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Facile Synthesis of Peptidyl Salicylaldehyde Esters and Its Use in Cyclic Peptide Synthesis

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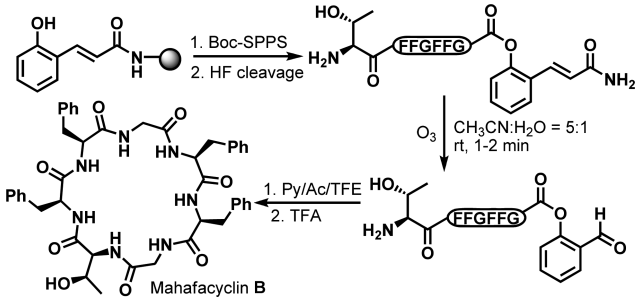
ABSTRACT



An efficient solid phase synthetic protocol for salicylaldehyde ester peptides is reported. With a Ser or Thr at the N-terminus, these salicylaldehyde ester peptides can be easily converted to Ser/Thr containing cyclic peptides.

Peptide head-to-tail cyclization generates an unusual class of peptides with a circular backbone structure. With their interesting structure and richness of pharmacology, cyclic peptides have always been attractive targets for synthetic chemistry.[1] The classical methods for peptide macrocyclization, which can be done either in solution or on resin, involve the activation of the carboxyl group of appropriately protected linear peptides. However, an inherent danger of enthalpic carboxyl activation is racemization at the C-terminal residue. The advent of chemoselective peptide ligation methods[2] has offered new ways for peptide macrocyclization. These ligation methods feature a prior capture step that brings the reacting C- and N-termini into close proximity, allowing spontaneous peptide bond formation in the subsequent step. With this entropy-driven activation scheme, the risks of racemization are minimized and the use of protection groups is not needed. In the late 1990s, Tam et al. first applied such methods in cyclic peptide synthesis.[3] Notably, the cysteine-based native chemical ligation (NCL)[4] was used by them to synthesize the macrocyclic cystine-knot peptides,[3c] which has now become the method of choice for preparing Cys-rich cyclic peptides. When the thiol-mediated ligation is followed by a desulfurization step, the method can be expanded to ligation at non-Cys residues.[5] However,

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(1) For reviews on cyclic peptides, see: (a) Clark, R. J.; Craik, D. J. *Biopolymers* **2010**, *94*, 414. (b) White, C. J.; Yudin, A. K. *Nat. Chem.* **2011**, *3*, 509. (c) Jiang, S.; Li, Z.; Ding, K.; Roller, P. *Curr. Org. Chem.* **2008**, *12*, 1502. (d) Lambert, J. N.; Mitchell, J. P.; Roberts, K. A. *J. Chem. Soc., Perkin Trans. 1* **2001**, *5*, 471. (e) Tam, J. P.; Wong, C. T. T. *J. Biol. Chem.* **2012**, *287*, 27020. (f) Davies, J. S. *J. Pept. Sci.* **2003**, *9*, 471.

(2) For reviews on peptide ligation methods, see: (a) Tam, J. P.; Yu, Q.; Miao, Z. *Biopolymers* **1999**, *51*, 311. (b) Hahn, M. E.; Muir, T. W. *Trends Biochem. Sci.* **2005**, *30*, 26. (c) Hackenberger, C. P.; Schwarzer, D. *Angew. Chem., Int. Ed.* **2008**, *47*, 10030. (d) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338. (e) Pattabiraman, V. R.; Bode, J. W. *Nature* **2011**, *480*, 471.

(3) (a) Botti, P.; Pallin, T. D.; Tam, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 10018. (b) Zhang, L. S.; Tam, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 2363. (c) Tam, J. P.; Lu, Y. A.; Yu, Q. T. *J. Am. Chem. Soc.* **1999**, *121*, 4316.

(4) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994**, *266*, 776.

(5) (a) Rohde, H.; Seitz, O. *Biopolym. (Pept. Sci.)* **2010**, *94*, 397. (b) Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064. (c) Botti, P.; Tchertchian, S. Patent WO 2006/133962, 2006. (d) Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem., Int. Ed.* **2008**, *47*, 6807. (e) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 8521. (f) Yang, R. L.; Pasunooti, K. K.; Li, F. P.; Liu, X. W.; Liu, C. F. *J. Am. Chem. Soc.* **2009**, *131*, 13592. (g) Ajish Kumar, K. S.; Haj-Yahya, M.; Olschewski, D.; Lashuel, H. A.; Brik, A. J. *Angew. Chem., Int. Ed.* **2009**, *48*, 8090. (h) Yan, L.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 523. (i) Thompson, R. E.; Chan, B.; Radom, L.; Jolliffe, K. A.; Payne, R. J. *Angew. Chem., Int. Ed.* **2013**, *52*, 9723. (j) Malins, L R.; Cergol, K. M.; Payne, R. J. *ChemBioChem* **2013**, *14*, 559.

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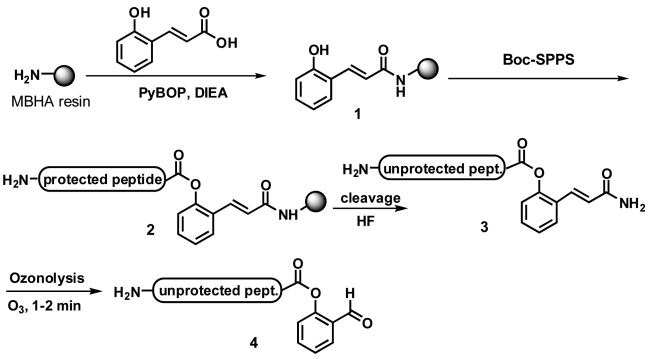
except for Ala and Val, which are derived from Cys and penicillamine respectively, few thiol-modified amino acids are commercially available, making it synthetically challenging to use this approach.

The aldehyde capture ligation by Tam et al. is one of the earliest ligation methods developed.[6] Conceptually similar to the imine formation-initiated ligation first proposed by Kemp,[7] this method makes use of an N-terminal Cys, Ser or Thr to react with a C-terminal glycoaldehyde ester peptide to initially form a relatively stable thiazolidine or oxazolidine ring. A subsequent intramolecular O−N acyl transfer forms a new peptide bond with a pseudoproline structure at the junction of the two peptide segments.[6] However, the difficulty of regenerating the native amino acid residue from the pseudoproline structure has limited the use of this method. Very recently, this problem was addressed by Li et al. by using an aromatic salicylaldehyde (SAL) ester in lieu of the glycoaldehyde ester.[8] The auxiliary benzylidene acetal group on the ligation product can be easily cleaved by acidic hydrolysis to generate native Ser/Thr. Since Ser and Thr are much more abundant than Cys, this ligation method holds good promise in the practice of protein and cyclic peptide synthesis.[9] As obviously seen from Li's work, a bottleneck in using this ligation scheme lies in the synthesis of the SAL ester peptides. Although they have demonstrated that the C-ter SAL ester can be introduced either though coupling a protected peptide-COOH with acetal-protected salicylaldehyde in solution[9a] or through phenolysis of resin-bound peptidyl-benzimidazolinone,[9b] the limitations of these methods are that the solution synthesis suffers from problems of inconvenience and racemization, which would limit its use to C-ter Gly or Pro, whereas the on-resin phenolysis method was inefficient and required long reaction time (16 h, as reported). Clearly, solid phase peptide synthesis (SPPS)[10] would be the preferred method for the synthesis of SAL ester peptides or their immediate precursors. An efficient SPPS method would greatly promote the use of Ser/Thr-based ligation. Herein we report a convenient solid phase synthesis method for SAL-ester peptides and its application in the head-to-tail cyclization of Ser or Thr-containing cyclic peptides.[9c,d]

Scheme 1. General Scheme for the Synthesis of C-Terminal Salicylaldehyde (SAL) Ester Peptide



The difficulty in the direct solid phase synthesis of peptide-SAL esters is mainly attributed to the liability of the phenolic ester and the reactivity of the aldehyde group. Fmoc chemistry is excluded because the phenolic SAL ester is labile to piperidine used in Fmoc SPPS. We thus focused our efforts on developing a Boc-based method. Since most protected forms of aldehyde are acid-sensitive and would not survive in the strong acidic conditions repetitively employed in Boc SPPS, we decided to use a latent group that would be a precursor to the aldehyde functionality. An ideal aldehyde precursor group must satisfy several criteria. (1) It should be compatible with the procedures of Boc SPPS. (2) After SPPS, the aldehyde group must be generated easily in a highly selective reaction without affecting other functional groups in the peptide. (3) It should contain a linker functional group for anchoring to the solid support.

After several attempts, we found that the commercially available (*E*)-3-(2-hydroxyphenyl)acrylic acid (i.e., *trans*-2-hydroxycinnamic acid) would fulfill these requirements. The carboxylic acid group can be used to couple itself to an amine-functionalized resin. The phenolic hydroxyl group can be used to elongate the peptide chain. Most importantly, ozonolysis of the C=C bond will afford an aldehyde group under very mild conditions and with excellent selectivity.

To test the feasibility of our proposal (Scheme 1), we coupled (*E*)-3-(2-hydroxyphenyl)acrylic acid to MBHA resin using PyBOP to prepare (*E*)-3-(2-hydroxyphenyl) acrylyl-MBHA **1** (Scheme 1). Ninhydrin test indicated completion of the coupling reaction in 1 h. Considering the relative low reactivity of the phenolic hydroxyl group, the loading of the first protected Boc-amino acid was run overnight at room temperature. Peptide chain elongation was effected using standard Boc SPPS protocols and monitored with ninhydrin test. The peptide was cleaved from the resin using either TFMSA/TFA or HF (Supporting Information). MS analysis clearly characterized the target peptide with a C-ter ester, **3**, formed with (*E*)-3-(2-hydroxyphenyl)acrylamide (Scheme 1). HPLC

(6) (a) Liu, C.-F.; Tam, J. P. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 6584. (b) Liu, C.-F.; Tam, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 4149. (c) Tam, J. P.; Miao, Z. *J. Am. Chem. Soc.* **1999**, *121*, 9013.

(7) For reviews, see: (a) Kemp, D. S. *Biopolymer* **1981**, *20*, 1793. (b) Coltart, D. M. *Tetrahedron* **2000**, *56*, 3449. For seminal literatures, see: (c) Kemp, D. S.; Wang, S. W.; Busby, S., III; Hugel, G. *J. Am. Chem. Soc.* **1970**, *92*, 1043. (d) Kemp, D. S.; Vellaccio, J. F. *J. Org. Chem.* **1975**, *40*, 3003. (e) Kemp, D. S.; Gratten, J. A.; Reczek, J. *J. Org. Chem.* **1975**, *40*, 3465. (f) Kemp, D. S.; Kerkman, D. J.; Leung, S. L.; Hanson, G. *J. Org. Chem.* **1981**, *46*, 490.

(8) Li, X. C.; Lam, H. Y.; Zhang, Y. F.; Chan, C. K. *Org. Lett.* **2010**, *12*, 1724.

(9) (a) Lam, H. Y.; Zhang, Y. F.; Liu, H.; Xu, J. C.; Wong, C. T. T.; Xu, C.; Li, X. C. *J. Am. Chem. Soc.* **2013**, *135*, 6272. (b) Zhang, Y. F.; Xu, C.; Lam, H. Y.; Lee, C. L.; Li, X. C. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 6657. (c) When we were about to submit our manuscript, a new paper from the Li group was published online on the synthesis of the SAL ester peptides, which is very similar to ours: Wong, C. T. T.; Lam, H. Y.; Song, T.; Chen, G.; Li, X. *Angew. Chem., Int. Ed.* **2013**, DOI: 10.1002/anie.201304773. (d) The SPPS method for the SAL ester peptides reported herein is also the subject of a patent application by us: Liu, C.-F.; Zhao, J. US. Pat. Appl. 61/595.853, 2012.

(10) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.

profiles of the crude products (Supporting Information) indicated that the purity of the target peptide was in the range of 50−90%. Figure 1 shows two typical examples of synthesized peptide acrylamide-phenolic esters **3**. A quantitative experiment was done on one of the ester peptides, H-**SIPLFPI**-O-2-PhCH=CHCONH$_2$, and the efficiency of this protocol was shown with a 60% isolated yield based on the MBHA resin loading (Supporting Information). This indicates that the phenolic ester linkage between the C-terminal carboxyl and (*E*)-3-(2-hydroxyphenyl)-acrylamide is fully compatible with Boc SPPS.

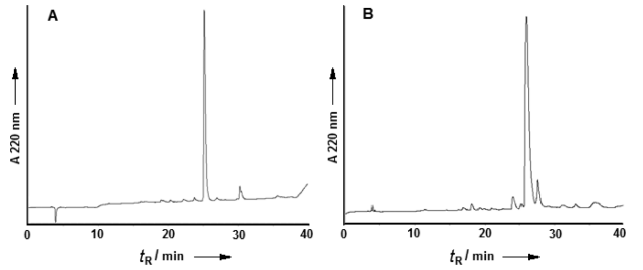


Figure 1. HPLC profiles of the crude peptides after cleavage. (A) H-**GVGFA**-O-2-PhCH=CHCONH$_2$ and (B) H-**SIPLFPI**-O-2-PhCH=CHCONH$_2$. The major peak is the desired product.

The key step of this strategy is the ozonolysis of the C=C bond to generate the aldehyde group. We first carried out ozonolysis using THF as the solvent, followed by addition of dimethylsulfide as the reductant. Although ozonolysis on H-**GVGFA**-OPhCH=CHCONH$_2$ generated the desired peptide-SAL ester **4** in 1−2 min at room temperature (Scheme 1), the solubility of most peptides in THF is poor. In this regard, a miscible water/organic solvent system would be ideal for small-to-medium sized peptides. It was reported that miscible water/organic solvent mixtures could be used as the media for ozonolysis of C=C bond in small organic compounds.[11] Further optimization demonstrated that the mixture of acetonitrile and water (5:1) was the best solvent for ozonolysis of our peptides. Using this method, 16 different C-terminal SAL ester peptides were prepared in good to excellent yields (Table 1). Ozone is a powerful oxidant, and very short reaction time is needed to oxidize the C=C bond. Under the conditions used, it did not cause any significant damage to the peptides under investigation (Table 1). The obtained SAL ester peptides were purified by reverse-phase HPLC under normal conditions and characterized by MS (Supporting Information).

With now an efficient method for solid phase synthesis of C-terminal SAL ester peptides in hand, we proceeded to apply it to the synthesis of cyclic peptides. Cyclization was performed on linear SAL ester peptides with a Ser or Thr residue at the N-terminus, **4** (Table 2 heading scheme). A typical example was a cyclic peptide cyclo[SGLFGFAG]

(11) Schiaffo, C. E.; Dussault, P. H. *J. Org. Chem.* **2008**, *73*, 4688.

Table 1. Synthesis of C-Terminal Salicylaldehyde Ester Peptides via Ozonolysis

3 **4**

entry	peptide sequence	yield (%)[a]
1	GVGFA	82
2	SGLYGFAG	85
3	SGLFGFAG	60
4	TGLFGFAG	75
5	SGQAG	89
6	LGFAG	80
7	Ac-LGFAG	65
8	SLSL	60
9	Ac-ASPLFA	76
10	LEQKGFS	45
11	VGTESFA	56
12	SGKAFL	72
13	SFLFA	67
14	TFFGFFG	55
15	SIPLFPI	71
16	SKSIPLFPI	42

[a] Isolated yield after HPLC purification on 5−10 mg reaction scale.

(entry 4, Table 2) prepared from its linear precursor, H-**SGLFGFAG**-OPhCHO. The linear peptide was first dissolved in 2,2,2-trifluoro ethanol (TFE) followed by addition of pyridine/acetic acid (1:1) mixture, a condition previously employed for oxazolidine formation in the earlier work by Tam's lab.[6c] To our delight, in 1 h at room temperature, the cyclization of this peptide proceeded cleanly to give a bicyclic *N*,*O*-benzylidene acetal **5** as the major product as seen from the HPLC profile of the reaction mixture (peak 1 in Figure 2, panel A). Remarkably, the concentration of the linear peptide used for cyclization was 10 mM, which is rather high for an intramolecular reaction, and no significant side products were observed (Figure 2, panel A). The cyclic product **5** was purified by HPLC and further treated with a cocktail solution of TFA/H$_2$O/*i*-Pr$_3$SiH (95: 2.5:2.5) to generate the final cyclic peptide **6** with a natural Gly-Ser peptide bond at the cyclization junction in excellent yield. In a similar manner, cyclization of H-**SGKAFL**-OPhCHO also led to a clean reaction (Figure 2, panel B), and the isolated cyclic acetal product was subsequently deprotected to afford cyclo[SGKAFL] with Leu-Ser at the cyclization junction (entry 1, Table 2). It is worth mentioning that, although both cyclization and deprotection steps gave high conversion yield in HPLC, the isolated yield was reduced significantly (see Table 2) because of attrition on the small amount (milligram scale) of material during HPLC purification.

As the aldehyde-mediated cyclization reaction is very clean, it is actually unnecessary to isolate the cyclic *N*,*O*-benzylidene acetal product **5** before the TFA deprotection

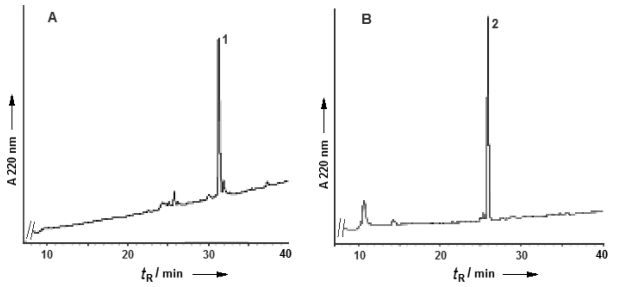


Figure 2. HPLC monitoring of the cyclization reaction after 1 h. (A) Cyclization of H-**SGLFGFAG**-OPhCHO (peak 1: its cyclic *N*,*O*-benzylidene acetal product **5**, *m*/*z* $[M + H]^+$ found 841.32, calcd 841.40). (B) Cyclization of H-**SGKAFL**-O-PhCHO (peak 2: its cyclic *N*,*O*-benzylidene acetal product **5**, *m*/*z* found 708.28, calcd 708.38).

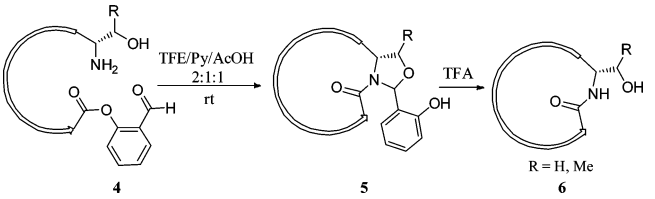
step. The solvent was removed under vacuum, and the treatment of TFA on the residue yielded the cyclic end product **6** directly. Mahafacyclin B, a natural cyclic heptapeptide with antimalarial activity isolated from the latex of Jatropha mahafalensis[12a] (entry 7, Table 2), was also synthesized using our methodology, and its identity was verified by MS and NMR analysis (Supporting Information). A recent total synthesis of mahafacyclin B[12b] employing soluble tag-assisted cyclization required more than 10 solution synthesis and purification steps. In comparison, only three purification steps were needed in our case. This clearly demonstrated the convenience and robustness of our methodology for the synthesis of cyclic peptides such as mahafacyclin B.

Because of high strains of the small ring structure, cyclization of H-**SLSL**-OPhCHO in the synthesis of cyclo-[SLSL] (entry 6, Table 2) had to be done at very high dilutions to minimize cyclodimerization, a side reaction inherently associated with small cyclic peptides.[1b] Here, cyclodimerization involved two consecutive ligation steps, the first intermolecular and the second intramolecular, forming a symmetric dimer structure. Significant cyclodimerization was observed even at 0.1 mM concentration of the linear SAL ester precursor (Figure S44, Supporting Information).

A number of other cyclic peptides ranging from 5 to 9 residues in ring size and with different cyclization junctions were prepared by using our method (Table 2). These results indicate that the cyclization reaction can tolerate a wide range of Xaa-Ser/Thr junctions, including the sterically hindered Ile-Ser (entry 8, Table 2), which is consistent with earlier results reported by Li et al.[8,9a−9c] As a negative control, no cyclization occurred with H-**LGFAG**-OPhCHO (entry 6, Table 1), which does not have an N-terminal Ser/Thr.

(12) (a) Baraguey, C.; Blond, A.; Cavelier, F.; Pousset, J. L.; Bodo, B.; Auvin-Guette, C. *J. Chem. Soc., Perkin Trans. 1* **2001**, 2098. (b) Fujita, Y.; Fujita, S.; Okada, Y.; Chiba, K. *Org. Lett.* **2013**, *15*, 1155.

Table 2. Synthesis of Ser or Thr-Containing Cyclic Peptides from Unprotected Peptide-O-2-PhCHO[a]



entry	sequence	yield (%)[b]
1	cyclo[SGKAFL]	39
2	cyclo[SFLFA]	35
3	cyclo[TGLFGFAG]	63
4	cyclo[SGLFGFAG]	65
5	cyclo[SGLYGFAG]	52
6	cyclo[SLSL][c]	20
7	cyclo[TFFGFFG]	56
8	cyclo[SKSIPLFPI][d]	29

[a] Unless otherwise indicated, cyclization was performed for 1 h at 10 mM concentration of the linear SAL ester peptide. [b] Isolated yield for two steps. [c] Concentration for cyclization was 0.05 mM. [d] Cyclization reaction was performed overnight at 2 mM of the linear precursor.

In summary, a convenient method for the synthesis of C-terminal salicylaldehyde ester peptides was developed. The key design of this method is the ozonolysis reaction on an aldehyde precursor, peptidyl-OPhCH=CHCONH$_2$, which is easily obtained from Boc SPPS. This method makes the peptide SAL esters readily available for Ser/Thr-mediated aldehyde capture ligation.[8] It was further demonstrated that the peptide SAL esters so-prepared can be easily converted to Ser/Thr-containing cyclic peptides through SAL capture ligation. A possible limitation of this method resides in the susceptibility of certain amino acids such as Cys, Met and Trp to ozonolysis, which would restrict its application scope. Nevertheless, these amino acids are of the lowest natural abundance, and our strategy certainly provides a new and efficient approach to the synthesis of salicylaldehyde ester peptides. It is simple to use because no tedious purification and characterization steps are needed. In addition, all the reagents used in this protocol are commercially available. Clearly, our method not only provides a powerful approach to cyclic peptide synthesis but also will spur renewed interest in the aldehyde-based capture ligation schemes for protein chemical synthesis in the future.

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Supporting Information Available. Experimental procedures and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.