SYNTHESIS OF A COUMARIN-HISTONE CONJUGATE FOR HAT FLUORESCENT ASSAY

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Abstract : To synthesize molecular reporters of histone acetyltransferases (HATs), we studied the on-resin modifications of the histone H4 peptide with the fluorescent coumarin motif. The compound was synthesized using the solid phase Fmoc chemistry. The efficacy of *tert*-butylthio and *para*-methoxytrityl as Cys protecting groups was investigated. Different reaction conditions were tested for deprotection efficiency. Following deprotection, the fluorescent chromophore coumarin was coupled to the peptide at the Cys site. This work is important for developing new fluorescent assays to study the enzymatic activities of HATs and to screen HAT inhibitors in a high-throughput fashion.

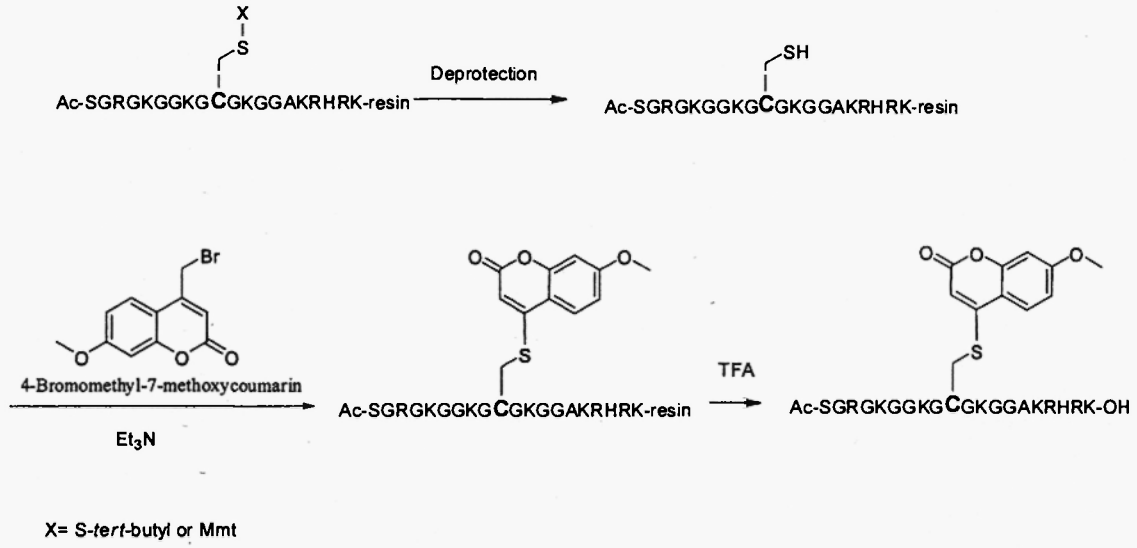
Introduction

Histone acetyltransferases (HATs) are epigenetic enzymes that catalyze the transfer of the acetyl group from acetyl coenzyme A to the ε-amino group of lysine residues in a histone or non-histone protein substrate.[1,2] An increasing body of evidence shows that HAT activities are deregulated in a number of cancers which indicate that HAT inhibitors may be of significance as new cancer chemotherapies.[3,4] However, currently no effective assay tools are available for studying HAT activities in vivo or for the high-throughput screening of HAT inhibitors.[5,6] Herein, we designed and synthesized a substrate-based construct containing the histone H4 peptide and a fluorescent probe coumarin. The fluorescence of coumarin is extremely sensitive to its local microenvironment,[7] and thus it can be used to detect the acetylation reaction catalyzed by HATs. Such a fluorescent reporter will be useful for enzymatic studies as well as for drug discovery.

The peptide fragment is derived from the *N*-terminus of histone H4. This *N*-terminal tail is the substrate of several HATs such as p300 and TIP60.[2,8] The amino acid sequence is as follows: H-Ser-Gly-Arg-Gly-Lys-Gly-Gly-Lys-Gly-**Leu**-Gly-Lys-Gly-Gly-Ala-Lys-Arg-His-Arg-Lys-OH. To introduce labeling, we changed the 10th amino acid residue Leu to Cys. In this way, the sulfurhydryl group of the Cys can be used for the region-specific modification with fluorescent probes such as coumarin. The new sequence is: H-Ser-Gly-Arg-Gly-Lys-Gly-Gly-Lys-Gly-**Cys**-Gly-Lys-Gly-Gly-Ala-Lys-Arg-His-Arg-Lys-OH.

The Fmoc chemistry has been recruited as a powerful method for solid phase peptide synthesis and the use of different side chain protecting groups makes it feasible to label a synthetic peptide at specific sites.[9,10] The successful synthesis of our fluorescent constructs strongly depends on the removal efficacy of the protecting group on the Cys residue. Herein, we studied two orthogonal Cys protecting groups: *tert*-butylthio (*tert*-butyl-S) and *p*-methoxytrityl (Mmt) for their use in introducing fluorescent labels (Scheme 1). In particular, we tested the removal efficiency of the two

protecting groups under different conditions. Our results demonstrated that the Mmt group is a more versatile thiol protecting group than the *tert*-butyl-S in organic solvents.



Scheme-1 : Synthesis of the fluorescent H4-coumarin reporter.

Results and Discussion

The synthesis of the modified H4 peptides was performed on Wang resin using the Fmoc solid phase chemistry. For each amino acid coupling, a reactant mixture containing 4 eq. of amino acid and *N*-hydroxybenzotriazole (HOBt) and 2-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used as coupling reagents. *N*-Methylmorpholine (NMM) was used as the activating base. Following each amino acid coupling, the Fmoc group was removed with 20% piperidine in DMF. Cys10 was coupled as Fmoc-Cys(S-tBu)-OH or Fmoc-Cys(Mmt)-OH. In most cases, the *N*-terminal amino group was capped by reacting with acetic anhydride.

Deprotection of *tert*-butyl-S-Cys

Wang resin does not swell in aqueous media. However, the deprotection of *tert*-butyl-S on Cys needs to be performed in aqueous solution due to the limited solubility of common reducing reagents, e.g., dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP), in organic solvents.[11] To balance the two elements, we used a mixed solution containing DMF and HEPES buffer (pH 8.0) so that the Wang resin can swell sufficiently while the deprotection reaction proceeds effectively.

After the peptide sequence was assembled (the *N*-terminus was Fmoc capped), the resin was treated with DTT solution (200 mM) for 4 h at room temperature. After washing and drying in vacuum, the peptide was cleaved with the trifluoroacetic acid (TFA) solution. The product was subjected to MALDI analysis. On the mass spectrum, the main peak is at 2293 Da which corresponds to the peptide with the *tert*-butyl-S attached. We did not observe any peak at 2024 Da, i.e. the peptide with *tert*-butyl-S removed. Therefore, the *tert*-butyl-S on the Cys was not deprotected by treatment with DTT.

We then tested TCEP for its ability to remove the *tert*-butyl-S group. The reaction was conducted by immersing the peptide-resin in a DMF/HEPES solution (2:1, v/v). The reaction time was increased to 21 h. Following TFA cleavage,

the peptide was analyzed by MALDI MS. A minor peak at 2024 Da was observed for the desired peptide product. The yield of deprotection was 10% based on its relative abundance on the mass spectrum.

To improve the deprotection efficiency, the reaction temperature was increased to 37 °C and the reaction time was 22 h and 37 h. However, no deprotected peptide was detected on the mass spectra. Therefore, higher temperature did not give apparent beneficial effect for the deprotection reaction. The reason for the poor deprotection efficiency may be due to limited swellability of the Wang resin in the aqueous buffer and the deficient reactivity of DTT or TCEP when the organic solvent DMF was present.

Deprotection of Mmt-Cys

Next, we tested if the Mmt can be used as a better protecting group than the *tert*-butyl-S group to introduce side-chain modifications on the cysteine residue. Again, the peptide was synthesized on Wang resin and the *N*-terminal amino group was acetylated.

The Mmt group is sensitive to dilute TFA which will not affect other protecting groups.[12] A 2% TFA solution was used and the deprotection was followed directly by the modification using the fluorescent coumarin group. After TFA cleavage from the resin, the peptide product was analyzed by MALDI-MS. The high molecular ion peak at 2213 Da was indicative of the peptide modified by the fluorescent group. Another peak at 2401 Da showed that two fluorescent groups were attached to the peptide. This may be due to the removal of a different side chain protecting group during the Mmt deprotection, thus suggesting that the Mmt deprotection conditions need to be further optimized.

Taken together, our study showed that the deprotection of *tert*-butyl-S-Cys with DTT or TCEP in an organic solvent was relatively difficult. Reducing ability of TCEP seems to be better than that of DTT, but still not high enough to completely remove the S-*tert*-butyl group. Previous reports showed that *tert*-butyl-S-Cys can be deprotected in shorter peptides, indicating the possibility that the deprotection reaction may be sequence dependent.[13,14] On the other hand, the use of Mmt for the protection of the Cys sulfurhydryl group seems to be versatile. The Mmt group can be removed effectively under mild acidic condition which allows the next-step modification to be conducted directly. Therefore, Mmt is a more reliable protecting group than S-*tert*-butyl in organic systems and offers a better resolution for on-resin modifications of synthetic peptides.

Experimental

Materials: Fmoc-L-amino acids and pre-loaded Wang resin were purchased from Novabiochem. HBTU, HOBT, DMF, DCM, TFA and ether were purchased from Fisher Scientific; DTT and TIS were purchased from Sigma-Aldrich; and TCEP was purchased from PIERCE.

Peptides were synthesized on a PS3 peptide synthesizer using the Fmoc strategy. HBTU and HOBT and NMM were the activating reagents for amino acid coupling. After each round of amino acid coupling, Fmoc group was removed with 20% piperidine in DMF (10 ml) for 5min and the step was repeated once. The *N*-terminus of the peptide was acetylated with acetic anhydride (2 ml) and pyridine (20 equiv) for 1 h.

For the deprotection of the *tert*-butyl-S group on the cysteine residue, the peptide on resin (10.7 mg) was allowed to react with DTT (200 mM, 30.85 mg) or TCEP (200 mM, 67.6 mg) in 1 ml of mixed solution containing DMF and HEPES aqueous buffer (1 M, pH = 8.0) at different ratios. The reaction was conducted in a 1.5-ml Eppendorf tube with shaking.

For the deprotection of the Mmt group on the cysteine residue, the peptide-loaded resin (0.02 mmol) was treated with a solution of 2% TFA and 5% TIS in dichloromethane (10 ml) for 10 min with shaking in a plastic tube. Then the mixture was filtered off and the peptide resin was washed twice with DCM. The above deprotection and washing steps were repeated 5 times. The peptide resin was finally washed with DCM and DMF. For the fluorescent group modification, 0.1 mmol of 4-bromomethyl-7-methoxycoumarin was dissolved in 9 ml DMF and 1 ml Et_3N. The mixture was then added into the peptide-resin and allowed to react overnight. On the next day, the liquid was aspirated and the resin was washed with DMF and DCM and ether.

The final peptide was cleaved off the resin for 4 h with a cleavage solution containing 95%TFA, 2.5% TIS and 2.5% H_2O. Cold ether was added to precipitate the crude peptide. The white precipitate was washed with ether 3 times. The precipitate was dried in a stream of nitrogen and then dissolved in H_2O for mass analysis.

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