Synthesis and Validation of Acetyl- and Malonyl-CoA Analogs for the Study of Substrate Specificity and Mechanism of Acyltransferases

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Synthesis and Validation of Acetyl- and Malonyl-CoA Analogs for the Study of Substrate Specificity and Mechanism of Acyltransferases

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Acyltransferases (ATs) are seen in a wide variety of metabolic pathways. These include enzymes in the polyketide synthase pathways, fatty acid biosynthesis, and secondary metabolism[1,2]. The products from these enzymes often include pharmaceutically important natural products[1,2,3]. ATs can transfer a wide range of moieties, such as acetyl, malonyl, succinyl, and longer-chain fatty acyl groups using coenzyme A (CoA)[4]. One such example of an AT is type III chloramphenicol acetyltransferase (CATIII) from *E.coli*. CATIII attaches the acetyl moiety of acetyl-CoA to a hydroxyl group on the antibiotic chloramphenicol[5,6], thus conferring chloramphenicol resistance to bacterial cells via O-acetylation[6]. The structure of CATIII with chloramphenicol bound had already been solved and refined at 1.75 Å resolution[6], which revealed that CATIII is a homo-trimer stabilized by hydrogen bonding, and chloramphenicol binds CATIII in a deep pocket located at the boundary between adjacent subunits of the trimer[6]. However, much is still unknown about how CATIII interacts with its second substrate, acetyl-CoA. We believe that by co-crystallizing acetyl-CoA analogs with chloramphenicol and CATIII, the structure of CATIII with both of its substrates bound can be solved via x-ray crystallography, which makes elucidation of enzyme-substrate interactions possible.

In the reaction catalyzed by CATIII, the reactivity of the thioester sulfur on the acetyl-CoA substrate allows for transfer of the acetyl moiety to chloramphenicol. However, the labile nature of the thioester sulfur makes the study of enzyme-substrate interactions difficult. In this study, we used two types of CoA-related analogs. One is the analog with the reactive sulfur atom replaced by an oxygen atom, and the other with the reactive sulfur atom replaced by an –NH group. We believe that the reduced reactivity of these analogs will allow us to capture the transition-state complex during the aforementioned co-crystallization, thereby enabling us to elucidate the enzyme-substrate interactions of CATIII.

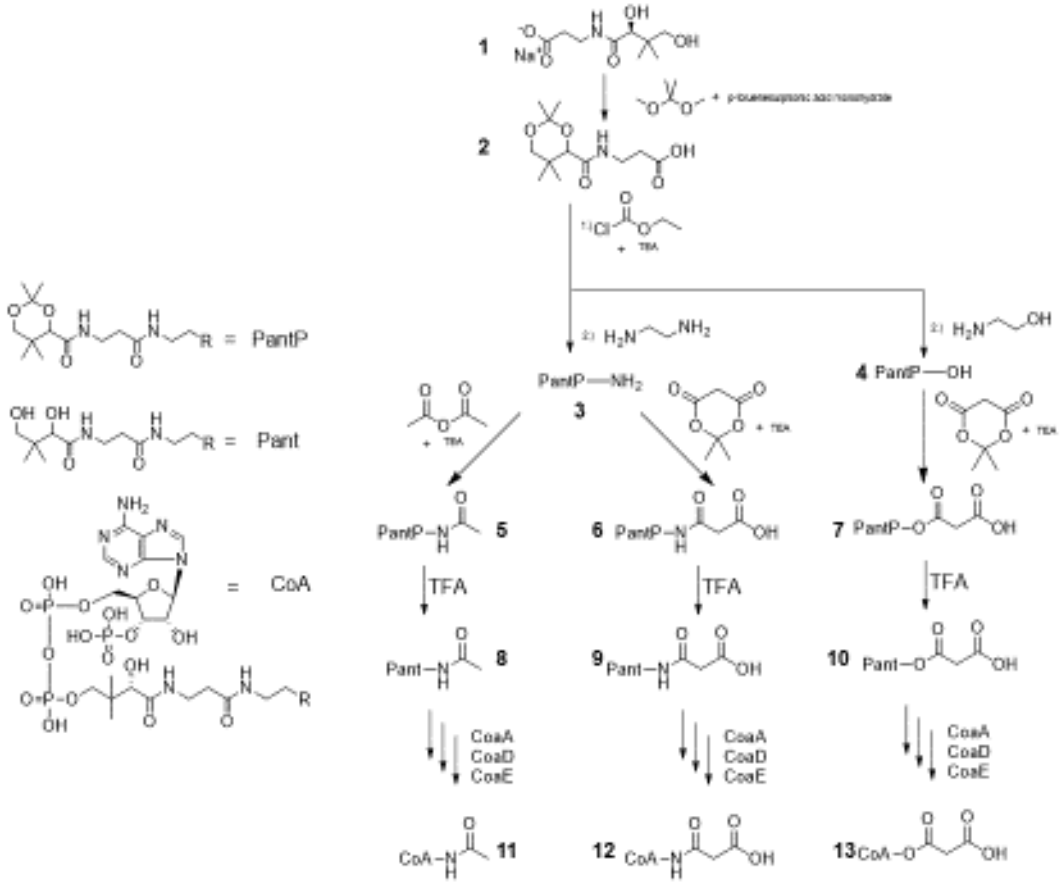
We hereby present the synthetic scheme to generate CoA-related analogs **11-13** (**Scheme 1**, **Table 1**). **1** is reacted with 2,2-dimethoxypropane and p-toluenesulphonic acid monohydrate to yield **2**. In order to form **3**, **2** is reacted with ethyl chloroformate, triethylamine (TEA) and ethylene diamine; in order to form **4**, **2** is reacted with ethyl chloroformate, TEA and ethanolamine. Acylated pantetheine acetonides are formed when **3** is reacted with TEA and acetic anhydride (forming **5**)/meldrum's acid (forming **6**), or when **4** is reacted with TEA and meldrum's acid (forming **7**). These acetonides are deprotected with trifluoroacetic acid (TFA), forming **8, 9 & 10**, and chemo-enzymatically converted to CoA-related analogs (**11, 12 & 13**).

Table 1. 1-13 Nomenclatures

1	Sodium pantothenate
2	Pantothenate acetonide
3	Amino(dethia)-pantetheine acetonide

4	Oxy(dethia)-pantetheine acetonide
5	Acetyl-amido(dethia)-pantetheine acetonide
6	Malonyl-amido(dethia)-pantetheine acetonide
7	Malonyl-oxy(dethia)-pantetheine acetonide
8	Acetyl-amido(dethia)-pantetheine
9	Malonyl-amido(dethia)-pantetheine
10	Malonyl-oxy(dethia)-pantetheine
11	Acetyl-amido(dethia)-CoA
12	Malonyl-amido(dethia)-CoA
13	Malonyl-oxy(dethia)-CoA

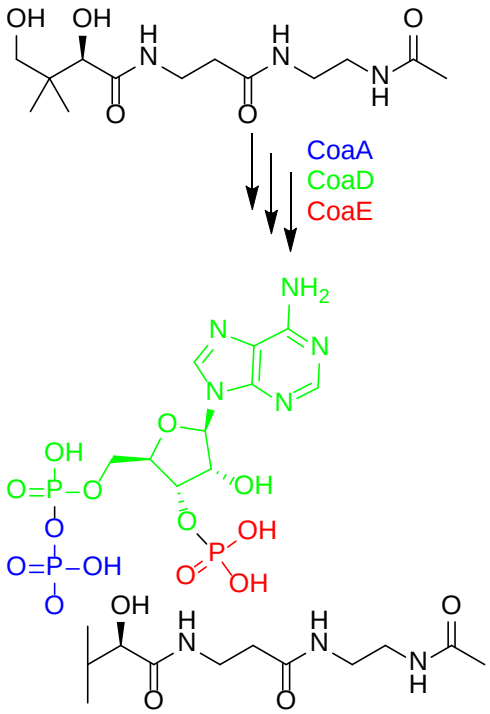
Scheme 1. Synthetic Scheme for CoA-related Analogs



It is worth noting that analogs **11-13** were ultimately synthesized from their respective pantetheines **8-10** via chemo-enzymatic conversion. For example, in the synthesis of **11,** pantothenate kinase (CoaA) phosphorylates pantothenate, phosphopantetheine

adenylyltransferase (CoaD) attaches an adenylyl group from ATP to the 4'-phosphopantethine, and dephospho-CoA kinase (CoaE) phosphorylates the dephospho-CoA to form CoA (**Scheme 2**).

Scheme 2. Chemo-enzymatic conversion of 8 to 11



acetyl-amido(dethia)-CoA

One challenge in this study is the spontaneous decarboxylation of **6** to form **5**. We initially tried to synthesize **6** using reagents shown in **Table 2a** below, with toluene as the solvent and the reaction taking place at 75℃ overnight. However, subsequent analysis by liquid chromatography-mass spectrometry (LC-MS) confirmed the presence of **5**, instead of **6**, which indicates that the malonyl moiety in **6** has likely undergone decarboxylation. To optimize the reaction, we switched the solvent to acetonitrile (**Table 2b**), which is more polar. In addition, we modified the reaction conditions such that the reaction was run at room temperature for 4 hours, and under such conditions, we successfully detected the presence of **6**, with minimal decarboxylation present. We also tried to slightly heat up the reaction mixture to ~40℃ using water bath to help facilitate the reaction progress. The reaction was run for 2 hours and we successfully detected the presence of **6**, without substantial decarboxylation.

Table 2a		**Table 2b**	
Amino(dethia)-pantetheine acetonide	100mg	Amino(dethia)-pantetheine acetonide	100mg
Meldrum's acid in toluene	100mg	Meldrum's acid in acetonitrile	100mg
TEA	0.5ml	TEA	0.5ml

Another challenge in this study is that it's hard for us to obtain crystals that yield a good diffraction pattern in x-ray crystallography. Previously, we have already set up crystal trials to figure out the optimal conditions for CATIII crystallization. In these trials, we did have observed some "good" crystals (e.g.: diamond-shaped), which enabled us to set up crystal trays under the corresponding solution conditions and loop those crystals. Currently, our lab is trying to further explore those solution conditions to obtain crystals that yield a better diffraction pattern, so that we can solve the structure of CATIII co-crystallized with chloramphenicol and acetyl-CoA analogs. So far, we have been able to solve a crystal structure of CATIII to a resolution of 1.8 Å in complex with chloramphenicol and acetyl-oxy(dethia)-CoA, another analog synthesized in the lab. We are looking to optimize this condition to yield structures with acetyl-amido(dethia)-CoA bound as well.

Structural information of CATIII will enable us to perform site-directed mutagenesis to study how mutant CATIII interacts with acetyl-CoA. In addition, we will also perform kinetic assays to determine how tightly CATIII binds acetyl-CoA, and how fast catalysis occurs.

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