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Postsynthetic Modification of Unprotected Peptides via *S*-Tritylation Reaction

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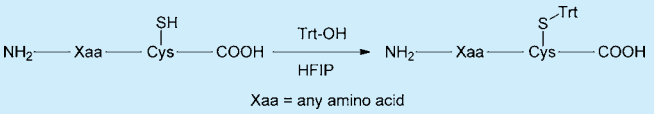
Supporting Information

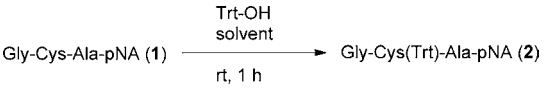
ABSTRACT: Tritylation using trityl alcohol (Trt−OH) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) is a convenient and efficient procedure that can offer *S*-protection of the Cys located in fully unprotected peptides. The procedure simply requires Trt−OH and HFIP to selectively promote *S*-tritylation in the presence of peptide nucleophilic functionalities.



The native chemical ligation (NCL) reaction is a useful and powerful addition to the peptide chemist's tools for synthesizing large peptides or proteins.[1] In NCL, the sulfhydryl group of *N*-terminal Cys residues serves to capture a peptide thioester and is involved in a subsequent intramolecular *S*−*N* acyl transfer to generate a native peptide bond. However, there is a limitation to the applicability of NCL; i.e. Cys must be present at the ligation site (Xaa-Cys, Xaa: any amino acid), although it is a relatively rare amino acid in nature. A ligation−desulfurization approach[2] using a thiolated building block, i.e. α, β, and γ thiol-derived amino acids, enables us to extend the repertoire of accessible ligation sites, for example, Xaa-Ala, Xaa-Phe, Xaa-Lys, or Xaa-Val.[3] In order to achieve chemoselective desulfurization of the thiolated building block at the ligation site, the other sulfhydryl group(s) of Cys, if any, in the ligated molecule must be protected. Thus, desulfurization in the presence of Cys is performed by resorting to the use of orthogonal protecting groups on Cys. For this purpose the acetamidomethyl (Acm) group is frequently employed in both Boc and Fmoc strategies.[4] However, Cys(Acm) has to be incorporated beforehand into peptide segments which are scheduled for the subsequent NCL reactions. This leads to narrowing the availability of ligation sites located in target molecules because Cys(Acm) is no longer able to participate in NCL. Therefore, if a protecting group is selectively introduced into the sulfhydryl function of Cys, which has already fulfilled its role in NCL, the accessibility of ligation sites can be retained. In numerous conjugation procedures, the chemical modification of the sulfhydryl group on Cys is often achieved via nucleophilic substitution or Michael addition by using haloacetyl or maleimide reagents, respectively.[5] On the other hand, there is little in the literature dealing with the introduction of a protecting group into the Cys sulfhydryl group(s) located in a fully unprotected peptide, where alkyl halides such as 4-methylbenzyl chloride or benzyl bromide were employed in the presence of tertiary amines[6] or molecular sieves[7] as bases, respectively. Besides *S*-alkylation, these methods are known to be accompanied by an undesired alkylation of unprotected functional groups, especially that of the amino function.[8]

In the present study, we tried to chemoselectively introduce the Trt group into Cys sulfhydryl groups by using trityl alcohol (Trt−OH) in an appropriate solvent. First, the utility of trifluoroacetic acid (TFA) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) as a reaction solvent generating an active species, a Trt cation, was examined by employing a model peptide, Gly-Cys-Ala-*p*NA (*p*NA, *p*-nitroaniline). The procedure simply constituted dissolving the model peptide in TFA or HFIP and then adding Trt−OH into the solution. The mixture was stirred at room temperature for 1 h. The results were quantified using the area percent of absorbance of each compound at 340 nm (Table 1, Table S1).

Table 1. Efficiency of the *S*-Tritylation Reaction in Different Solvents



entry	solvent	Trt−OH (equiv)	yield[a]
1	HFIP	1.1	96.6
2	HFIP	3.0	98.0
3	TFA	1.1	61.4
4	TFA	3.0	87.7

[a]Yields were determined by RP-HPLC (340 nm).

In both solvent systems, no modification with the amino function was observed even upon adding an excess of Trt−OH (3.0 equiv). When using 1.1 equiv of Trt−OH, *S*-tritylation in HFIP was almost quantitatively completed in 1 h, while that in TFA did not lead to completion even when the reaction time was extended. This was probably due to the equilibrium between *S*-tritylation and de-Trt in TFA, as an excess of Trt−

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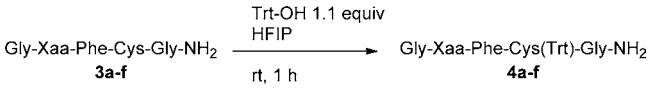
OH (3.0 equiv) in TFA could displace the balance of the reaction in favor of *S*-tritylation. To drive the reaction in TFA to end point, therefore, a large excess of Trt−OH or removal/dilution of TFA is required.[9] Thus, the yield evaluation of the reaction mixture in TFA had to be done after removal of TFA, while that in HFIP could be directly performed using RP-HPLC. In view of this, we concluded that HFIP possesses adequate acidity to produce the Trt cation and not cleave the Trt group on Cys. In addition to *S*-tritylation on Cys, the Trt cation is also liable to react with and modify those amino acids containing electron-rich functional groups such as Tyr, Trp, Met, and His, although it is expected to act as a weak, nonaggressive, electrophile due to its steric hindrance.

Next, we investigated the sulfhydryl reactivity in the presence of other nucleophilic functional groups on model peptides having susceptible amino acids listed in Table 2. Peptides were treated using Trt−OH (1.1 equiv) in HFIP to alkylate the Cys sulfhydryl group in the respective sequences at room temperature for 1 h (Table 2, Table S2).

Table 2. Efficiency of the *S*-Tritylation Reaction in the Presence of Peptide Nucleophilic Functionalities



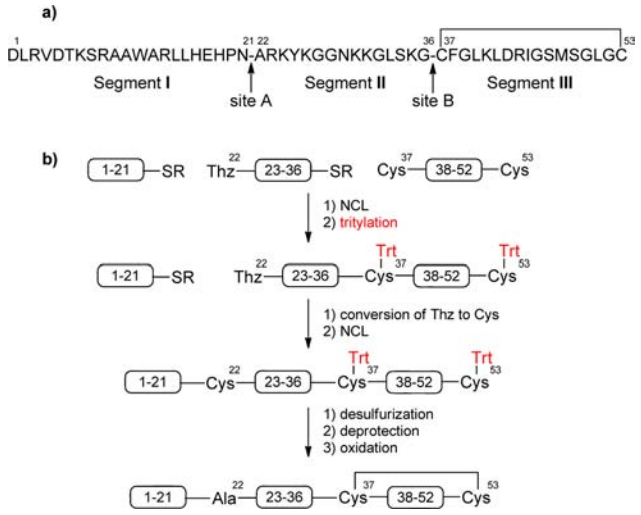
entry	peptide **3**	Xaa	yield[a]	Di-Trt[b]
1	**3a**	Trp	96.2	0.9
2	**3b**	Tyr	97.5	–[c]
3	**3c**	Ser	96.3	–
4	**3d**	His	94.2	2.4
5[d]	**3d**	His	91.3	–
6	**3e**	Glu	96.0	–
7	**3f**	Met	97.9	–

[a]Yields were determined by RP-HPLC (220 nm). [b]Di-Trt represents Gly-Xaa(Trt)-Phe-Cys(Trt)-Gly-NH_2. [c]–: Not detectable. [d]This reaction was performed using Trt−OH (3.0 equiv) in TFA.

As had been expected, *S*-tritylation proceeded smoothly without any significant modification on the susceptible residues except that on His. However, this was of no matter as the Trt group was found to be introduced into the τ-nitrogen of the imidazole moiety of His. This byproduct could be readily converted to the intact His by treatment with TFA. In fact, it was not produced when tritylation was performed in TFA. From these results, we were able to confirm that tritylation using Trt−OH in HFIP is a convenient and efficient procedure that can offer *S*-protection for Cys located in fully unprotected peptides without any side reactions.

The utility of tritylation using Trt−OH in HFIP was demonstrated by synthesizing rat C-type natriuretic peptide-53 (CNP-53).[10] In the stepwise SPPS, the desired peptides are always contaminated more or less by amino acid deletion and terminated products which are extremely difficult to separate, especially when the size of the peptides increases beyond 40−50 amino acid residues. In contrast, the segment condensation strategy by NCL is one of the most promising procedures for excluding such side products because assembly of the entire molecule can be carried out with purified and well-characterized segments. Thus, CNP-53 was divided into three segments **I**, **II**, and **III**, in the range of around 20 amino acid residues, corresponding to (1−21), (22−36), and (37−53), respectively (Scheme 1).

Scheme 1. (a) Structure of Rat CNP-53; (b) Synthetic Strategy for Rat CNP-53



When performing NCL, the Cys residue is required at the ligation site. In the target molecule of CNP-53, however, only one Cys residue located at position 37 in segment **III** is available for NCL at site B (Gly^{36}-Cys^{37}). To perform the peptide-chain assembly at site A (Asn^{21}-Ala^{22}) by Ala ligation involving a ligation−desulfurization approach, a Cys residue was introduced at position 22 instead of Ala in segment **II**.

In order to achieve chemoselective desulfurization, NCL at Asn^{21}-Cys^{22} followed by the conversion of Cys^{22} to Ala^{22} should be carried out prior to NCL at Gly^{36}-Cys^{37} or under the auspices of protecting groups on Cys^{37} and Cys^{53}. In the former case a thioester surrogate such as *S*-protected *N*-alkylcysteine[11] is required in segment **II**, while assembly of segment (**II** + **III**) cannot be performed by NCL because there is no sulfhydryl functionality at the *S*-protected Cys^{37} in the latter case. Therefore, we applied the present tritylation procedure using Trt−OH in HFIP to introduce the Trt group at $Cys^{37,53}$ in segment (**II** + **III**) obtained after NCL at Gly^{36}-Cys^{37}. This enables the sequential assembly of the ordinary thioester peptides via NCL onto the *C*-terminal peptide (Scheme 2). The two *N*-terminal thioester peptides **5** and **6** were synthesized using an ABI 433A peptide synthesizer on a Boc-Asn(Xan)-SCH_2CH_2CO-Leu-MBHA resin and a Boc-Gly-SCH_2CH_2CO-$[Arg(Tos)]_3$-MBHA resin, respectively, using in situ neutralization protocols of coupling with Boc-amino acid/1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HCTU)/6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt)/*N*,*N*-diisopropylethylamine (DIEA) (4/4/4/6 equiv) in 1-methyl-2-pyrrolidinone (NMP). The *C*-terminal peptide **7** was assembled on a Boc-Cys(4-MeBzl)-PAM resin. The peptide resins were treated by HF in the presence of *p*-cresol/butanedithiol/$MeONH_2$·HCl[12] (for **5**) or *p*-cresol (for **6** and **7**) at −2 to −5 °C for 1 h to remove all the protecting groups except for the thiazolidine (Thz) group used to protect the *N*-terminal Cys residue in thioester peptide **6**. The crude products were purified by RP-HPLC to give highly pure peptides in 10% yield for (1−21)-thioester **5**, 39% yield for (22−36)-thioester **6**, and 31% yield for (37−53)-peptide **7**.

Next, NCL between peptides **6** and **7** at Gly^{36}-Cys^{37} was carried out in 200 mM phosphate buffer (pH 8.0) containing 6 M guanidine·HCl (Gnd·HCl) in the presence of 2% (v/v)

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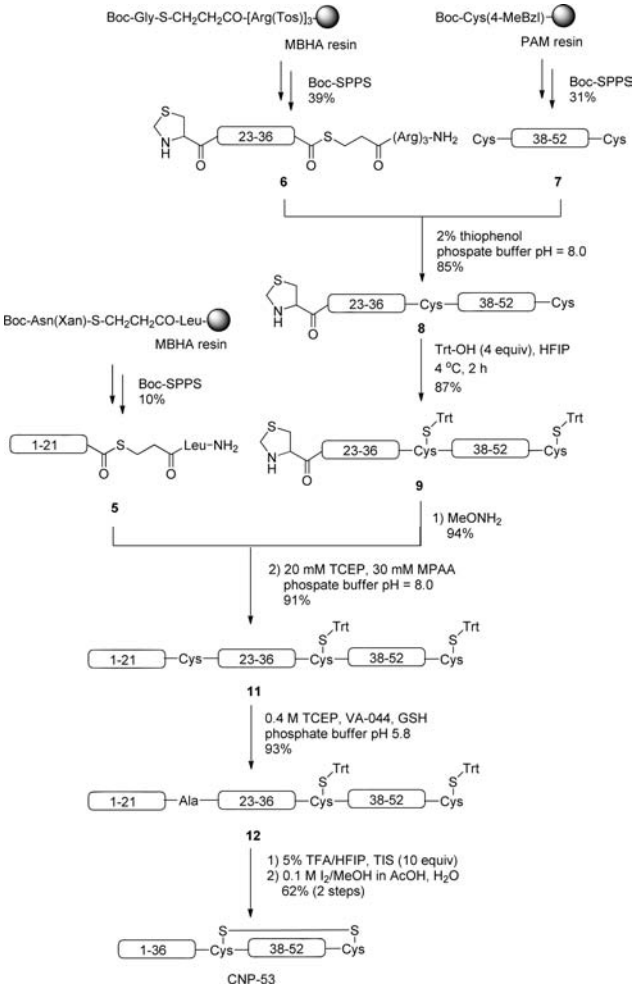
Scheme 2. Synthetic Route for Rat CNP-53



thiophenol, and the ligated product **8** and Trt−OH (4 equiv) were dissolved in HFIP at 4 °C to quantitatively afford [Thz^{22}, Cys(Trt)^{37,53}]-(22−53) (**9**) (Figure 1). The Trt group on Cys remained intact throughout RP-HPLC purification and lyophilization using the 0.1% TFA/CH_3CN system. Following

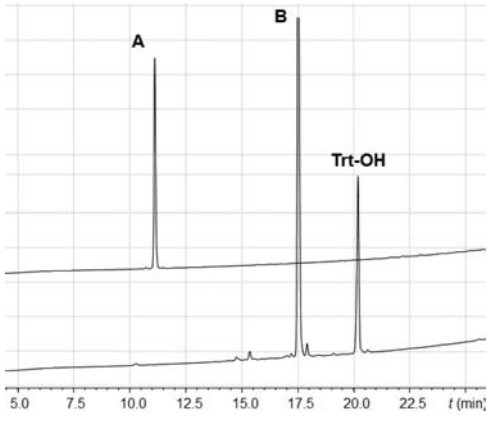


Figure 1. Tritylation of [Thz^{22}]-(22−53) **8** in HFIP. (A) [Thz^{22}]-(22−53) **8**. (B) [Thz^{22}, Cys(Trt)^{37,53}]-(22−53) **9**. HPLC conditions: column, YMC-ODS AA12S05−1546WT (4.6 mm × 150 mm); elution, 10−80% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

ring opening of the Thz structure by $MeONH_2$·HCl to generate the *N*-terminal Cys residue,[13] the final NCL between **5** and [Cys^{22}, Cys(Trt)^{37,53}]-(22−53) was performed in 200 mM phosphate buffer (pH 8.0) containing 6 M Gnd·HCl in the presence of 20 mM tris(2-carboxyethyl)phosphine (TCEP) and 30 mM 4-mercaptophenylacetic acid (MPAA) to give the ligated product **11**, which was subjected to desulfurization using TCEP, glutathione (GSH), and VA-044 at 40 °C to quantitatively convert Cys^{22} to Ala^{22} to yield [Cys(Trt)^{37,53}]-CNP-53 (**12**). Removal of the Trt group in **12** followed by disulfide formation or the iodine-mediated oxidation afforded CNP-53.

In summary, tritylation using Trt−OH in HFIP is a convenient and efficient procedure that can offer *S*-protection on Cys located in fully unprotected peptides. The procedure simply requires Trt−OH and HFIP to selectively promote *S*-tritylation in the presence of peptide nucleophilic functionalities. The utility of the method was demonstrated by synthesizing CNP-53 with the aid of Ala ligation in which chemoselective desulfurization was performed with the peptide having postsynthetic *S*-Trt protection. Moreover, since the method makes possible efficient tritylation on peptides with a thioester moiety (Figure S12), it facilitates the thioester method in which *S*-protection on Cys is definitely required[14] as well as a tandem ligation approach in which the *N*-terminal Cys(Trt) is substitutable for Thz. By resorting to the present method, we are also trying to assemble a whole molecule of protein by coupling synthetic peptides and recombinant proteins.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, characterization, and spectroscopic and chromatographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779. (b) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338−351. (c) Monbaliu, J. C. M.; Katritzky, A. R. *Chem. Commun.* **2012**, *48*, 11601−11622. (d) Raibaut, L.; Ollvier, N.; Melnyk, O. *Chem. Soc. Rev.* **2012**, *41*, 7001−7015.

(2) (a) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526−533. (b) Wan, Q.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 9248−9252.

(3) (a) Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064−10065. (b) Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem., Int. Ed.* **2008**, *47*, 6807−6810. (c) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 8521−8524. (d) Yang, R.; Pasunooti, K. K.; Li, F.; Liu, X. W.; Liu, C. F. *J. Am. Chem. Soc.* **2009**, *131*, 13592−13593. (e) Harpaz, Z.; Siman, P.; Kumar, K. S. A.; Brik, A. *ChemBioChem* **2010**, *11*, 1232−1235. (f) Siman, P.; Karthikeyan, S. V.; Brik, A. *Org. Lett.* **2012**, *14*, 1520−1523. (g) Malins, L. R.; Cergol, K. M.; Payne, R. J. *ChemBioChem* **2013**, *14*, 559−563.

(4) Pentelute, B. L.; Kent, S. B. H. *Org. Lett.* **2007**, *9*, 687−690.

dx.doi.org/10.1021/ol502773v | *Org. Lett.* 2014, 16, 5740−5743

(5) (a) Chalker, J. M.; Bernardes, G. J. L.; Lin, Y. A.; Davis, B. G. *Chem.—Asian J.* **2009**, *4*, 630−640. (b) Takaoka, Y.; Ojida, A.; Hamachi, I. *Angew. Chem., Int. Ed.* **2013**, *52*, 4088−4106.
(6) Zhang, R.-H.; Xu, X.-J.; Tang, Y.-Q.; Hojo, H.; Aimoto, S. *Science in China* **1994**, *37*, 932−939.
(7) Calce, E.; Leone, M.; Monfregola, L.; De Luca, S. *Org. Lett.* **2013**, *15*, 5354−5357.
(8) Monfregola, L.; Leone, M.; Calce, E.; De Luca, S. *Org. Lett.* **2012**, *14*, 1664−1667.
(9) Photaki, I.; Taylor-Papadimitriou, J.; Sakarellos, C.; Mazarakis, P.; Zervas, L. *J. Chem. Soc. C* **1970**, 2683−2687. (b) Büllesbach, E. E.; Danho, W.; Helbig, H. J.; Zahn, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1980**, *361*, 865−873.
(10) Kojima, M.; Minamino, N.; Kangawa, K.; Matsuo, H. *FEBS Lett.* **1990**, *276*, 209−213.
(11) Hojo, H.; Onuma, Y.; Akimoto, Y.; Nakahara, Y. *Tetrahedron Lett.* **2007**, *48*, 25−28.
(12) Taichi, M.; Kimura, T.; Nishiuchi, Y. *Int. J. Pept. Res. Ther.* **2009**, *15*, 247−253.
(13) Bang, D.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2004**, *43*, 2534−2538.
(14) Aimoto, S. *Pept. Sci.* **1999**, *51*, 247−265.

dx.doi.org/10.1021/ol502773v | *Org. Lett.* 2014, 16, 5740−5743