly, the cyclic peptide product from OPA cyclization could be further modified with DMAC or N-maleimide derivatives for constructing new architecture and incorporating functional moieties. In this regard, N-maleimide-OPA bifunctional linker offers a simple way to conjugate amine-containing biomolecules to the cyclic peptide obtained from OPA cyclization. Overall, we can anticipate that this OPA cyclization method can be applied for the synthesis of various functional cyclic/bicyclic peptides, peptide conjugates and branched peptides in both chemical biology study and drug discovery. The operational simplicity and high efficiency of OPA peptide cyclization will also potentially provide a new tool for construction of DNA-encoded cyclic peptide library.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge via the Internet at http://pubs.acs.org at DOI:
Detailed experimental procedures and spectral for all cyclic peptides.

AUTHOR INFORMATION

Corresponding Author

* xuechenl@hku.hk

Author Contributions

Notes

The authors declare no competing financial interest.

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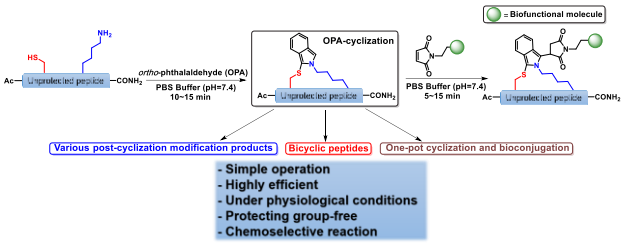

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