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Photolabile Protection for One-Pot Sequential Native Chemical Ligation

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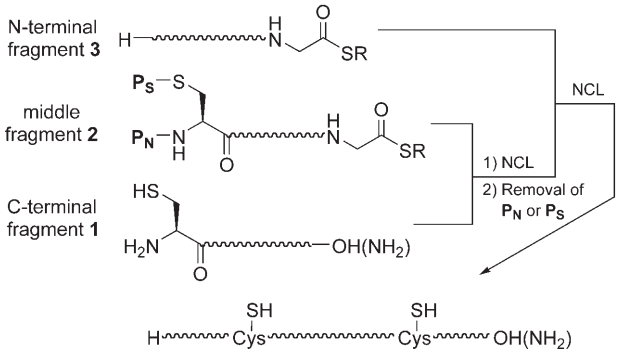
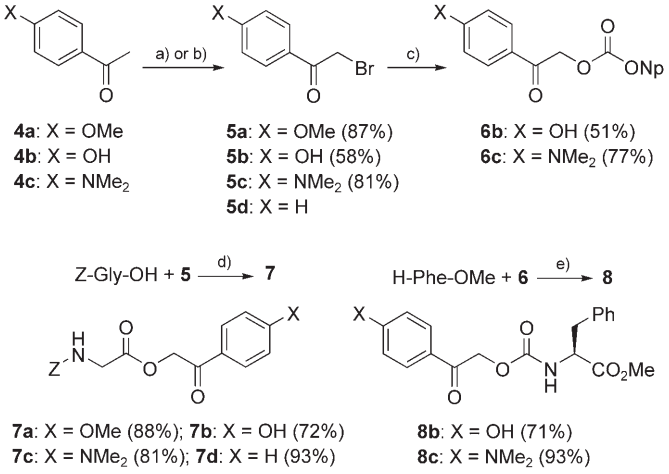
The strategy of native chemical ligation (NCL) has widespread application in the chemical synthesis of proteins.[1] This methodology features chemoselective amide formation between a thioester and an N-terminal cysteine through attack of the Cys sulfhydryl group on the thioester followed by intramolecular S-N acyl migration. Sequential NCL with more than one thioester fragment could potentially be an attractive method for constructing protein molecules (Scheme 1).[2,3] In this strategy, protection of the amino and/or sulfhydryl groups of the N-terminal Cys residue of the middle fragment **2** is needed to avoid the formation of undesired products such as cyclic peptides.[4] Recently, Kent's group reported an elegant one-pot sequential NCL approach for peptide synthesis in a purification-free manner using a thiazolidine derivative as N/S-protected Cys, which regenerates the Cys residues by the action of methoxyamine·hydrochloride.[5] The use of photolabile protecting groups in conjunction with acid- or base-sensitive groups provides a powerful means for selective manipulation of functional groups in synthetic chemistry.[6] Thus, we envisioned that a photolabile moiety should also serve as a useful protection for either the N^{α}-amino or the sulfhydryl group in a sequential NCL strategy, in which simple photoirradiation would probably allow consecutive NCL steps to be conducted in one-pot. Here we report the development of novel phenacyl-type photolabile protecting groups and their application to a purification-free one-pot sequential NCL strategy.



Scheme 1. Synthetic strategy for the preparation of peptides/proteins by using sequential native chemical ligation (NCL). Protection of the amino (P_N) or sulfhydryl (P_S) group in fragment **2** is necessary for the sequential NCL strategy.

Among several photoremovable groups, *o*-nitrobenzyl-type protecting groups have been shown to be useful in peptide chemistry.[7] However, such photodeprotection on an N-terminal Cys residue proceeds by a Norrish type II mechanism to release the N/S-unprotected Cys and an aldehyde, which then recombine to form an imine or thiazolidine moiety that fails to show the desired reactivity in the following NCL step. Photolabile phenacyl-type protection is a potential alternative to using nitrobenzyl groups due to their photosolvolytic character. Phenacyl-type groups with methoxy[8,9] or hydroxyl[10] substituents have received attention as photosensitive groups, in which the electron-donating substituents are responsible for the rapid release of parent molecules. This prompted us to examine the feasibility of $-CO_2H$ or $-NH_2$ protection of phenacyl-type groups with a more electron-donating *p*-dimethylamino substituent and the applicability of such protecting groups to one-pot sequential NCL strategies.

First, we evaluated the usefulness of 4-(dimethylamino)phenacyl (Map) esters. The requisite carboxylates **7** were prepared as shown in Scheme 2. Photolysis of **7** was conducted in EtOH by using a 100 W high-pressure Hg lamp ($h\nu > 300$ nm) at a concentration of 1 mM. Deprotection profiles of **7** are summarized in Table 1. Photolysis of the novel Map ester **7c** proceeded efficiently to complete the release of Z-Gly-OH at a rate two times faster than 4-hydroxyphenacyl ester **7b**, which has great potential as a "photo-cage". Although the ester **7c** did not show sufficient stability toward 20% piperidine in DMF, this



4a: X = OMe
4b: X = OH
4c: X = NMe_2

5a: X = OMe (87%)
5b: X = OH (58%)
5c: X = NMe_2 (81%)
5d: X = H

6b: X = OH (51%)
6c: X = NMe_2 (77%)

7a: X = OMe (88%); **7b**: X = OH (72%)
7c: X = NMe_2 (81%); **7d**: X = H (93%)

8b: X = OH (71%)
8c: X = NMe_2 (93%)

Scheme 2. Syntheses of protecting agents **5** and **6** and protected amino acid derivatives **7** and **8**: a) $CuBr_2$, EtOAc, reflux for **5a** and **5b**; b) Br_2, conc. H_2SO_4, then $(EtO)_2P(O)H$, Et_3N for **5c**; c) HCO_2Na, EtOH, reflux, then *p*-nitrophenylchloroformate, pyridine, CH_2Cl_2; d) 1,8-diazabicyclo[5.4.0]undec-7-ene, benzene; e) Et_3N, DMF for **8b**; Et_3N, pyridine for **8c**.

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Table 1. Photoinduced-deprotection of protected amino acid derivatives (**7** and **8**)

	Substrate (*p*-substituent)	Photolysis [*t*/min][a]	Yield [%][b]	$t^{1/2}$ [min][c]
1	**7a** (OMe)	90	22	n.d.[d]
2	**7b** (OH)	30	96	11
3	**7c** (NMe_2)	7	97	5.3
4	**7d** (H)	90	11	n.d.[d]
5	**8b** (OH)	70	93	19
6	**8c** (NMe_2)	20	95	7.5

[a] Each sample solution in EtOH (1 mM) was irradiated with a 100 W high-pressure Hg lamp ($h\nu > 300$ nm) at room temperature. [b] Determined by measurements of regenerated amino acid derivatives (Z-Gly-OH for **7** and H-Phe-OMe for **8**) by using reversed-phase HPLC analyses. [c] Determined by the disappearance of **7** or **8** by using HPLC analyses. [d] Not determined.

group could be used in the design of caged compounds as well as **7b**.

Next, we investigated the feasibility of the 4-(dimethylamino)phenacyloxycarbonyl (Mapoc) moiety for the protection of amines. To our knowledge, carbamates including the 4-hydroxyphenacyl unit have yet to be examined well for this purpose.[11] Phenylalanine-derived carbamates (**8b** and **8c**) were prepared according to the Scheme 2. Treatment of phenacyl bromides **5b** and **5c** with sodium formate followed by conversion to carbonate afforded phenacylcarbonates **6**. Treatment of H-Phe-OMe with **6** yielded *N*-protected derivatives **8**. Deprotection profiles of **8** under photoirradiation are also summarized in Table 1. Although the photolysis of **8b** or **8c** proceeded slowly as compared with that of the corresponding esters **7b** or **7c**, regenerated H-Phe-OMe was quantitatively recovered within 70 or 20 min, respectively. Mapoc carbamate **8c** also released the amine faster than **8b** did. Additionally, the Mapoc group was found to remain intact during acidic (TFA) or basic (20% piperidine in DMF) treatments commonly used in peptide chemistry. Furthermore, the half-life of the Map or Mapoc group in EtOH in normal daylight was 5.1 or 7.0 days, respectively; this shows that Map- or Mapoc-protected peptide can remain almost intact during usual experimental manipulations.

The utility of the Mapoc group in NCL was confirmed by its use in the syntheses of cyclic peptide **12** and a 32-residue human-brain natriuretic peptide (hBNP-32 **17**; Figures 1 and 2).[12] A "phototriggered intramolecular NCL" strategy was used for the preparation of **12**. As a model peptide, we chose a 12-mer peptide sequence (CSEFENEIIKQG) derived from the third extracellular loop region of the CXCR4-chemokine receptor.[13] The N^{α}-Mapoc thioester **9** was synthesized by Fmoc-based solid-phase protocols on a sulfonamide safety-catch linker.[14] The N^{α}-Mapoc group was introduced at the end cycle of the solid-phase protocol by using **6c** (5 equiv) in the presence of DIPEA (5 equiv). Completion of the protection with the Mapoc group was confirmed by a Kaiser ninhydrin test.[15] The protected resin was subjected to consecutive treatment for release of a thioester with iodoacetonitrile and with ethyl-3-mercaptopropionate to afford a fully protected thioester. TFA treatment of the protected thioester gave the N^{α}-Mapoc thioester **9**, which

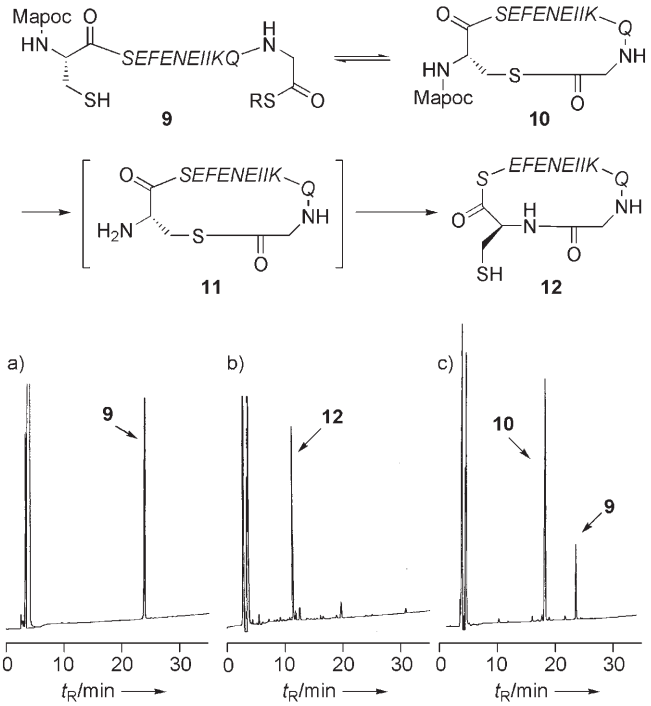


Figure 1. HPLC monitoring of phototriggered intramolecular NCL for the synthesis of cyclic peptide **12**: a) HPLC-purified **9**; b) phototriggered NCL ($t = 20$ min): **9** (0.2 mM) was photoirradiated at 25 °C in phosphate buffer (pH 7.6) containing guanidine·HCl (6 M) in the presence of 1% (*w*/*v*) sodium mercaptoethanesulfonate; c) Purified peptide **9** was dissolved in phosphate buffer (pH 7.6) containing guanidine·HCl (6 M) without photoirradiation. HPLC conditions: Cosmosil $5C_{18}$ARII column (4.6×250 mm) with a linear gradient of MeCN/0.1% aq. TFA (20:80–80:20 over 50 min) at a flow rate of 1.0 mL min^{-1}, detection at 220 nm. Generally, compound **11** is thought to be a real intermediate; however, it is rapidly converted to compound **12**. Since we cannot detect compound **11** by HPLC, it is shown in brackets.

was dissolved in phosphate buffer (pH 7.6) containing 6 M guanidine hydrochloride and 1% (*w*/*v*) sodium mercaptoethanesulfonate at a concentration of 0.2 mM. Photolysis (> 300 nm, 20 min) triggered an intramolecular NCL reaction to yield the desired cyclic peptide **12** in high purity. Without UV irradiation, no ligation product was obtained and reversible thiol **9**⇌thioester **10** exchange was observed.

Being encouraged by these results, we applied Mapoc protection to a one-pot peptide-chain assembly of hBNP-32 utilizing a sequential NCL strategy followed by disulfide-bond formation with DMSO. As shown in Scheme 3, hBNP-32 has two Gly-Cys sequences, and these Cys residues are connected to each other by a disulfide bond. Therefore, we prepared three peptide fragments consisting of a C-terminal segment **13**, a middle segment **14**, and an N-terminal segment **15** by Fmoc protocols. Synthesis of thioesters **14** and **15** was achieved by trithio-ortho ester methodology, in which a Gly trithio ester was anchored through a backbone amide linker (BAL) to a solid support.[16] Application of standard Fmoc protocols efficiently afforded the peptide thioesters. For the middle fragment **14**, the completed peptide resin with an unprotected N^{α}-amino group was treated with **6c** (5 equiv) in the presence of DIPEA (5 equiv). After 12 h reaction, a Kaiser test indicated that no free N^{α}-amino group remained on the resin. Treatment of

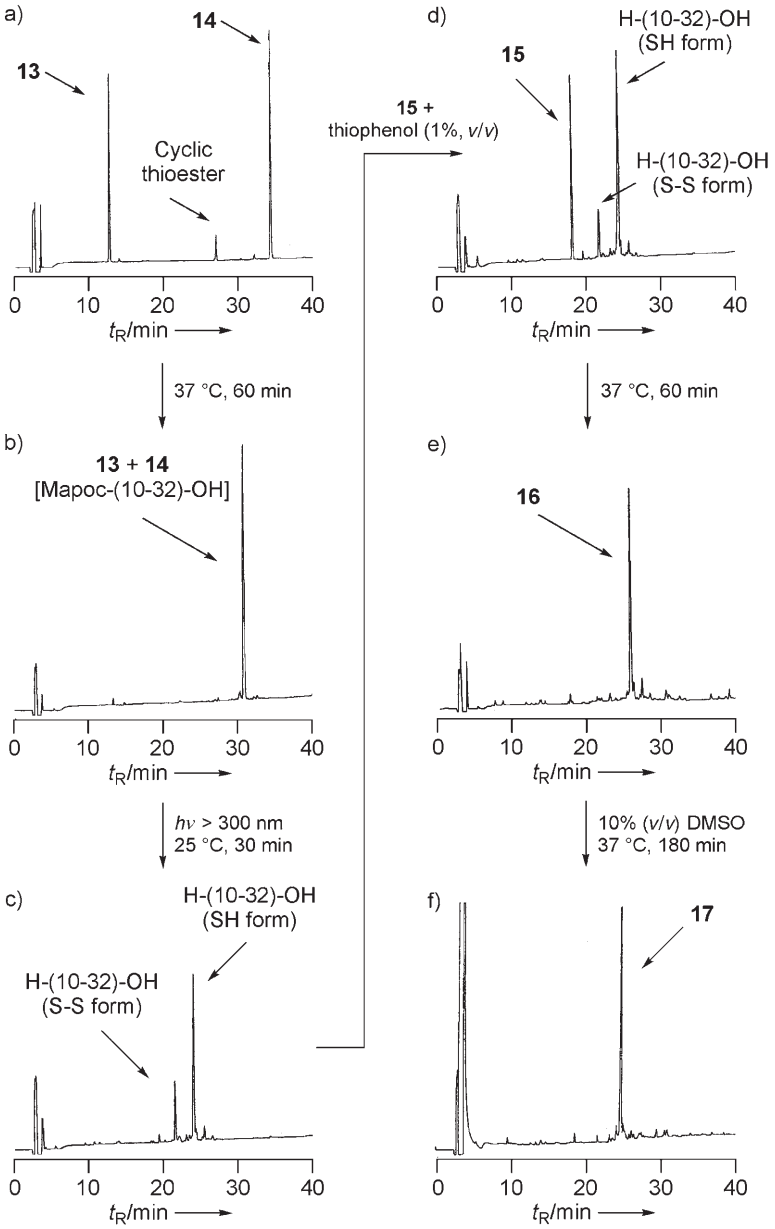
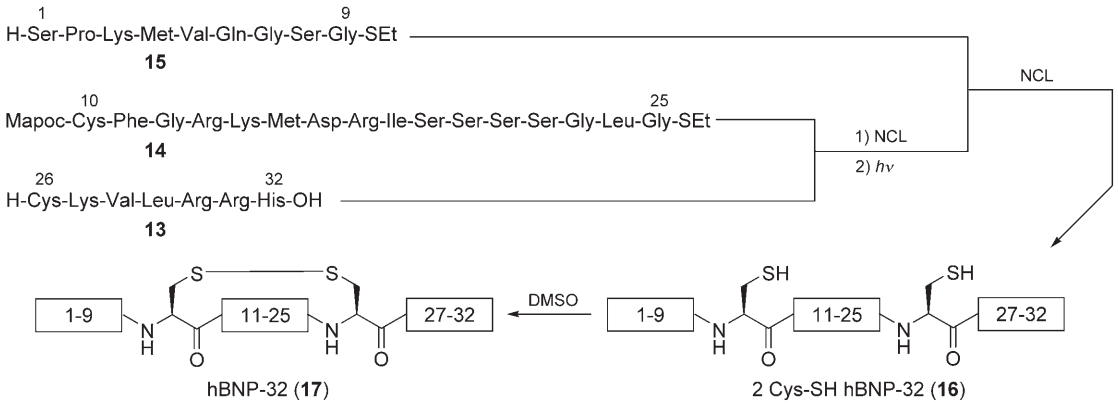


Figure 2. HPLC monitoring of one-pot sequential NCL reactions followed by disulfide-bond formation for the synthesis of hBNP-32 (**17**). a) The 1st NCL ($t = 0$ min): fragments **13** (1 mM) and **14** (1 mM) were ligated in phosphate buffer (pH 7.6) containing guanidine·HCl (6 M) in the presence of thiophenol (0.3%, *v/v*). b) The 1st NCL ($t = 60$ min). c) Photoinduced deprotection of the 1st NCL product ($t = 30$ min); the ligated product was photoirradiated ($h\nu > 300$ nm) in buffer for ligation. d) the 2nd NCL ($t = 0$ min): fragment **15** (1 mM) was coupled with the deprotected peptide in the presence of additional thiophenol (1%). e) the 2nd NCL ($t = 60$ min). f) formation of the disulfide bridge ($t = 180$ min); DMSO was added (10% *v/v*) to the ligation reaction mixture (0.33 mM peptide). HPLC conditions: Cosmosil $5C_{18}$ARII column (4.6×250 mm) with a linear gradient of MeCN/0.1% aq TFA (5:95–45:55 over 40 min) at a flow rate of 1.0 mL min^{-1}, detection at 220 nm.

the resulting resin with a TFA-based deprotection reagent yielded the N^{α}-Mapoc thioester **14**. Sequential NCL reactions were achieved in one pot without purification of the ligated intermediates (Figure 2). The initial ligation between the middle fragment **14** and the C-terminal fragment **13** was carried out in the presence of 0.3% (*v/v*) thiophenol in phosphate buffer (pH 7.6) containing 6 M guanidine hydrochloride at 37 °C for 1 h. The reaction proceeded quantitatively to yield the N^{α}-Mapoc ligated product (**13** + **14**, Figure 2a and b). Without purification, the coupled product was subjected to photoirradiation at 25 °C for 30 min (Figure 2c). This was followed by the second ligation with thioester **15** for 1 h in the presence of additional thiophenol (1% *v/v*) to afford the linear 2-Cys-SH hBNP-32 (**16**) with satisfactory purity (Figure 2d and e). Monitoring of the reaction progress by HPLC indicated that this sequence of reactions went to completion without any significant accompanying side reactions. The peptide solution was diluted threefold with phosphate buffer, followed by the addition of DMSO (10% *v/v*) to yield the disulfide-bridged hBNP-32 (**17**, Figure 2f).[17] HPLC purification of the crude material afforded purified **17** in 56% yield as calcu-



Scheme 3. Synthetic scheme for the preparation of hBNP-32 (**17**) by utilizing a one-pot sequential NCL followed by disulfide-bond formation with DMSO.

lated from peptide fragments employed in the sequential NCL reactions.

In this communication, we have presented novel 4-(dimethylamino)phenacyl-type photolabile protecting groups (Map and Mapoc) and demonstrated their unequivocal utility in peptide synthesis. In particular, the Mapoc unit represents a photosensitive protection group for amines that exhibits adequate stability against the acidic (TFA) and basic (20% piperidine/DMF) reagents commonly used in peptide chemistry. When used in conjunction with NCL reactions, the Mapoc group can be used for phototriggered intramolecular cyclizations. One-pot sequential NCL reactions can also be readily achieved. It is noteworthy that the one-pot synthesis presented herein could be conducted without either purification of ligated intermediates or readjustment of ligation conditions, such as pH. Finally, we believe the use of photolabile 4-(dimethylamino)phenacyl-type protection provides an efficient protocol for the synthesis of Cys-containing peptides/proteins and represents an alternative to Kent's thiazolidine-mediated approach. Extension of our methods to the preparation of proteins will be presented in due course.

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Keywords: native chemical ligation · peptides · proteins · synthesis design · thioesters

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