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Synthesis of H4 pantetheine adducts for histone acetyltransferase inhibition

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Abstract

Site-specific modifications of peptides provide a powerful tool for design of chemical probes and enzyme inhibitors. A convenient synthesis method was developed and used to produce H4K16-pantetheine bisubstrate analogs which could be employed as inhibitors of histone acetyltransferases *in vivo* and *in vitro*.

Keywords: bisubstrate inhibitor; histone acetyltransferases (HATs); H4; pantetheine.

Introduction

Histone acetyltransferases (HATs) catalyze the lysine acetylation on the core histones in eukaryotic chromatin. Histone acetylation is linked to gene transcription, DNA repair and is associated with several diseases, especially cancer (Pollard and Peterson, 1998; Timmermann et al., 2001; Turner, 2002; Khan and Khan, 2010; Selvi et al., 2010). Chemical inhibitors of HATs have been developed by different strategies and applied both as chemical probes and as potential anticancer therapeutics (Suzuki and Miyata, 2006; Zheng et al., 2008). Recently, bisubstrate inhibitors were found to show strong inhibition to HATs, such as Lys-CoA for p300 (Lau et al., 2000) and H4K16-CoA for Tip60 (Wu et al., 2009). However, the negatively charged phosphate groups in the CoA motif cause poor pharmacokinetics performance and restrict their applications *in vivo* (Cebrat et al., 2003). The CoA precursor called pantetheine was shown to exhibit good ability of penetrating the cell membrane and can be transformed to CoA by the endogenous CoA metabolic pathway (Meier et al., 2006). This advantage encouraged us to synthesize histone peptide K-pantetheine analogs that could be used to study inhibition of HATs, especially *in vivo*.

Results and discussion

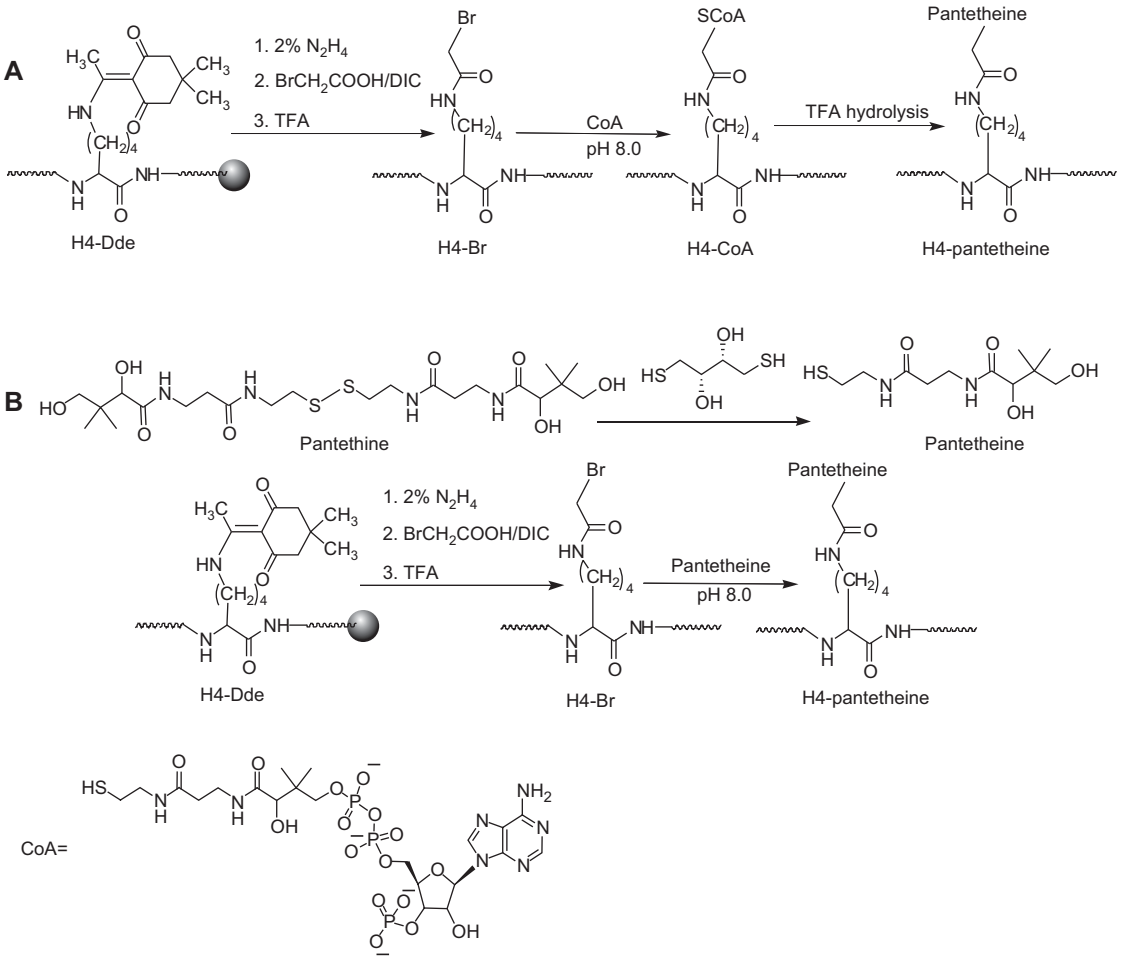
Solid phase Fmoc peptide chemistry was used to synthesize the chain of H4 peptide containing dimethyldioxocyclohexylidene (Dde) protecting group at *N*-ε-Lys 16. An effective hydrazine deprotection step was recruited to release free primary amine on H4K16 which can react with bromoacetic acid to form amide bond. Two different methods (Scheme 1) were studied to obtain H4K16-pantetheine. Although both strategies yielded the designed molecular weight peak on mass spectrometry, only directly conjugating pantetheine to H4K16 was confirmed as an effective route to create lysine-pantetheine modification on the tested peptides.

Hydrolysis of peptide-CoA product precursor

The structure of CoA has two phosphate groups between pantetheine and 3′-phosphate-adenosine (Scheme 1). The bond between phosphorus and oxygen of phosphate ester can be hydrolyzed by different methods (Nogowska, 2000; Kamerlin et al., 2008). Free H4-Br can be cleaved from resin by 95% trifluoroacetic acid (TFA) solution at room temperature, which means that the peptide backbone is stable in this condition. We attempted to hydrolyze H4(11–22) K16-CoA with TFA at different volumes, reaction times and temperatures. The reaction was monitored by MALDI-MS. However, MS spectra always showed a major peak at 1728.9 Da or 1830.6 Da which corresponded to the fragment H4 (11–22) K16-pantetheine (plus one phosphate or two phosphates) and the expected peak at 1666.9 Da as the minor one (Figure 1). These results indicate that the leaving group is a key to controlling the direction of hydrolysis. Our result showed OR′ (R′=adenosine 3′,5′-monophosphate) is a much better leaving group than OR (R=pantetheine peptide) in TFA hydrolysis. The phosphate ester bond between phosphate and H4(11–22) K16-pantetheine could be destroyed by stronger acidic condition or higher temperature. The harsh condition, however, could also break the peptide bond or the side chain of amino acid, making hydrolysis reaction even more complicated.

Direct coupling of pantetheine to peptide

Pantetheine synthesized by its dimeric form, pantethine, has been reported previously (Burns et al., 1991; Crawford et al., 2006). Dithiothreitol (DTT) has been used to reduce pantetheine and the process of reaction was monitored by HPLC. The retention time for each component was 18 min for DTT, 22 min for pantetheine, and 27 min for pantetheine under the gradient 5–40% (buffer A and buffer B described in experimental section) (Figure 2A). The reaction finished within 10 h. Following the HPLC purification, the glue-like product was obtained after lyophilization. ESI-MS ($M+Na^+$) *m/z* is 301 (calcd. for $C_{11}H_{22}N_2O_4S$: 278).



Scheme 1 Synthesis of peptide K-pantetheine adducts through two strategies: hydrolysis of the peptide-CoA (A) and directly conjugating pantetheine to peptide (B).

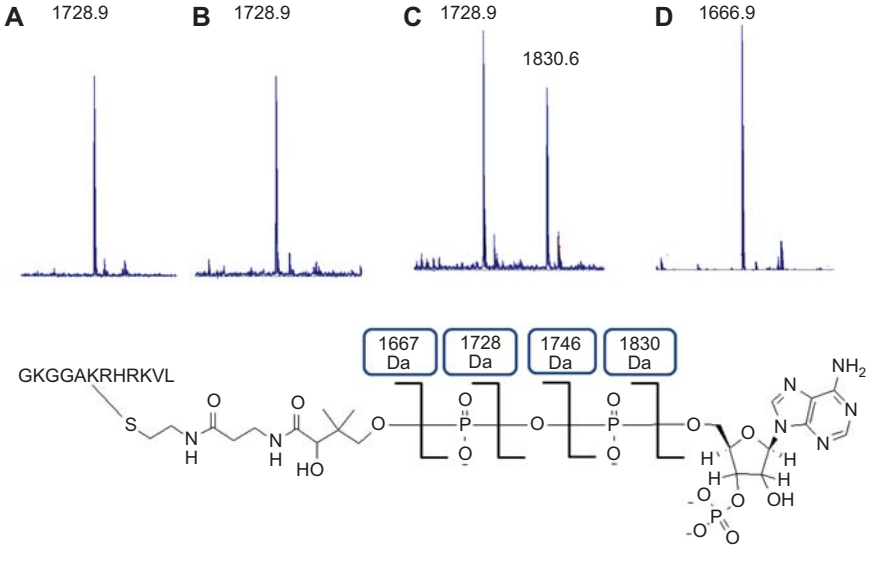


Figure 1 Mass spectroscopic data of two synthetic methods (only the major peaks shown).
(A), (B), and (C) Hydrolysis of peptide-CoA product precursor in TFA (w/v=1:20) at room temperature, 40°C and 60°C for 10 h. (D) Direct coupling of pantetheine to peptide at room temperature for 10 h.

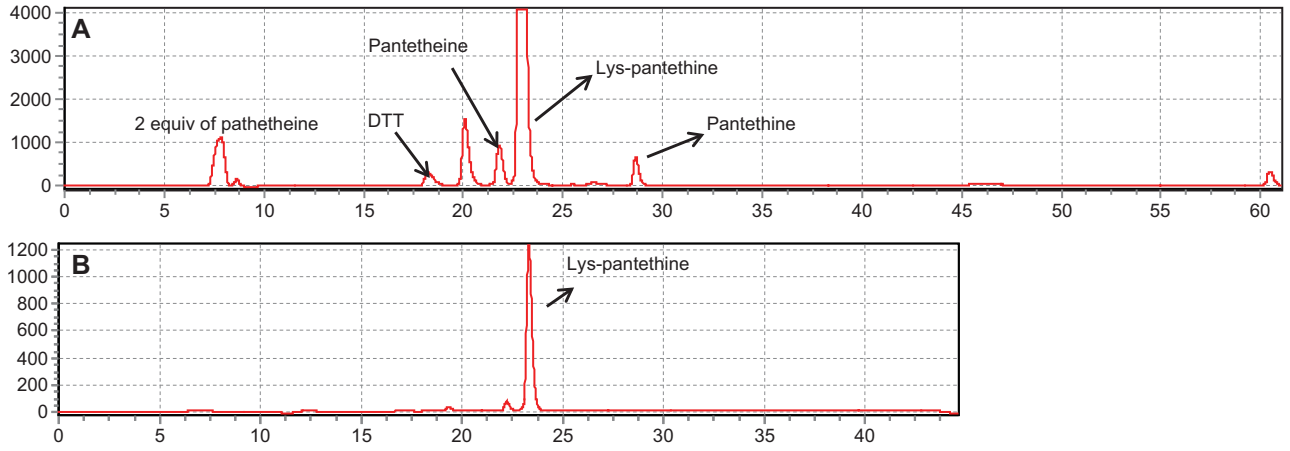


Figure 2 HPLC analysis of coupling pantetheine to lysine-Br.
(A) The reaction mixture. (B) Lysine-pantetheine after purification (retention time: 23.0 min).

The mechanism of conjugating pantetheine to peptide is a S_N2 reaction. The pantetheine thiolate (pH=8) acts as a nucleophile to attack H4-Br (the electrophile), resulting in H4 pantetheine, with bromide released as a leaving group. Because the weight of pantetheine is difficult to measure, the amount of pantetheine needs to be controlled carefully. Inappropriate amounts of pantetheine could complicate the purification process. From HPLC analysis, the product peak from the reaction largely overlapped with pantetheine if pantetheine was used up to five equivalents (eq) of peptide. To resolve this issue, the quantification of pantetheine can be done by equally separating the solution made after dissolving the glue-like pantetheine in ddH_2O. Another issue is that the solution should be made freshly because thiol group is unstable under basic condition. Here, Table 1 lists MS information of the four lysine-pantetheine analogs synthesized with different peptide lengths. Based on these data, the results matched well with the expected values.

Test of enzymatic inhibition

Following the synthesis, we measured the activities of pantetheine and the peptide-pantetheine compounds for their ability in blocking Tip60-catalyzed acetyltransferase reaction. The data showed that at 1 mM of concentration, most of these peptide-pantetheine compounds showed marginal inhibitory activities for Tip60 (Figure 3). As the peptide length becomes longer, the potency of inhibition becomes stronger. For example, H4(11–22)-pantetheine has the longest length and shows the strongest potency, with 43% of Tip60 activity inhibited at 1 mM of this compound. Nevertheless, the overall potencies of these compounds are weaker than the peptide-CoA compounds that we reported previously (Wu et al., 2009). These results therefore demonstrate that adenosine pyrophosphate is an indispensable element to achieve high potency of HAT inhibition.

Conclusions

In this paper, we compared two methods of peptide lysine-pantetheine synthesis: hydrolysis of the peptide-CoA and directly conjugating pantetheine to peptide. The second method was found to be a concise synthetic way to obtain lysine modification of peptide with pantetheine.

Experimental section

Materials

Fmoc-L-amino acids, HCTU [2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate], preloaded Wang resin, Rink amide resin were purchased from Novabiochem. DIC (*N*,*N*′-diisopropylcarbodiimide), DMF (dimethylformamide), DCM (dichloromethane), TFA, and ether were purchased from Fisher Scientific; DTT, pantethine, and TIS (triisopropylsilane) were purchased from Sigma-Aldrich.

Synthesis of pantetheine-histone analogs

Solid phase peptide synthesis was performed on a PS3 synthesizer using the Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] strategy. Preloaded Wang resins or Rink amide resins were used as solid phase.

Table 1 Sequences and molecular masses of H4-K16-pantetheine analogs.

Peptide-pantetheine	Peptide sequence	Formula	Calcd.	Found
Lysine-pantetheine	Ac-**K**-NH_2	$C_{21}H_{39}N_5O_7S$	505.6	505.2
H4(15–17)-pantetheine	Ac-A**K**R-NH_2	$C_{30}H_{56}N_{10}O_9S$	732.9	733.3
H4(14–18)-pantetheine	Ac-GA**K**RH-NH_2	$C_{38}H_{66}N_{14}O_{11}S$	927.1	927.4
H4(11–22)-pantetheine	Ac-GKGGA**K**RHRKVL-OH	$C_{71}H_{129}N_{26}O_{18}S$	1667.0	1666.9

Highlighted K is the residue to which pantetheine is attached.

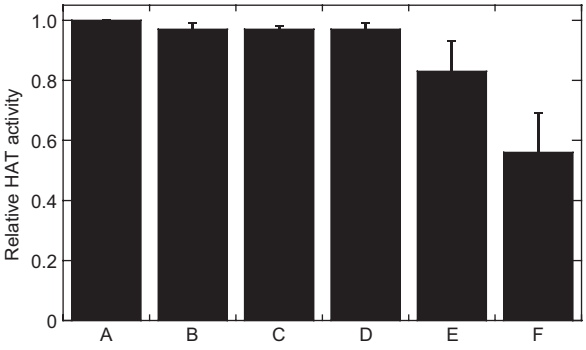


Figure 3 Activity of H4-pantetheine analogs for Tip60 inhibition. The reaction contained 200 μM of H4-20, 10 μM of ^{14}AcCoA and 100 nM of Tip60. The concentration of peptide-pantetheine analogs or pantetheine is 1 mM. (A) Without inhibitor, (B) pantetheine, (C) lysine-pantetheine, (D) H4(15–17)-pantetheine, (E) H4(14–18)-pantetheine, (F) H4(11–22)-pantetheine.

For the coupling of each amino acid, HCTU was used as activating reagents. Removal of Fmoc group was performed with 20% v/v piperidine/DMF. The N-terminal amino group was acetylated with acetic anhydride. After all the amino acids were coupled to the solid phase, Dde was removed with 2% hydrazine in DMF for 2 h. The resin was then treated with five eq of $BrCH_2COOH$ and five eq of DIC in DMF for 4 h. After washing and drying in vacuum, the bromo-labeled peptides were cleaved from the resin by treatment with 95% TFA, 2.5% H_2O and 2.5% TIS for 4 h. Crude products were precipitated with cold ether, and then purified with reverse-phased HPLC. All the bromopeptide products were characterized with analytical HPLC and MALDI-MS before the next-step reaction.

Directly conjugating pantetheine to peptide

For the linkage of pantetheine to the bromopeptide, a mixture of one eq bromopeptide and five eq pantetheine was dissolved in a minimum amount of sodium phosphate buffer (100 mM, pH=8). The mixture was allowed to stand in the dark for 16 h. The final bisubstrate products were purified with reverse-phased (RP) HPLC (C18, Varian) on a Varian Prostar HPLC system using linear gradients of H_2O/0.05% TFA (solvent A) vs. acetonitrile/0.05% TFA (solvent B). Analytical HPLC and MALDI-MS were used for characterization. The purified analogs were dissolved in ddH_2O and adjusted to neural pH using NaOH.

The pantetheine was synthesized by reducing pantetheine (bispantetheine). The reaction contained one eq pantetheine, five eq DTT in 50 mM Tris buffer (pH=8). After 16 h, the mixture was centrifuged at 20 000 *g* for 10 min. The supernatant was purified with RP-HPLC (C18, Varian) using linear gradients of H_2O/0.05% TFA (solvent A) vs. acetonitrile/0.05% TFA (solvent B). Analytical HPLC and MALDI-MS were used for characterization.

Hydrolysis of peptide-CoA

In the hydrolysis strategy, TFA was used to directly cleave the adenosine 3′-phosphate 5′-diphosphate from the CoA motif in the peptide-CoA conjugate (Scheme 1B). The reaction was tested for different times (2–16 h), several temperatures (25–60°C), and varying amounts of TFA. In the final step, TFA was removed by nitrogen blowing. After lyophilization, the mixture was dissolved in H_2O and analyzed by MALDI-MS.

Radioactive HAT assay

Radioactive acetyltransferase assays were conducted similarly as previously reported (Wu et al., 2009). [^{14}C]-labeled acetyl-CoA was used as donor and the N-terminal 20 amino acids of histone H4(H4–20) were used as HAT substrates. Recombinant Tip60 was expressed in BL21 (DE3) cells.

Acknowledgments

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