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Selective Coupling at the α-Amino Group of Cysteine Using Transfer Active-ester-condensation Technology to Synthesize a Linear Octadecapeptide

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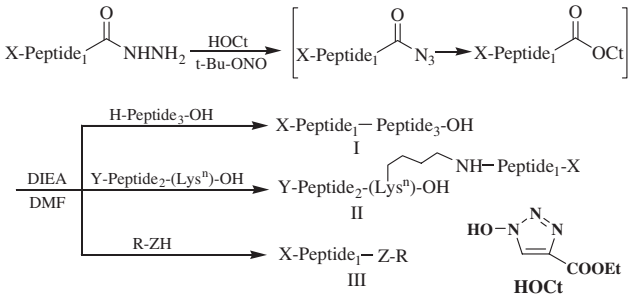
The peptide segment Fmoc-Ubiquitin$^{67–76}$-$NHNH_2$ was converted to the active ester Fmoc-Ubiquitin$^{67–76}$-OCt and reacted with an unprotected seven residue fragment (H-mNGF$^{110–117}$-OH) from murine Nerve Growth Factor, which contains a Cys at the N-terminus and a free ε-amino group at Lys^{115}, using transfer active-ester-condensation (TAEC) technology. The linear product H-Ubiquitin$^{67–76}$-murine Nerve Growth Factor$^{110–117}$-OH was formed exclusively through the native chemical ligation mechanism.

Transfer active-ester-condensation (TAEC) technology has previously been applied to peptide segment coupling in the synthesis of long chain peptides and branched peptides.[1,2] It is also used for the preparation of peptide C-terminal derivatives such as peptide thioesters, *N*-methyl-*N*-methoxyl amides and trifluoroethyl esters (Scheme 1).[3] Besides maintaining the advantageous features of the traditional azide method with respect to the use of minimal side chain protection in the segment condensation, TAEC enables high coupling yield, fast reaction rate under mild conditions, and can circumvent the problems faced in the azide approach, such as the requirement of low temperature, the limitation on solvent choice, low yield, and strong acid conditions.[4]

In TAEC, the peptide 1-hydroxy-4-ethoxycarbonyl-1,2,3-triazole (HOCt)[5] active ester (peptide-OCt), prepared from the related hydrazide through an azide intermediate, plays a key role in the reaction (Scheme 1). When the peptide-OCt reacts with an unprotected peptide segment containing His, Arg, Ser, and Glu, it couples selectively with the free $–NH_2$ and not with other potentially reactive side chain groups such as $–NHC(NH_2)=NH$, =NH, –OH, and –COOH.

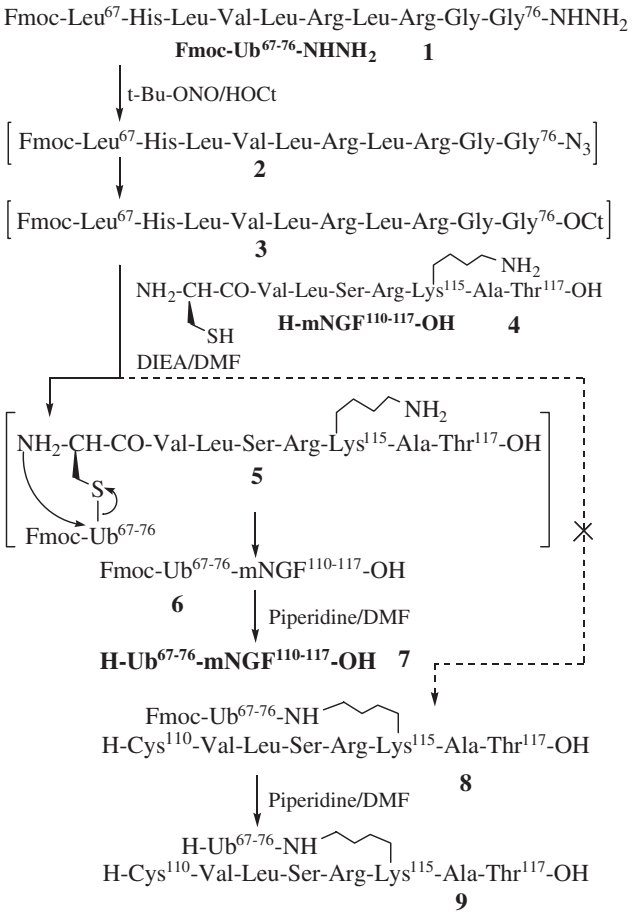
When the reagents R–SH and R–NH_2 react with the peptide-OCt respectively to make the related peptide C-terminal derivatives, the speed for the formation of peptide thioester is much faster than that of peptide amide. This phenomenon implies that besides the reaction selectivity between –NH_2 and other functional groups, the peptide-OCt should possess coupling selectivity between –SH and –NH_2 groups under TAEC conditions. If a peptide fragment contains a Cys at the N-terminus as well as other free –NH_2, the peptide-OCt might couple with the N-terminal Cys through the native chemical ligation mechanism.[6]



X, Y = Protecting group at N-terminus in the peptide fragments; -ZH = -SH, -NHOMe, -NH_2, -OH; Lys^n = Lys located at position n (n = 1, 2, 3...) in $Peptide_2$; Arg, His, Ser, Glu in $Peptide_1$, $Peptide_2$ and $Peptide_3$ did not need protection in TAEC; I = long chain peptide; II = branch peptide; III = peptide C-terminal derivatives.

Scheme 1. The general route of TAEC.

In this paper, the peptide H-Ubiquitin$^{67–76}$-murine Nerve Growth Factor$^{110–117}$-OH (H-Ub$^{67–76}$-mNGF$^{110–117}$-OH, Scheme 2, **7**) was chosen as the synthetic target, which contains a C-terminal decapeptide (H-Ub$^{67–76}$-OH) derived from ubiquitin (Ub)[7] and a seven residue fragment (H-mNGF$^{110–117}$-OH) from murine Nerve Growth Factor (mNGF).[8] H-mNGF$^{110–117}$-OH (Scheme 2, **4**), which contains a Cys^{110} at the N-terminus



Scheme 2. Synthesis of H-Ub$^{67–76}$-mNGF$^{110–117}$-OH by TAEC method.

Table 1. Analytical data on peptide segments and coupling products in Scheme 2

No.	HPLC /min	MS *m/z*	Calculated *m/z*
1[a]	23.6	1369.5	1369.67
4[a]	12.2	876.5	877.06
6 (**8**)	22.8	2214.4	2214.70
7 (**9**)[b,c]	10.8	1993.7	1992.45

[a]Synthesized with Fmoc SPPS. [b]Purified with HPLC, >99% purity in HPLC analysis. [c]AA analysis (Thr_1 0.88, Ser_1 0.83, Gly_2 2.16, Ala_1 1.00, Cys_1 0.45, Val_2 2.08, Leu_5 4.96, His_1 1.12, Lys_1 0.98, and Arg_3 3.12).

and an ε-NH_2 on the side chain of Lys^{115}, should be a good model to evaluate the selectivity of peptide-OCt coupling between the –SH of the N-terminal Cys and the amino groups under TAEC conditions.

The synthesis of H-$Ub^{67–76}$-$mNGF^{110–117}$-OH (**7**) is shown in Scheme 2. Fmoc-$Ub^{67–76}$-$NHNH_2$ (**1**), synthesized by Fmoc solid phase peptide, was converted to the related active ester Fmoc-$Ub^{67–76}$-OCt (**3**) through the azide intermediate **2** using *t*-Bu-ONO/HOCt in DMF. When the peptide H-$mNGF^{110–117}$-OH (**4**) reacted with **3**, a coupled product was isolated with 75% yield. FAB-MS (*m/z* 2214.4) of the product confirmed that the two peptide fragments had coupled in a 1:1 ratio. After removal of the Fmoc group with piperidine/DMF, the final product was separated by HPLC, and characterized by both FAB-MS (*m/z* 1993.7) and amino acid (AA) analysis (Table 1).

In Scheme 2, even though **4** contained two potentially reactive free amino groups, i.e., α-NH_2 on the N-terminal Cys^{110} and ε-NH_2 on the side chain of Lys^{115}, the analytical data in Table 1 showed that only one Fmoc-$Ub^{67–76}$-OH (**1**) coupled with one H-$mNGF^{110–117}$-OH (**4**), and the coupling product could be **6** or **8**, and the final product could be the linear peptide **7** or the branched peptide **9**. The analytical results shown in Table 1 were consistent with formation of either **7** or **9**, or a mixture of them, although the latter possibility seemed unlikely since the product appeared homogeneous under a number of different HPLC conditions.

Identification of the product was carried out by N-terminal sequencing. The results (Table 2) showed that the N-terminal AA residue was Leu exclusively rather than Leu and Cys since the quantity of Leu was 1028.60 picomole, while that of Cys was 1.74 picomole only; and the second AA was His (106.13 picomole) rather than Val (0.13 picomole).[9] If the final synthetic peptide was **9** or a mixture of **7** and **9**, both Leu and Cys should be found in similar amount as the N-terminal residues, and His and Val should be in similar amount as the second residues. Therefore, the structure of the final product was H-$Ub^{67–76}$-$mNGF^{110–117}$-OH (**7**).

During the reaction shown in Scheme 2, the thioester intermediate **5** was produced preferentially because the –SH group on Cys^{110} possessed much stronger reactivity under TAEC conditions than –NH_2, then the α-NH_2 on Cys^{110} replaced the –SH by formation of the stable amide bond. Both the ortho effect and the formation of a pentacyclic transition state between the –SH and the α-NH_2 on Cys^{110} could benefit the formation of **6** rather than **8**, and produced the single product **7** rather than the mixture of **7** and **9**. Thus, the linear product **7** was formed exclusively via the intermediate **5** through the native chemical ligation mechanism (Scheme 2).[6]

Table 2. *N*-terminal AA analysis results of the final product H-$Ub^{67–76}$-$mNGF^{110–117}$-OH (**7**)[a]

N-terminal AA	*C* /min	R_1 /min	R_2 /min	Q_1 /pmol	Q_2 /pmol
Leu	19.42	19.48	19.13	**1028.60**	0.11
Cys	13.50	13.52	13.37	1.74	6.98
His	8.57	8.48	8.55	0.34	**106.13**
Val	14.83	14.88	14.55	2.52	0.13

[a]Applied Biosystems 477A Protein Sequencer Chromatogram equipment was used; *C*: Retention time of standard AA; R_1, R_2: Retention time of the AA cleaved from the N-terminus of the product **7** in the first and second cycle respectively; Q_1, Q_2: N-terminal AA quantity of the product **7** in the first and second cycle respectively.

The target linear peptide H-$Ub^{67–76}$-$mNGF^{110–117}$-OH was synthesized using TAEC technology successfully. By combining the features of the Fmoc-peptide-OCt active ester and the high reactivity of the –SH on the N-terminal Cys together, TAEC could control peptide fragment condensation at the α-NH_2 of the N-terminal Cys selectively and exclusively through the native chemical ligation mechanism even though the peptide fragment contained other amino groups. The principle established in this work may be a valuable precedent for future work in peptide and protein synthesis.

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References and Notes

1 P. Wang, R. Layfield, M. Landon, R. J. Mayer, R. Ramage, *Tetrahedron Lett.* **1998**, *39*, 8711.
2 P. Wang, R. Ramage, R. Layfield, M. Landon, R. J. Mayer, *J. Pept. Res.* **1999**, *53*, 673.
3 P. Wang, K. T. Shaw, B. Whigham, R. Ramage, *Tetrahedron Lett.* **1998**, *39*, 8719.
4 a) J. Meienhofer, in *The Peptides Analysis, Synthesis, Biology*, ed. by E. Gross, J. Meienhofer, Academic Press, New York, **1979**, Vol. 1, pp. 197–239. b) Y. S. Klausner, M. Bodanszky, *Synthesis* **1974**, 549.
5 a) L. Jiang, A. Davison, G. Tennant, R. Ramage, *Tetrahedron* **1998**, *54*, 14233. b) N. Robertson, L. Jiang, R. Ramage, *Tetrahedron* **1999**, *55*, 2713.
6 a) P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776. b) S. Aimoto, *Biopolymers (Pept. Sci.)* **1999**, *51*, 247. c) J. P. Tam, J. Xu, K. D. Eom, *Biopolymers (Pept. Sci.)* **2001**, *60*, 194. d) Y. Ma, Y. Zhao, *Chin. Sci. Bull.* **2003**, *48*, 1.
7 W. M. Bonner, C. L. Hatch, R. S. Wu, in *Ubiquitin*, ed. by M. Rechsteiner, Plenum, New York, **1988**, pp. 157–172; D. J. Katzmann, G. Odorizzi, S. D. Emr, *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 893.
8 A. M. Colangelo, N. Finotti, M. Ceriani, L. Alberghina, E. Martegani, L. Aloe, L. Lenzi, R. Levi-Montalcini, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 18658.
9 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

