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A Novel On-resin Synthesis of *C*-Terminally Amidated Peptides

Kalle Kaljuste and James P. Tam*

Department of Microbiology and Immunology, Vanderbilt University. A5519 MCN, 1161 21st Ave S. Nashville, TN 37232-2363, USA

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Abstract: An efficient method for solid-phase synthesis of peptide alkyl- and aryl amides is developed based on cleavage of peptide-thioester linker, HS-$(CH)_2$-CO-Nle, by a silver ion-amine complex. The metal ion-assisted acyl transfer reaction is usually completed in less than 1 h with high yields. This method is particularly suitable for preparing peptide aryl amides which are difficult to synthesize by other methods and also can be adopted for the combinatorial synthesis of nonpeptide amide libraries.

Peptides modified on the *C*-terminus are useful for enhancing biological potency as well as proteolytic stability. Furthermore, modifications of the *C*-terminus with a structural marker would allow these peptides to be used as probes in biological assays, chromogenic substances or enzyme substrates. These peptide substrates often contain arylamide bonds. Methods to amidate by the weakly nucleophilic aryl amines at the *C*-terminus by solid phase synthesis are limited because most resins for peptide synthesis are designed for preparing *C*-terminal acid or carboxamide.[1] Oxime resin, which contains an activated linkage, has been used for preparing several peptide alkyl and aryl amides.[2]

We have developed a method for the synthesis of various *C*-terminally amidated peptides on thioester resin[3]. Thioester linkage is relatively stable under standard Boc solid phase peptide synthesis conditions, and the thioester resin has been widely used to afford peptide thioesters for segment ligation.[4] Direct amidation of thioesters by nucleophilic alkyl amines at elevated temperature and under prolonged reaction conditions has been achieved.[5] However, milder conditions can be used based upon silver ion-assisted inter- or intramolecular aminolysis of thioesters. Such a method involves complexation of metal ions with the amine nitrogen atom and the sulfur atom of the thioester followed by *S,N*-acyl shift.[6a] By this mechanism, the thioester linker on a solid support can be displaced by a wide variety of amines, including weakly nucleophilic aryl amines, thereby providing a methodology to modify peptides more efficiently.

Peptides were synthesized on a newly developed thioester resin derived from aminomethyl-polystyrene resin.[7] The norleucine residue was incorporated as a spacer and also served as an internal standard for amino acid analysis. The S-trityl-3-mercaptopropionic acid moiety was coupled to the aminomethyl resin as a nondetachable linker.[8] After acidic removal of the S-trityl protecting groups, the mercapto moiety of the linker

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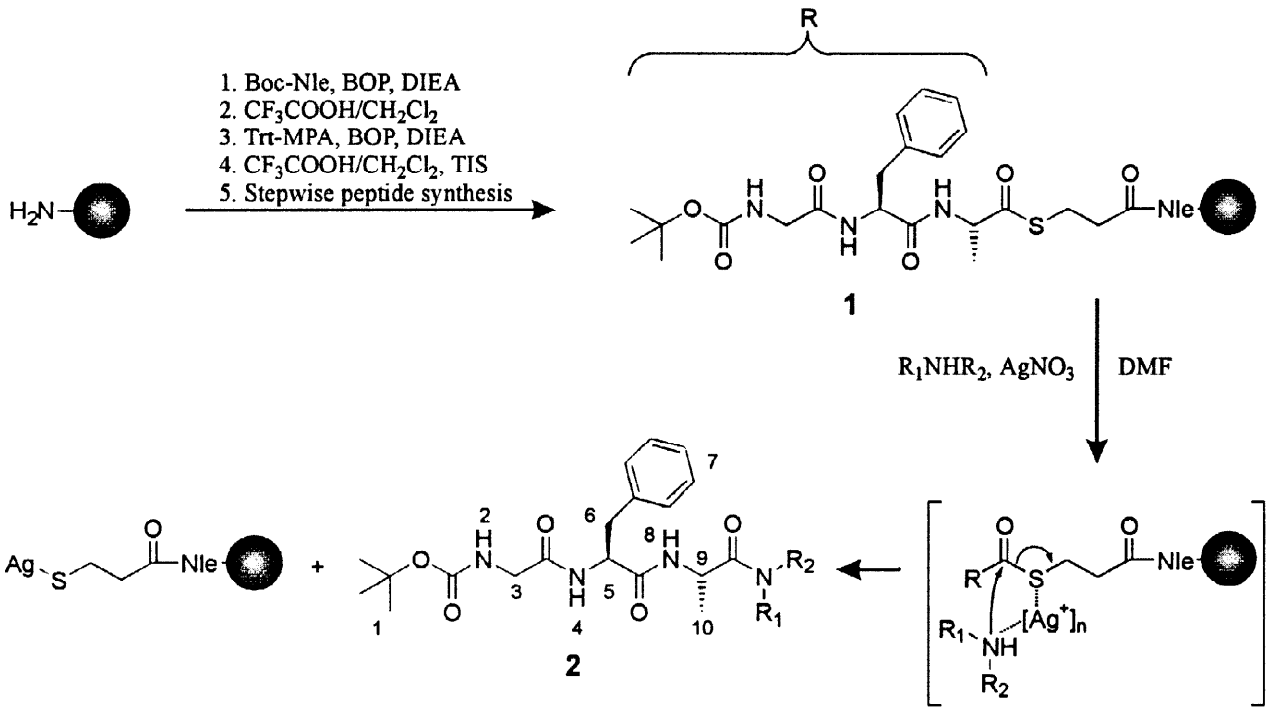


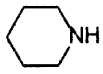
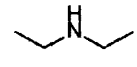
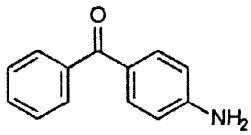
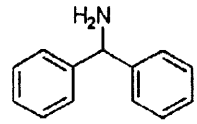
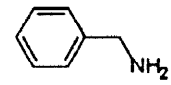
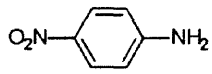
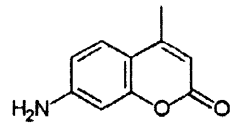
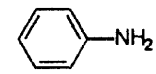
● - aminomethyl-polystyrene resin; BOP - Benzotriazole-1-yl-phosphonium-hexafluorophosphate; TIS - triisopropylsilane; MPA - 3-mercaptopropionic acid; DIEA - diisopropylethylamine

Scheme 1. On-resin synthesis of *C*-terminally amidated peptides from a thioester solid support

is sufficiently nucleophilic to affect complete thioesterification with Boc-amino acid BOP/HOBt in 1h. For the cleavage of the peptides and simultaneous amidation, the silver-amine complexes were typically used in excess to achieve improved reaction rates.[9] Under these conditions, acyl transfer reaction proceeds quickly in dimethylformamide, most reactions being 90 % complete within 1h with a purity greter than 50%. Notably, this method gives good amidation yields with weakly nucleophilic aryl amines. These compounds, **2d**, **2g** and **2h**, Table 1, are frequently used for labeling of peptides to be used in cross-linking experiments and for preparation of chromogenic or fluorogenic enzyme substrates.[10a-c] Classical solution amidation methodology typically requires base-catalyzed acylation and long reaction times due to the low reactivity of these aryl amines. With sterically hindered secondary amines, such as diphenylamine and dicyclohexylamine, low yields were obtained. Also, the reaction with 2-naphtylamine failed due to the low solubility of the silver-amine complex in DMF.

Racemisation of *C*-terminal amino acid residue is dependent on the basicity of the amines and the excess being used. For weak amines, **2d**, **2g**, **2h**, the reaction proceeds even in 10 fold excess without racemization of the *C*-terminal amino acid residue as determined by HPLC analysis of peptide and amino acid analysis and derivatization with Marfey's reagent.[11] For unhindered basic amines, **2a-2c**, racemization up to 45% was detected when a 3 fold-excess of amine over the silver salt was used. Decreasing the amine/$AgNO_3$ molar ratio to 1:1 helped to suppress the racemization to <5%. Racemization usually decreases in nonpolar solvents.

Table 1. Product yields according to HPLC analysis in the cleavage of the Boc-Gly-Phe-Ala-Mpa-Nle-aminomethyl resin **1** by the silver ion-amine complexes in DMF for 1h

Comp.	Amine	Yield % HPLC	Comp.	Amine	Yield % HPLC	Comp	Amine	Yield % HPLC
2a		97	**2b**		91	**2c**		92
2d		79	**2e**		82	**2f**		95
2g[12]		50	**2h**		75	**2i**		96

However, when the reaction was conducted in the CH_2Cl_2 using CF_3COOAg as a metal ion similar level of racemization was detected. Also, oxidation of aryl amines under these conditions was more severe, delivering colored solutions and higher levels of impurities in the crude product.

The importance of the silver-amine complex in improving the reaction rates was demonstrated by control experiments. Reactions performed with **2d-2i** at identical conditions did not afford any desired amidated products in 1h. The proposed mechanism of Ag^+-amine-thioester complexation and the subsequent *S,N*-acyl transfer has been well documented, and two equivalents of Ag^+ ions are generally required.[6a,b] The Ag^+- amine complex show the greatest stabilty in apolar solvents, such as CH_2Cl_2 under which cleavage/amidation occur nearly quantitative in <0.5 h. However, many Ag^+- amine complexes have poor solubility in CH_2Cl_2.

Our method requires that the amino functional group of the peptide resins be protected, thus an additional deprotection step is required at the completion of the synthesis. Recently, based upon the same principle, we have shown an on-resin cyclization of a peptides with a free α-amine which has been used for the synthesis of cyclic peptides with good yield and very low levels of oligomerization.[3]

In summary, we have demonstrated that the thioester linker can be efficiently displaced by alkyl and aryl amines by silver ion-assistance providing an efficient route for the synthesis of *C*-terminally amidated peptides on the solid phase. The method is compatible with the Boc-protocol and can be easily adopted for the synthesis of cyclic peptides and small-molecule amide libraries on solid support.

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7. S-trityl-3-mercaptopropionic acid and all the amino acids, except for the thioester bond formation, were coupled by dicyclohexylcarbodiimide/1-hydroxybenzotriazole activation using 2 equivalents of amino acids. For the thioester bond formation 3 equivalents of amino acid was coupled by using BOP/HOBt/DIEA activation for 1 h. After the reaction a small sample of the resin was removed and tested for the presence of mercapto groups with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) in DMF, containing 1% DIEA.

8. S-trityl-3-mercaptopropionic acid; 45 mmol (3.9 ml) 3-mercaptopropionic acid and 50 mmol (7 ml) triethylamine were dissolved in 150 ml CH_2Cl_2, followed by addition of 50 mmol (13.9g) of trityl chloride in 3 portions. The solution was mixed overnight and extracted with 4x60 ml 0.5M citric acid solution, 5x60 ml concentrated $NaHCO_3$ solution and dried over $MgSO_4$. The solvent was evaporated, residue dissolved in $CHCl_3$ and submitted to silica gel column chromatography. Yield 12.2g, 78%.

9. Typical procedure: 3 molar equivalents of amine and 3 equivalents of silver nitrate were dissolved in 100 µl DMF and this silver-amine complex solution was added to a 5 mg of peptide-resin (0.44mmol/g) and shaken for 1h (For the weakly nucleophilic amines, 4-nitroaniline, 7-amino-4-methylcoumarine and 4-aminobenzophenone 10 equivalents of amine and 3 equivalents of $AgNO_3$ were used). The resin was filtered and silver ions were precipitated by addition of 300 µl of methanol and 30 µl 2M NaCl solution. The AgCl precipitate was removed, solvent evaporated, residue dissolved in MeOH and submitted for HPLC analysis. Products were analysed by MALDI-TOF mass spectroscopy.

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12. **Compound 2g:** ^{1}H-NMR, 300 Mhz, (numbers in bold style indicate the ^{1}H atoms as marked on Scheme 1.) ($(CD_3)_2SO/TMS_{int}$); δ= 10.52 (s,1H, N<u>H</u>-C-$(CH)_2$), 8.46 (d, *J*=7Hz, 1H, **8**), 8.25 (d, *J*=9Hz, 2H, O_2N-$(C\underline{H})_2$), 8.01 (d, *J*=8Hz, 1H, **4**), 7.89 (d, *J*=9Hz, 2H, NH-C-$(C\underline{H})_2$), 7.16-7.26 (m, 5H, **7**), 6.97 (t, 2H *J*=6Hz, **2**), 4.53-4.58 (m, 1H, **6**), 4.42 (qt, 1H, *J*=7Hz, **9**), 3.56 (dd, 1H, *J*=4Hz & 13Hz, **3**) & 3.45 (dd, 1H, *J*=4Hz & 13Hz, **3**), 3.03 (dd, 1H, 4Hz & 14Hz, **6**) & 2.79 (dd, 1H, 9Hz & 14Hz, **6**), 1.37 (d, *J*=7 Hz, 3H, **10**), 1.35 (s, 9H, **1**).