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A Reversed Thioester Analogue of Acetyl-Coenzyme A: An Inhibitor of Thiolase and a Synthon for Other Acyl-CoA Analogues

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Abstract: We have previously reported a general synthetic approach to analogues of coenzyme A (CoA) and CoA esters using a combination of enzymatic and nonenzymatic reactions (Martin et al. *J. Am. Chem. Soc.* **1994**, *116*, 4660). We report here the extension of this method to a CoA ester analogue **1c** in which the orientation of the thioester is reversed. A key to this synthesis is the use of a trithioortho ester as a protected thioester. The reversed thioester analogue **1c** is a time-dependent inhibitor of thiolase, apparently forming an acyl enzyme in which the CoA moiety rather than an acetyl moiety is covalently attached to an active site nucleophile. This analogue also serves as a general synthon for analogues having other functionality at the site of the thioester group. This has been applied to the synthesis of a reversed thioester analogue of succinyl-CoA **6** and hydroxamate **7** and hydrazide **8** analogues of acetyl-CoA, analogues which are not available by the previously described methodology. The hydroxamate and hydrazide analogues are potent inhibitors of the enzyme citrate synthase. The reversed thioester analogue of acetyl-CoA may have useful applications in enzymology and permits the ready access to a range of additional CoA analogues modified in the thioester moiety.

We have previously reported a general synthetic approach to analogues of coenzyme A (CoA) **1a** and CoA esters using a combination of enzymatic and nonenzymatic reactions.[1,2] This method involves enzymatic synthesis of a general CoA analogue synthon **1b** having a thioester linkage in place of the amide bond nearest the thiol group (Figure 1). For synthetic convenience, the thiol group is replaced with a methyl group.[2] An aminolysis reaction is then performed to reform the amide bond present in CoA and to introduce the functionality of interest in place of the thiol group (Scheme 1). This synthetic methodology provides a versatile approach to manipulation of the CoA or CoA ester molecule in the region of the thiol or acylthio group, which is the site of reactivity in almost all enzyme-catalyzed reactions involving CoA. This method has now been used to prepare a number of CoA and CoA ester analogues, and these analogues have been useful in mechanistic and structural studies of CoA ester-utilizing enzymes.[3−5] These studies have included determination of the structures of enzyme−inhibitor complexes to provide insights into the nature of enzyme-transition state complexes,[3,4] structural analysis of a short hydrogen bond in an enzyme−inhibitor complex,[3,4] and determination of the stereochemistry of the tetrahedral inter-

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(1) Martin, D. P.; Drueckhammer, D. G. *J. Am. Chem. Soc.* **1992**, *114*, 7287.

(2) Martin, D. P.; Bibart, R. T.; Drueckhammer, D. G. *J. Am. Chem. Soc.* **1994**, *116*, 4660.

(3) Usher, K. C.; Remington, S. J.; Martin, D. P.; Drueckhammer, D. G.; *Biochemistry* **1994**, *33*, 7753.

(4) Schwartz, B. M.; Drueckhammer, D. G.; Usher, K. C.; Remington, S. J. *Biochemistry* **1995**, *34*, 15459.

(5) Schwartz, B. M.; Drueckhammer, D. G. *J. Am. Chem. Soc.* **1996**, *118*, 9826.

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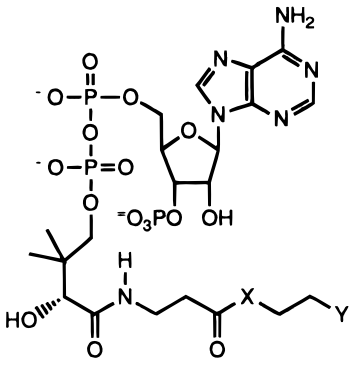
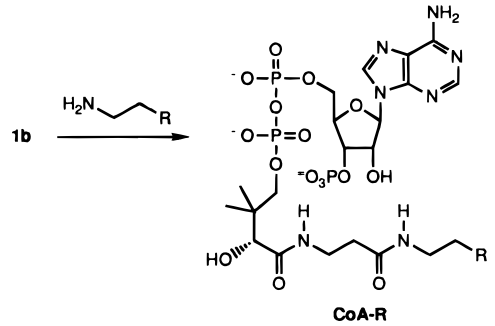


1a X = NH, Y = SH (CoASH)
1b X = S, Y = CH_3
1c X = NH, Y = CH_2–C(=O)–S-CH_3

Figure 1. Structures of Coenzyme A **1a**, CoA synthon **1b**, and "reversed thioester" acyl CoA analogue **1c**.

Scheme 1



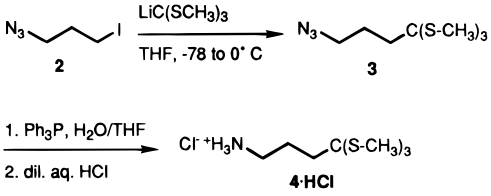
mediate or transition state in the reactions of acetyl-CoA dependent acetyltransferases.[5]

Previously prepared analogues of acetyl-CoA have included a noncleavable ketone isostere,[1,2] stable mimics of the nucleophilic enol and enolate forms of acetyl-CoA,[2−4] and mimics of the tetrahedral intermediate or transition state in acetyl transfer.[5] Reported here is the synthesis of a new and unique acyl-CoA analogue **1c** in which the orientation of the thioester is reversed. **1c** was prepared from the previously reported analogue **1b**, using a trithioortho ester as a protected thioester in the aminolysis reaction. **1c** is a time-dependent inhibitor of thiolase, apparently forming an unnatural acyl-enzyme in which the CoA moiety is covalently linked to an active site cysteine. Studies using this analogue provide a novel analysis of the various contributions to catalysis of acetyltransfer. **1c** has also been used as a synthon for the introduction of additional functional groups in the synthesis of a reversed thioester analogue of succinyl-CoA **6** and hydroxamate **7** and hydrazide **8** analogues of acetyl-CoA. The hydroxamate and hydrazide analogues are potent competitive inhibitors of citrate synthase, and the hydroxamate analogue is also a modest inhibitor of malate synthase.

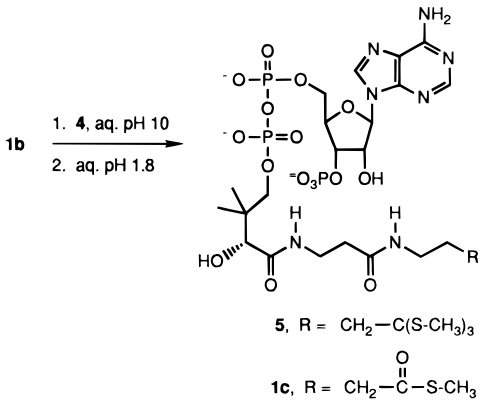
Results

Synthesis of CoA Analogues. The reversed thioester analogue **1c** was prepared as shown in Schemes 2 and 3. The amine **4** was prepared (Scheme 2) starting from 1-azido-3-iodo propane **2**, which was prepared according to literature procedures.[6] Reaction of **2** with the lithium salt of tris(methylthio)-methane at −78° gave 1-azido-4,4,4-tris(methylthio)butane **3**

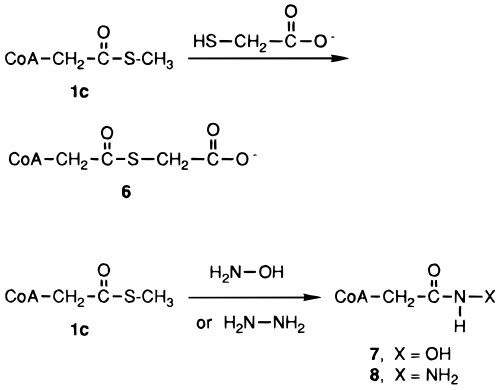
Scheme 2



Scheme 3



Scheme 4



in 91% yield.[7] Reduction of the azide using triphenylphosphine in aqueous THF gave amine **4** in 77% yield, which was isolated as the hydrochloride salt by extraction into dilute HCl.[8] Reaction of **4** with **1b** (Scheme 3) gave the trithioortho ester CoA analogue **5**. Hydrolysis of **5** in dilute aqueous HCl at pH 1.8 yielded the reversed thioester CoA analogue **1c**, which was isolated in 82% yield from **1b**.

The reversed succinate thioester **6**, the hydroxamate **7**, and the hydrazide **8** analogues were prepared from **1c** as shown in Scheme 4. The reactions were carried out using a 2.0 M concentration of mercaptoacetic acid, hydroxylamine, or hydrazine, respectively. The formation of **6** was complete in 20 h, and formation of **7** and **8** were complete within 3 h at room temperature as observed by analytical reverse-phase HPLC. Purification by reverse-phase HPLC gave **6** in 56% yield, **7** in 61% yield, and **8** in 55% yield.

CoA Analogues as Inhibitors of CoA Ester-Utilizing Enzymes. Incubation of the acetoacetyl-CoA thiolase from *Alcaligenes eutrophus* with **1c** resulted in time dependent inactivation of the enzyme. The rate and extent of inactivation increased with increasing concentration of **1c** (Figure 2). A reciprocal plot of 1/(rate of inactivation) vs 1/[**1c**] (not shown)

(6) Pearson, W. H.; Bergmeier, S. C.; Chytra, J. A. *Synthesis* **1990**, 156. Khoukhi, M; Vaultier, M.; Carrié, R. *Tetrahedron Lett.* **1986**, *27*, 1031.

(7) Barbero, M.; Cadamuro, S.; Degani, I.; Dughera, S.; Fochi, R. *J. Chem. Soc., Perkin Trans. I* **1993**, 2075.

(8) Knouzi, M.; Vaultier, M.; Carrié, R. *Bull. Soc. Chem. Fr.* **1985**, 815.

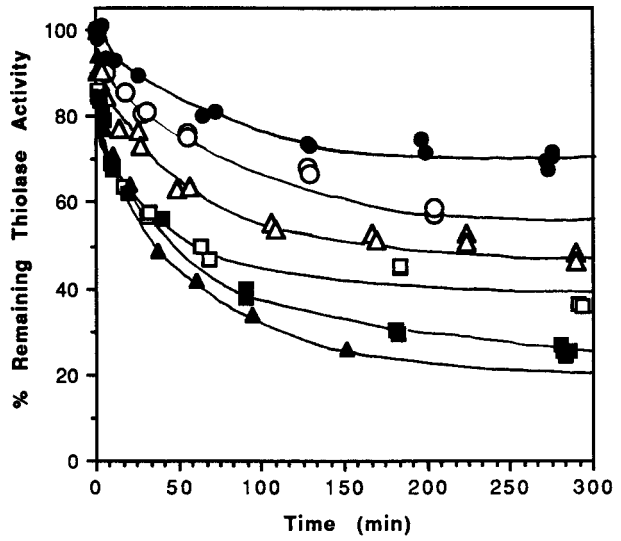


Figure 2. Time-dependent inhibition of thiolase in the presence of **1c**: (●) 10.4 μM, (○) 20.8 μM, (△) 41.6 μM, (□) 83.2 μM, (■) 111 μM, and (▲) 180 μM. Assays were conducted as described under Experimental Procedures.

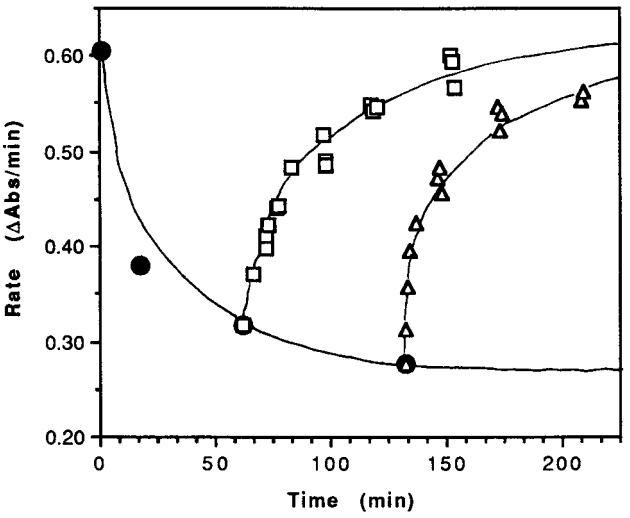


Figure 3. Time-dependent reactivation of thiolase after incubation with 1c. (●) indicates activity immediately after dilution into buffer, (□) indicates activity after a specified time after dilution into buffer, (△) indicates activity after dilution into buffer containing 106 μM hydroxylamine. Assays were conducted as described under Experimental Procedures.

gave an estimated K_I of 100 μM and a V_{max} for inactivation of 2×10^{-3} s^{-1}. Inclusion of CoA in the inactivation reaction resulted in partial protection from inactivation, with 60 μM CoA causing a 2-fold decrease in the rate of inactivation by 117 μM **1c**. Low concentrations of thiol had no measurable effect on the rate or final extent of inactivation, as identical results were obtained in the presence and absence of 5 mM mercaptoethanol. Upon dilution of the inhibited enzyme sample, thiolase activity was regained at a rate of 4×10^{-4} s^{-1} (Figure 3). Dilution into a buffer containing 106 μM hydroxylamine resulted in a rate of reactivation at least 2-fold greater than the rate when diluted into buffer alone (Figure 3).

Analogues **1c** and **6** were also tested as inhibitors of other acyltransferase enzymes, with results summarized in Table 1. **1c** exhibited competitive inhibition of carnitine acetyltransferase with a K_i of 110 μM, about double the K_m for the natural substrate acetyl CoA. No time-dependent inhibition was observed upon incubation of the enzyme with **1c** in the presence or absence of carnitine. In addition, no thiomethane was detected by reaction with DTNB upon incubation of the enzyme with **1c** and carnitine. Carnitine acetyltransferase was also incubated with **1c** in the presence of thiocarnitine,[9] but again no time-dependent inhibition was observed. Analogue **1c** was a very poor inhibitor of chloramphenicol acetyltransferase, with <25% inhibition observed in the presence of 200 μM **1c** (K_m for acetyl-CoA = 32 μM), and no time dependent inhibition was observed in the presence or absence of chloramphenicol. The reversed thioester analogue of succinyl-CoA **6** was tested as an inhibitor of the enzymes succinyl-CoA synthetase and succinyl-CoA:3-oxoacid CoA transferase. No time dependent inactivation of either enzyme was observed, but **6** was a competitive inhibitor of succinyl-CoA synthetase, with a K_i of 40 μM (K_m for acetyl-CoA is 7 μM). No inhibition of succinyl-CoA:3-oxoacid CoA transferase was observed, though the concentrations of inhibitor used were well above the relatively high K_m of 6.5 mM for succinyl-CoA.

Table 1. Enzyme Inhibition by Reversed Thioester Substrate Analogs

enzyme	K_m[a] (μM)	K_i (**1c** or **6**)[b] (μM)
thiolase	350[c]	100[d]
carnitine acetyltransferase	50	110
chloramphenicol acetyltransferase	32	>400
succinyl-CoA synthetase	7	40
CoA transferase	6500[e]	>300

[a] K_m for acetyl-CoA with thiolase and carnitine and chloramphenicol acetyltransferases, K_m for succinyl-CoA with succinyl-CoA synthetase and succinyl-CoA:3-oxoacid CoA transferase. [b] K_i for **1c** with thiolase and carnitine and chloramphenicol acetyltransferases, K_i for **6** with succinyl-CoA synthetase and CoA transferase. [c] In thiolase catalyzed exchange of radiolabeled CoA into acetyl-CoA, K_m for condensation is 1−2 mM (ref 14). [d] K_I for time-dependent inhibition ($k_{inact} = 2 \times 10^{-3}$ s^{-1}). [e] Reference 16.

Table 2. Affinity of Citrate Synthase for Acetyl-CoA Analogs

compd	K_i (or K_m)[a] (M)
CoA-S-C(O)-CH_3 (**13**)	1.6×10^{-5} (K_m)
CoA-CH_2-CO_2^--(H^+)[b] (**14**)	1.6×10^{-8}
CoA-CH_2-C(O)-NH_2[b] (**15**)	2.8×10^{-8}
CoA-CH_2-C(O)-NH-OH (**7**)	6×10^{-9}
CoA-CH_2-C(O)-NH-NH_2 (**8**)	3.7×10^{-7}

[a] At pH 8.0. [b] Reference 2.

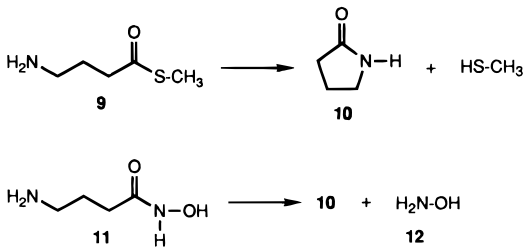
The hydroxamate **7** and hydrazide **8** analogues were tested as inhibitors of citrate synthase. The K_i values, which represent dissociation constants for **7** or **8** from the citrate synthase−oxaloacetate complex, are shown in Table 2. The hydroxamate **7** was found to be a potent competitive inhibitor, with a K_i 2700 times lower than the K_m for acetyl CoA. No slow onset of inhibition was detected. The hydrazide **8** was also found to be a competitive inhibitor, with a K_i 43-fold lower than the K_m for acetyl CoA. The hydroxamate **7** was also tested as an inhibitor of malate synthase and was found to be a competitive inhibitor with a K_i of 5 μM, 5-fold lower than the K_m for acetyl-CoA.

Discussion

Synthesis of CoA Analogues. While the methodology previously developed in this lab for the synthesis of CoA ester analogues has proven quite versatile, the analogues described here pose special synthetic problems. Analogue **1c** could not be prepared directly by previous methods as the amine nucleophile **9** needed for the aminolysis reaction of **1b** as in Scheme 1 would cyclize to the lactam **10** (Scheme 5) rather than

(9) Duhr, E. D.; Mauro, J. M.; Clennan, E. L.; Barden, R. E. *Lipids* **1983**, *18*, 382.

Scheme 5



undergoing the desired reaction. This problem is also encountered in the synthesis of **6**. Likewise, attempted synthesis of the hydroxamate analogue **7** by aminolysis of **1b** with **11** resulted in cyclization of the amine to the lactam **10**, releasing hydroxylamine **12** (Scheme 5) which reacted rapidly with the thioester of **1b** to give an undesired product. Synthesis of the hydrazide **8** could also face cyclization problems as observed with **11** and has the added complication of the potentially nucleophilic acylhydrazine functionality. The extension of our methodology as described here allows for the facile, routine synthesis of these acyl-CoA analogues not previously available.

The key to the synthesis of analogue **1c** was the use of a trithioortho ester as a protected thioester. The synthesis of several tris(methylthio)ortho esters by reaction of the corresponding alkyl halides with the lithium salt of tris(methylthio)-methane has been recently reported,[7] and the conversion of **2** to **3** followed a similar procedure (Scheme 2). Slow generation of the lithium salt of tris(methylthio)methane at low temperature and subsequent dropwise addition of **2** was found to be critical for high yield of **3**. The aminolysis reaction of **1b** with **4** to form **5** (Scheme 3) proceeded as in the synthesis of other CoA analogues.[2] The insolubility of **5** in anhydrous organic solvents precluded the hydrolysis of the trithioortho ester using fluoboric acid in DMSO or THF with very low water concentration as described previously.[7] Instead, the trithioortho ester **5** was readily converted to the thioester **1c** by acidifying the aqueous solution of **5** to pH 1.8 with aqueous HCl followed by incubation at room temperature. The reaction was complete in 20 h, and the conditions were compatible with other functionality in the CoA molecule, as no significant side products were observed by analytical reverse-phase HPLC. The overall yield of 82% from **1b** to **1c** is similar to reported yields in the hydrolysis procedure using fluoboric acid.[7]

The utility of **1c** as a CoA analogue synthon was demonstrated by the syntheses of the reversed succinate thioester **6** and the hydroxamate **7** and hydrazide **8** analogues (Scheme 4). The synthesis of **6** by thioester exchange demonstrates that the simplest reversed thioester **1c** can be used to prepare the equivalent reversed thioesters corresponding to CoA esters of different acids. This permits preparation of reversed thioesters specific to a CoA ester utilizing enzyme of interest. The syntheses of **7** and **8** demonstrate the use of **1c** for the introduction of other functionality at the site of the thioester. The method shown in Scheme 4 is quite efficient and may be useful for the introduction of other nucleophilic functionality at this position.

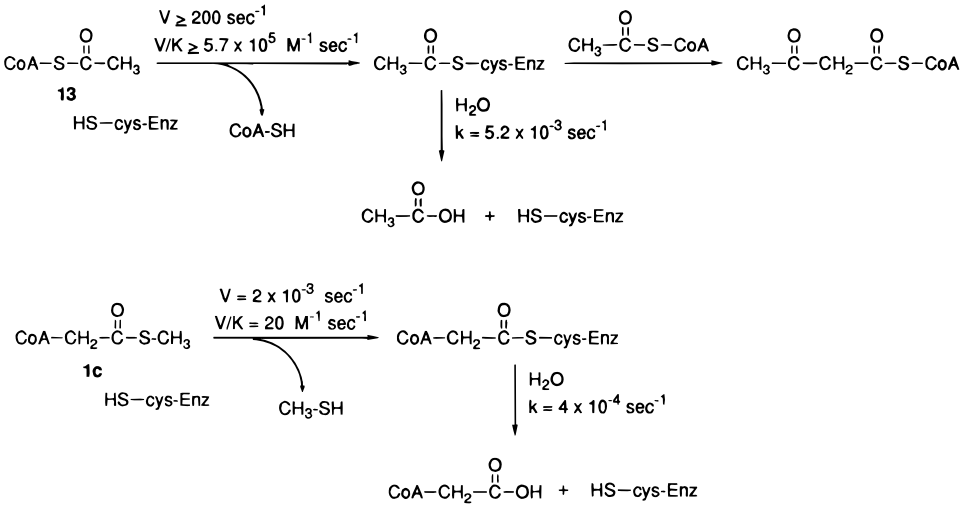
Enzyme Inhibition Studies with Reversed Thioester 1c. The methyl thioester **1c** is the simplest reversed thioester isostere of a CoA ester. It is actually an isomer of propionyl-CoA rather than acetyl-CoA, differing from propionyl-CoA in that the methylene group and sulfur atom on each side of the carbonyl group are interchanged. As many acetyl-CoA utilizing enzymes accept propionyl-CoA as an alternate substrate,[10,11] **1c** was viewed as a potential probe of the acetyl-CoA dependent acetyl transferases. **1c** was expected to bind to these enzymes similarly to formation of the normal Michaelis complex with acetyl-CoA. However, the reversed orientation of the thioester could make this analogue unreactive in acyl transfer or it could undergo acyl transfer with the resulting covalent attachment being to the CoA moiety rather than an acetyl group.

Studies of **1c** as a potential enzyme inhibitor were conducted with the thiolase from *Alcaligenes eutrophus*, which catalyzes the reversible reaction shown in Scheme 6.[12,13] In the initial step, an acetyl group is transferred from acetyl-CoA **13** to an active site residue identified as Cys-89 in the highly homologous thiolase from *Zoogloea ramigera*.[12,14] This acetyl enzyme reacts with a second equivalent of acetyl-CoA to form the product acetoacetyl-CoA. The rate constant for acetyl-enzyme formation with the *Zoogloea* thiolase has been determined to be at least 200 s^{-1}, 2.8-fold faster than the overall condensation reaction.[14] In a much slower reaction, the acetyl-enzyme may be hydrolyzed to form acetate and free enzyme, with a rate constant of 5.2×10^{-3} s^{-1}. When **1c** was incubated with thiolase, inactivation of the enzyme was observed. The inactivation displayed saturation kinetics, with an estimated K_I of 100 μM and a rate of inactivation of 2×10^{-3} s^{-1} when extrapolated to saturating inhibitor. This is consistent with transfer of the CoA moiety to the active site nucleophilic cysteine, in a reaction which is 10^5-fold slower than acetyl-enzyme formation from acetyl-CoA. Partial protection from inactivation was observed when CoA was added at a concentration near the K_m for CoA of 40−50 μM observed in thiolase-catalyzed exchange of radiolabeled CoA into acetyl-CoA.[14] This provides further evidence for inactivation due to reaction of **1c** in the thiolase active site. Inactivation did not go to completion but approached a steady value of activity which decreased with increasing inhibitor concentration. Upon dilution of the inactivated enzyme, reactivation was observed at a rate of 4×10^{-4} s^{-1}. This is consistent with hydrolysis of the acyl-enzyme, at a rate more than 10-fold slower than hydrolysis of the natural acetyl-enzyme intermediate. Reactivation occurred at a greater rate when the inactivated enzyme was diluted into an aqueous solution of hydroxylamine. This further supports the formation of a thioester-linked intermediate with the enzyme, as thioesters are especially reactive toward hydroxylamine.[15] The extent of enzyme inactivation at equilibrium represents the ratio of the rates of acylation and deacylation of the enzyme at each concentration of **1c**.

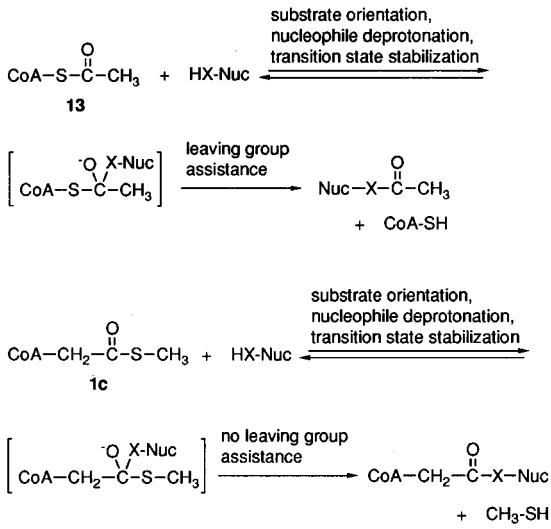
Several factors are expected to facilitate acetyl-enzyme formation by thiolase. These include binding of acetyl-CoA in position for acetyl transfer to the thiol group of Cys-89, deprotonation of the nucleophilic thiol group either prior to or in concert with initiation of acetyl transfer, stabilization of the tetrahedral intermediate or transition state, and stabilization and/or protonation of the thiolate leaving group (Scheme 7). Thiolase is also expected to effectively bind **1c** in position for acyl transfer, as the necessary positioning of **1c** in the active

(10) Billhardt, U. M.; Stein, P.; Whitesides, G. M. *Bioorg. Chem.* **1989**, *17*, 1.
(11) Brandange, S.; Josephson, S.; Mahlen, A.; Morch, L. *Acta Chem. Scand. B* **1984**, *38*, 695.
(12) Davis, J. T.; Chen, H.-H.; Moore, R.; Nishitani, Y.; Masamune, S.; Sinskey, A. J.; Walsh, C. T. *J. Biol. Chem.* **1987**, *262*, 90.
(13) Peoples, O. P.; Sinskey, A. J. *J. Biol. Chem.* **1989**, *264*, 15293.
(14) Thompson, S.; Mayerl, F.; Peoples, O. P.; Masamune, S.; Sinskey, A. J.; Walsh, C. T. *Biochemistry* **1989**, *28*, 5735.
(15) Bolanowski, M. A.; Earles, B. J.; Lennarz, W. J. *J. Biol. Chem.* **1984**, *259*, 4934.

Scheme 6



Scheme 7



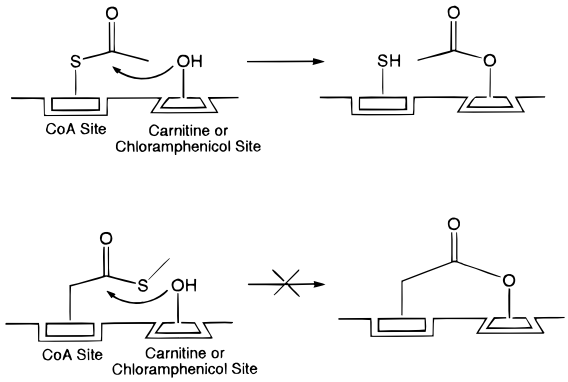


Figure 4. Top: representation of the reaction catalyzed by carnitine acetyltransferase and chloramphenicol acetyltransferase. Bottom: representation of anticipated bisubstrate adduct formation by carnitine acetyltransferase or chloramphenicol acetyltransferase in the presence of **1c** and carnitine or chloramphenicol, respectively.

site should be very similar to that for acetyl-enzyme formation from acetyl-CoA. The shorter C−C vs C−S bond lengths and conformational differences may have some effects on this proper positioning, though other acetyl-CoA analogues having this CH_2 for S substitution are potent inhibitors of citrate synthase.[2−4] Deprotonation of the nucleophilic thiol group should also be efficient in the complex with **1c**. The structures of the tetrahedral intermediates or transition states appear very similar for the two reactions, thus the enzyme may also provide some stabilization of the tetrahedral intermediate in the reaction with **1c**. However, the leaving group in reaction with **1c** is in the wrong position relative to the natural reaction for stabilization and/or protonation by the enzyme. It is also possible that the methanethiolate leaving group is bound in the active site in such a way that its expulsion is impeded by the enzyme. It is these leaving group factors that are probably responsible for the much slower acylation of the enzyme by **1c** relative to acetyl enzyme formation with acetyl-CoA.

A rate constant for the uncatalyzed (base catalyzed) acyl transfer reaction of a CoA ester with a thiol in aqueous solution of 0.18 M^{-1} s^{-1} has been determined at pH 8.2.[16] The second-order rate constant of 20 M^{-1} s^{-1} for reaction of thiolase with **1c** is only about 200-fold greater than this uncatalyzed reaction, while the corresponding second-order rate constant for acetyl-enzyme formation from acetyl-CoA (5.7×10^5 M^{-1} s^{-1}) is more than 10^4-fold faster than reaction with **1c** and more than 10^6-fold faster than the uncatalyzed reaction. The maximum rate of enzyme inactivation at saturating **1c** corresponds to an effective thioester concentration of only about 10 mM in the complex of **1c** with thiolase, relative to the second-order rate constant for the uncatalyzed reaction. This analysis illustrates that thiolase is a very poor catalyst for the autoacylation reaction with **1c**, relative to its catalysis of acetyl enzyme formation from acetyl-CoA.

It was anticipated that the reversed thioester analogue **1c** might react similarly with other acetyl transferases such as carnitine acetyltransferase and chloramphenicol acetyltransferase (Figure 4). Chase and Tubbs reported the alkylation of CoA by bromoacetyl-carnitine in the active site of carnitine acetyltransferase to form a covalent bisubstrate-adduct which inhibited the enzyme.[17] It was expected that displacement of methanethiol from **1c** by the carnitine hydroxyl group catalyzed by carnitine acetyltransferase could lead to a similar strong-binding CoA-carnitine bisubstrate adduct. As with thiolase, the transition state for acyl transfer would appear to have some similarities to that for the natural reaction and thus might be stabilized by the enzyme, perhaps facilitating acyl transfer. However, no time-dependent inhibition was observed, and no methanethiol was detected by reaction with DTNB, indicating that no acyl transfer

(16) Moore, S.; Jencks, W. P. *J. Biol. Chem.* **1982**, *257*, 10893.

(17) Chase, J. F. A.; Tubbs, P. K. *Biochem. J.* **1969**, *111*, 225.

of the CoA moiety to the carnitine hydroxyl occurred. **1c** also exhibited no time dependent inhibition of chloramphenicol acetyltransferase in the presence of chloramphenicol. The improper positioning of the leaving group, which results in a greatly decreased reaction rate with thiolase, apparently precludes any measurable reaction with the carnitine and chloramphenicol acetyltransferases.

The analogue of carnitine having the nucleophilic hydroxyl group replaced with a thiol group was prepared. This thiol-carnitine analogue has been shown to be accepted as a substrate for carnitine acetyltransferase, with a V_{max} of 5.7 s^{-1} (compared to 126 s^{-1} with the natural substrate, carnitine) and V/K of 3×10^4 M^{-1} s^{-1} (compared to 6×10^5 M^{-1} s^{-1} for the natural substrate).[9] It was anticipated that carnitine acetyltransferase might catalyze acyl transfer from **1c** to the more nucleophilic thiol group of the carnitine analogue to form a bisubstrate inhibitor. However, no time-dependent inhibition was observed, indicating that no significant acyl transfer occurred.

The enzyme succinyl-CoA:3-oxoacid CoA transferase catalyzes the transfer of the CoA moiety between succinate and a 3-oxoacid.[16,19,20] The initial step in catalysis is the reaction of an enzyme carboxylate group at the thioester of succinyl-CoA, displacing CoA to form a mixed anhydride between the enzyme and succinate. Similar reaction with the succinyl CoA analogue **6** or with **1c** could form a structure having the CoA moiety linked to the enzyme via a mixed anhydride. However, the absence of time dependent inhibition with either analogue indicates that such a reaction did not occur.

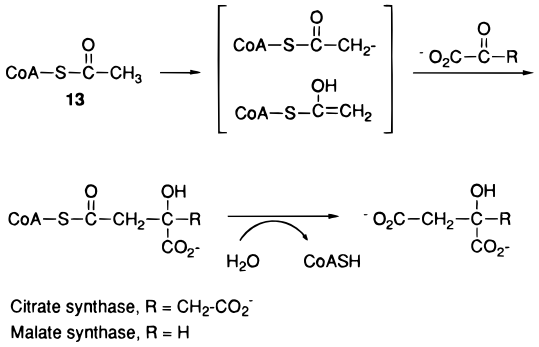
It is unclear why the reversed thioester inactivates thiolase by an apparent acyl-transfer reaction while not inactivating any of the other enzymes studied. Of possible significance is the differences in inherent nucleophilicity of the initial acyl acceptors used by these enzymes. While uncatalyzed acyl transfer from a thioester to a thiol occurs fairly readily (see above), such uncatalyzed acyl transfer to a hydroxyl nucleophile would appear to be much slower. An estimate for the rate of uncatalyzed acyl transfer from a thioester to a hydroxyl nucleophile may be obtained from the rate constant for hydrolysis of acetyl-CoA. The reported rate constant for acetyl-CoA hydrolysis in aqueous solution of $\leq 2 \times 10^{-7}$ s^{-1} corresponds to a second-order rate constant of $\leq 4 \times 10^{-9}$ M^{-1} s^{-1}.[18] This is more than 10^7-fold slower than the corresponding reaction with a thiol nucleophile and demonstrates that very large acceleration of this reaction would be necessary for observation of any reaction of **1c** catalyzed by the chloramphenicol or carnitine acetyltransferases. The lower inherent reactivity of the carboxylate nucleophile relative to a thiolate could similarly provide an explanation for the lack of time dependent inhibition of succinyl-CoA:3-oxoacid CoA transferase, in contrast to thiolase. Thus while proper positioning of thioester and nucleophile may be sufficient for modest acyl transfer to the thiol group of thiolase, it is not sufficient for acyl transfer to the substrate hydroxyl group in the reactions of chloramphenicol and carnitine acetyltransferases or the carboxylate group of succinyl-CoA:3-oxoacid CoA transferase. These nucleophilicity arguments do not explain the lack of acyl transfer to the thiol group of thiocarnitine by carnitine acetyltransferase. It is possible that the carnitine acetyltransferase does not efficiently generate a thiol(ate) nucleophile, since size, acidity, and other features of the thiol group are very different from its natural hydroxyl substrate. Alternatively, inherent nucleophilicity of the acyl acceptor may not be important. Instead thiolase may have a fortuitously placed acidic residue which assists in expulsion of the leaving group from the reversed thioester despite the incorrect leaving group trajectory, while the other enzymes may not have such an acidic group. Current data does not permit distinction between these and other possible explanations.

The results of inhibition studies with the reversed thioester **1c** has some analogy to efforts to mimic enzyme catalysis using simple model systems[21] and catalytic antibodies.[21−23] While these approaches to enzyme mimetics have shown substantial success, they have fallen far short of the efficiency of enzymes. Difficulties in achieving enzyme-like efficiency may stem from an inability to provide for catalysis of all aspects of the reaction. While catalytic antibodies may be efficient at stabilization of one or more high energy species along a reaction coordinate, efforts to build in functional groups for acid and/or base catalysis have had only partial success.[22] The reactions of CoA ester utilizing enzymes with reversed thioester analogues described here indicate that substrate orientation and stabilization of the tetrahedral intermediate, in the absence of all aspects of acid/base catalysis, are not sufficient for efficient catalysis, even with the remaining elements of transition state stabilizing power of a natural enzyme. This is further supported by the many examples of the elimination of enzyme activity by mutation of a single active site acidic or basic group, even though the substrate orientation and many aspects of transition state stabilizing power of the enzyme is presumably preserved.[24−27] The results of inhibition studies with the reversed thioester **1c** thus offer an additional approach to analyzing the importance of various factors to the catalytic power of enzymes.

Enzyme Inhibition Studies with Hydroxamate 7. Synthesis and inhibition studies with the hydroxamate analogue of acetyl-CoA **7** were inspired by numerous literature reports of hydroxamates as enzyme inhibitors. Hydroxamate analogues of peptides are potent inhibitors of thermolysin and other zinc-containing proteases.[28−30] Mechanistic and structural studies indicate that the inhibition is due to the formation of a bidentate complex in which both the hydroxyl and carbonyl oxygens of the hydroxamate functionality are coordinated to the zinc ion.[29,30] Similar coordination to zinc ion is observed in the structure of the complex of a class II aldolase with a hydroxamate-containing substrate analogue inhibitor.[31] Hydroxamate compounds are also potent inhibitors of the zinc-containing enzyme carbonic anhydrase, though in this case the zinc ion is coordinated only to the ionized nitrogen atom of the hydroxamate.[32] A hydroxamate analogue of D-xylose is a very potent inhibitor of xylose isomerase, exhibiting a K_i at least 10^6-fold lower than the K_m

(18) Gilbert, H.; Lennox, B.; Mossman, C.; Carle, W. *J. Biol. Chem.* **1981**, *256*, 7371.
(19) Whitty, A.; Fierke, C. A.; Jencks, W. P. *Biochemistry* **1995**, *34*, 11678.
(20) Rochet, J.-C.; Bridger, W. A. *Protein Science* **1994**, *3*, 975.
(21) Kirby, A. J. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 707.
(22) Jacobsen, J. R.; Schultz, P. G. *Curr. Opinion Struct. Biol.* **1995**, *5*, 818.
(23) MacBeath, G.; Hilvert, D. *Chem. Biol.* **1996**, *3*, 433.
(24) Palmer, M. A. J.; Differding, E.; Gamboni, R.; Williams, S. F.; Peoples, O. P.; Walsh, C. T.; Sinskey, A. J.; Masamune, S. *J. Biol Chem.* **1991**, *266*, 8369.
(25) Roberts, J. R.; Mitchell, G. A.; Miziorko, H. M. *J. Biol. Chem.* **1996**, *271*, 24604.
(26) Man, W. J.; Li, Y.; O'Connor, C. D.; Wilton, D. C. *Biochem. J.* **1991**, *280*, 521.
(27) Sampson, N. S.; Kass, I. J. *J. Am. Chem. Soc.* **1997**, *119*, 855.
(28) Nishino, N.; Powers, J. C. *Biochemistry* **1978**, *17*, 2846. Grobelny, D.; Poncz, L.; Galardy, R. E. *Biochemistry* **1992**, *31*, 7152.
(29) Holmes, M. A.; Matthews, B. W. *Biochemistry* **1981**, *20*, 6912.
(30) Izquierdo-Martin, M.; Stein, R. L. *J. Am. Chem. Soc.* **1992**, *114*, 325.
(31) Dreyer, M. K.; Schulz, G. E. *J. Mol. Biol.* **1996**, *259*, 458.
(32) Scolnick, L. R.; Clents, A. M.; Liao, J.; Crenshaw, L.; Hellberg, M.; May, J.; Dean, T. R.; Christianson, D. W. *J. Am. Chem. Soc.* **1997**, *119*, 850.

Scheme 8



for glucose. The crystal structure of the enzyme−inhibitor complex revealed bidentate coordination to an active site magnesium ion in a fashion similar to the metal coordination in the zinc proteases, with the carbonyl oxygen also coordinated to a second active site magnesium ion.[33] Coordination of the hydroxamate moiety to magnesium ion is also responsible for potent inhibition of the enzymes enolase[34] and ketol-acid reductoisomerase[35] by substrate analogues containing a hydroxamate functionality. Hydroxamates have also been shown to be potent inhibitors of certain enzymes in which metal coordination is not involved in formation of the inhibitor complex, including triosephosphate isomerase[36] and D-glucose-6-phosphate isomerase.[37]

The hydroxamate **7** was tested as an inhibitor of the enzymes citrate synthase and malate synthase, which catalyze the reactions shown in Scheme 8.[3,4,38,39] Malate synthase requires magnesium ion for activity[38] while citrate synthase requires no metal ions.[3,4,39] The hydroxamate **7** was found to be a very potent inhibitor of citrate synthase, with a K_i 2700 times lower than K_m for acetyl-CoA. In contrast, only modest inhibition of malate synthase by **7** was observed, with a K_i only 5-fold lower than the K_m for acetyl-CoA. As with other metal ion-containing enzymes, the 5-fold enhanced binding of **7** to malate synthase, relative to acetyl-CoA binding, is probably due to coordination of the hydroxamate to the metal ion. While the nature of the coordination complex is unknown, the modest inhibition is consistent with metal coordination by only the carbonyl oxygen atom. Such coordination is similar to the expected binding mode of acetyl-CoA, as proposed for the related divalent metal dependent acetyl-CoA utilizing enzyme HMG-CoA lyase.[40] The enhanced binding of **7** relative to acetyl-CoA may be due to the greater electron density and resulting better metal coordination of the hydroxamate carbonyl oxygen relative to the carbonyl oxygen of acetyl-CoA.

The mechanism and structure of citrate synthase has been extensively studied using synthetic CoA analogues as transition state mimics.[2−4] The previously reported carboxylate analogue **14**, proposed to mimic the enol or enolate intermediate, was found to bind to the enzyme with a K_i 1000-fold lower than the K_m for acetyl CoA at pH 8 (Table 1).[2] The corresponding primary amide analogue **15** was found to bind with a K_i 570 times lower than the same K_m.[2] The hydroxamate analogue **7** is an even better inhibitor and is the best inhibitor of citrate synthase at pH 8 prepared thus far. The hydrazide analogue **8** exhibited modest inhibition with a K_i 43 times lower than K_m.

Structures of the complexes of hydroxamate inhibitors of several enzymes including thermolysin,[29] triosephosphate isomerase,[36] xylose isomerase,[33] and fuculose aldolase[31] have provided valuable insights into the mechanisms of these enzymes. Similar studies are likely to be valuable with acetyl-CoA utilizing enzymes, using the hydroxamate analogue **7**. In structural studies of the complex of citrate synthase with the amide analogue **15**, the orientation of the amide in the active site has never been certain as the nitrogen and oxygen atoms are not clearly distinguishable in the X-ray structure.[3,4] With the hydroxamate analogue **7**, the nitrogen has the readily identifiable hydroxyl oxygen atom attached which should provide a clear picture of the orientation of binding. Structural studies of the citrate synthase complex with **7** may help to further clarify the mechanism of citrate synthase.

Conclusion. The work described here has solved some limitations in the previously described method for the synthesis of CoA analogues. Analogues are now accessible having a wider range of functionality in place of the thioester moiety. This methodology has been useful in the design of novel inhibitors of thiolase and citrate synthase. This methodology should facilitate further studies of mechanism and structure−activity relationships in CoA ester-utilizing enzymes using synthetic CoA analogues as probes.

Experimental Section

General Experimental. Thiolase (*Alcaligenes eutrophus*) was a gift of Dr. David Martin of Metabolix, Inc. Other enzymes were from Sigma. Reagents were obtained from Aldrich, Sigma, or Mallinckrodt and used as supplied. Methylene chloride (CH_2Cl_2), pyridine, and acetonitrile (CH_3CN) were distilled from calcium hydride, and tetrahydrofuran (THF) was distilled from sodium. For ^{1}H NMR experiments, TMS (0 ppm) and HOD (4.8 ppm) were used as internal references when $CDCl_3$ and D_2O were used as solvents, respectively. For ^{13}C experiments, $CDCl_3$ (central peak at 77 ppm) was used as a reference. 200 MHz ^{1}H NMR and 50 MHz ^{13}C experiments were performed using a Varian Gemini 200 spectrometer, and 400 MHz ^{1}H and 100 MHz ^{13}C NMR experiments were performed using a Varian XL-400. Analytical and preparative scale HPLC experiments were performed using a Perkin-Elmer 250 HPLC with a PE LC-235 diode array detector and a gradient of methanol (solvent B) in aqueous potassium phosphate (solvent A: 50 mM, pH 4.5 analytical; 10 mM, pH 4.5 preparative). Analytical HPLC was done on a Rainin Microsorb C-18 column (4.6 mm × 25 cm) with monitoring at 215 and 260 nm. Compounds were eluted with a flow rate of 1 mL/min with 5% solvent B for 2 min, followed by a linear gradient to 60% solvent B over 12 min, and then maintenance at 60% solvent B. Preparative scale HPLC was done on a Rainin Microsorb C-18 column (21.4 cm × 25 cm) with monitoring at 215 and 280 nm and a flow rate of 10 mL/min with solvent gradients as noted below for individual compounds. Mass spectral analysis was performed at the University of Riverside Mass Spectrometry Facility, Riverside, CA. Elemental analysis was performed by E + R Microanalytical Laboratory, Madison, NJ. Kinetic experiments were performed using a Shimadzu Model UV-1201 spectrophotometer using the supplied software. K_i values were determined by computer fitting of data to the equation $v = (V_{max}[S])/(K_m(1 + [I]/K_i) + [S])$.[41] The concentrations of CoA analogue solutions were determined using $\epsilon_{260} = 15\,400\ M^{-1}\ cm^{-1}$.

(33) Allen, K. N.; Lavie, A.; Petsko, G. A.; Ringe, D. *Biochemistry* **1995**, *34*, 3742.
(34) Wedekind, J. E.; Poyner, R. R.; Reed, G. H.; Rayment, I. *Biochemistry* **1994**, *33*, 9333.
(35) Aulabaugh, A.; Schloss, J. V. *Biochemistry* **1990**, *29*, 2824.
(36) Zhang, Z.; Sugio, S.; Komives, E. A.; Liu, K. D.; Knowles, J. R.; Petsko, G. A.; Ringe, D. *Biochemistry* **1994**, *33*, 2830.
(37) Bonnette, C.; Salmon, L.; Gaudemer, A. *Tetrahedron Lett.* **1996**, *37*, 1221.
(38) Durchschlag, H.; Biedermann, G.; Eggerer, H. *Eur. J. Biochem.* **1981**, *114*, 255.
(39) Karpusas, M.; Branchaud, B.; Remington, S. J. *Biochemistry* **1990**, *29*, 2213. Kurz, L. C.; Drysdale, G. R.; Riley, M. C.; Evans, C. T.; Srere, P. A. *Biochemistry* **1992**, *31*, 7908.
(40) Hruz, P. W.; Anderson, V. E.; Miziorko, H. M. *Biochim. Biophys. Acta* **1993**, *1162*, 149.
(41) Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103.

1,1,1-Tris(methylthio)-4-azidobutane (3). To a solution of trismethylthiomethane (1.92 mL, 14.5 mmol) in THF (15 mL) at −78 °C under N_2 was added *n*-butyllithium (5.8 mL, 14.5 mmol, 2.5 M in hexane) dropwise. After stirring for 2 h at −78 °C, a solution of 1-azido-3-iodopropane **2**[6] (3.06 g, 14.5 mmol) in THF (15 mL) that had been precooled to −78 °C was added dropwise via cannula. When addition was complete, the reaction mixture was stirred an additional 3.5 h at −78 °C whereupon it turned a bright orange color. The reaction was then placed in a cold room at 6 °C for an additional 3.5 h of stirring whereupon the reaction mixture became a deep burgundy color. The reaction was poured into saturated aqueous ammonium chloride (100 mL) and extracted with ether (2 × 100 mL). The combined ether extracts were washed successively with 5% aqueous NaOH (50 mL), H_2O (2 × 50 mL), and saturated aqueous NaCl (1 × 20 mL) and dried over $MgSO_4$, and the solvent removed by rotary evaporation to leave compound **3** as a yellow oil (3.13 g, 91%) that was used without further purification. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.92−2.00 (m, 4H), 2.10 (s, 9H), 3.33−3.42 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.95, 24.63, 35.09, 51.22, 70.56.

1,1,1-Tris(methylthio)-4-aminobutane hydrochloride (4·HCl). To a solution of **3** (11 g, 46.4 mmol) in THF (50 mL) containing H_2O (1 mL, 56 mmol) was added triphenylphosphine (12.16 g, 46.4 mmol). After 15 min the reaction mixture became warm and was placed in an ice bath. The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was poured into ether (100 mL) and extracted with 1% HCl (3 × 100 mL). The combined aqueous extracts were washed with ether (50 mL) and lyopholized to give **4**·HCl (8.8 g, 77%) as an off-white powder. ^{1}H NMR (200 MHz, D_2O): δ 2.03 (m, 4H), 2.13 (s, 9H), 3.05 (t, 2H, J = 6.9 Hz). ^{13}C NMR (100 MHz, CD_3OD): 12.97, 24.54, 35.57, 40.52, 71.65. FABMS *m*/*z* 212 (M^+ for free base). Anal. Calcd for $C_7H_{18}ClNS_3$: C, 33.78; H, 7.70; N, 5.63; S, 38.65. Found: C, 34.07; H, 7.55; N, 5.60; S, 38.57.

Tris(methylthio)methyldethia-CoA (5). **4**·HCl (2.5 g, 10 mmol) was dissolved in 80 mL of H_2O. The pH was adjusted to 10.5 with aqueous NaOH, and the solution extracted 3 × 60 mL ether. The combined ether extracts were concentrated by rotary evaporation to a yellow oil which was dissolved in acetonitrile (5 mL) and added to a solution of **1b** (140 mg, 182 μmol) in H_2O (2.5 mL) which had been adjusted to pH 10 with aqueous NaOH. The pH was readjusted to 10, and the reaction was stirred at room temperature and monitored for product formation via analytical HPLC (retention time = 19.95 min, coeluted with amine **4**, product formation was followed by monitoring at 260 nm, at which only **5** absorbs). When the reaction was complete (20 h), the reaction mixture was poured into H_2O (30 mL), the pH adjusted to 10 with aqueous NaOH, and excess amine was extracted with ether (6 × 30 mL), with the pH readjusted to 10 with aqueous NaOH after each extraction as necessary. Preparative HPLC (5 min 5% A followed by a linear gradient to 60% A over 35 min and then maintenance at 60% B, product elutes between 40 and 50 min) yielded pure **5** (90% yield), aliquots of which were removed for mass spec. and NMR analysis. λ_{max} = 260 nm. ^{1}H NMR (as potassium salt, 400 MHz, D_2O): δ 0.77 (s, 3H), 0.90 (s, 3H), 1.63−1.72 (m, 2H), 1.83−1.89 (m, 2H), 2.03 (s, 9H), 2.43 (t, 2H, J = 6.4 Hz), 3.12 (t, 2H, J = 6.8 Hz), 3.40−3.50 (m, 2H), 3.55 (dd, 1H, J = 4.4, 9.6 Hz), 3.83 (dd, 1H, J = 4.4, 9.6 Hz), 4.01 (s, 1H), 4.22 (br s, 2H), 4.57 (br s, 1H), 6.16 (d, 1H, J = 3 Hz), 8.33 (s, 1H), 8.59 (s, 1H). FABMS: *m*/*z* 900 $[M - H^+]^-$.

Methylthiocarbonyldethia-CoA (1c). Methanol was removed from the preparative HPLC elutant containing **5** via rotary evaporation, the aqueous solution (40 mL) was adjusted to pH 1.8 with concentrated aqueous HCl, and the reaction monitored via analytical HPLC (retention time = 14.5 min). When the reaction was complete (22 h), the pH was adjusted to 7 with aqueous NaOH, and the solvent removed by lyopholization. Preparative HPLC (5 min 5% A followed by a linear gradient to 35% A over 35 min, product eluted between 25 and 35 min) yielded **1c** (126 mg, 149 μmol, 82% yield from **1b**). The product was pure as indicated by analytical HPLC and by ^{1}H NMR. λ_{max} = 260 nm. ^{1}H NMR (as potassium salt, 400 MHz, D_2O): δ 0.69 (s, 3H), 0.82 (s, 3H), 1.67−1.76 (m, 2H), 2.18 (s, 3H), 2.36 (t, 2H, J = 6.6 Hz), 2.53 (t, 2H, J = 7.2 Hz), 3.06 (t, 2H J = 6.8 Hz), 3.38 (t, 2H, J = 5.6), 3.49 (dd, 1H, J = 4.8, 10.0 Hz), 3.76 (dd, 1H, J = 4.8, 10.0 Hz), 3.95 (s, 1H), 4.17 (br s, 2H), 4.52 (br s, 1H), 6.17 (d, 1H, J = 5.6 Hz), 8.25 (s, 1H), 8.51 (s, 1H). FABMS: *m*/*z* 822 $[M - H^+]^-$. HRMS (FAB): $[M - 2H^+ + Na^+]^-$ calcd for $C_{24}H_{38}N_7O_{17}NaP_3S$ *m*/*z* 844.1156, found 844.1110. $[M - 3H^+ + 2Na^+]^-$ calcd for $C_{24}H_{37}N_7O_{17}Na_2P_3S$ *m*/*z* 866.0975, found 866.0930.

(Carboxymethylthio)carbonyldethia-CoA (6). To a solution of **1c** (5 mg, 6.1 μmol) in 0.1 M potassium phosphate buffer, pH 7 (2.5 mL) was slowly added mercaptotacetic acid (200 μL, 2.9 mmol), and the pH adjusted with 1 M aqueous NaOH as necessary to maintain a pH of 7. The reaction was stirred under nitrogen and monitored for product formation via analytical HPLC (retention time = 12.44 min). When the reaction was complete (20 h), preparative HPLC (5 min 5% A followed by a linear gradient to 40% A over 25 min and then maintenance at 40% A, product elutes between 25 and 33 min) yielded pure **6** (56% yield). λ_{max} = 260 nm. ^{1}H NMR (as potassium salt, 400 MHz, D_2O): δ 0.71 (s, 3H), 0.84 (s, 3H), 1.72−1.80 (m, 2H), 2.38 (t, 2H, J = 6.4 Hz), 2.60 (t, 2H, J = 7.2 Hz) 3.09 (t, 2H, J = 6.8 Hz), 3.39 (t, 2H, J = 6.4 Hz), 3.48−3.55 (m, 3H), 3.72−3.81 (m, 1H), 3.95 (s, 1H), 4.19 (br s, 2H), 4.54 (br s, 1H), 6.15 (d, 1H, J = 6 Hz), 8.30 (s, 1H), 8.85 (s, 1H). HRMS (FAB): $[M - H^+]^-$ calcd for $C_{25}H_{40}N_7O_{19}P_3$ *m*/*z* 866.123, found 866.119.

N-Hydroxycarboxamidodethia Coenzyme A (7). Aqueous hydroxylamine (1 mL, 4 M) that had been adjusted to pH 10 with aqueous NaOH was added to **1c** (6.4 mg, 7.8 μmol) in 1 mL of H_2O that had been adjusted to pH 10 with aqueous NaOH. The reaction was monitored by analytical HPLC (retention time = 11.2 min) and was complete in 3 h. The pH was adjusted to 4.5 and the product purified by preparative HPLC (5 min 5% A followed by a linear gradient to 35% A over 35 min, product eluted between 16 and 21 min) to yield pure **7** (3.9 mg, 61%). λ_{max} = 260 nm. ^{1}H NMR (as potassium salt, 400 MHz, D_2O): δ 0.68 (s, 3H), 0.80 (s, 3H), 1.62−1.72 (m, 2H), 2.07 (t, 2H, J = 7.4 Hz), 2.36 (t, 2H, J = 6.4 Hz), 3.06 (t, 2H J = 7.4 Hz), 3.37 (t, 2H, J = 6.4), 3.48 (dd, 1H, J = 4.4, 9.6 Hz), 3.73 (dd, 1H, J = 4.4, 9.6 Hz), 3.94 (s, 1H), 4.16 (br s, 2H), 4.52 (br s, 1H), 6.11 (d, 1H, J = 6.4 Hz), 8.25 (s, 1H), 8.50 (s, 1H). HRMS (FAB): $[M - H^+]^-$ calcd for $C_{23}H_{38}N_8O_{18}P_3$ *m*/*z* 807.1517, found 807.1505.

N-Aminocarboxamidodethia Coenzyme A (8). Aqueous hydrazine (2.5 mL 4 M) that had been adjusted to pH 9.5 with aqueous NaOH was added to **1c** (9.5 mg, 11.6 μmol) in 2.5 mL of H_2O that had been adjusted to pH 9.5 with aqueous NaOH. The reaction was monitored by analytical HPLC (retention time = 10.9 min) and was complete in 3 h. Excess hydrazine was removed by rotary evaporation, the pH adjusted to 4.5, and the product purified by preparative HPLC (5 min 5% A followed by a linear gradient to 25% A over 40 min, product eluted between 17 and 25 min). Lyophilization gave **8** (5.2 mg, 55%). λ_{max} = 260 nm. ^{1}H NMR (as potassium salt, 400 MHz, D_2O): δ 0.68 (s, 3H), 0.80 (s, 3H), 1.65−1.68 (m, 2H), 2.10−2.19 (m, 2H), 2.36 (t, 2H, J = 6.4 Hz), 3.05 (t, 2H, J = 6.8 Hz), 3.37 (t, 2H, J = 6.4), 3.48 (dd, 1H, J = 4.0, 10.0 Hz), 3.74 (dd, 1H, J = 4.0, 10.0 Hz), 3.92 (s, 1H), 4.16 (br s, 2H), 4.52 (br s, 1H), 6.11 (d, 1H, J = 6.0 Hz), 8.24 (s, 1H), 8.50 (s, 1H). HRMS (FAB): $[M - H^+]^-$ calcd for $C_{23}H_{39}N_9O_{17}P_3$ *m*/*z* 806.1677, found 806.1685.

Inhibition Studies of Citrate Synthase. For inhibition studies of citrate synthase with the acetyl CoA analogues **7** and **8**, assays were conducted in Tris buffer (0.1 M, pH 8), containing DTNB (0.1 mM), oxaloacetate (0.5 mM), citrate synthase (0.03 units, from porcine heart), and acetyl CoA (8 μM to 40 μM). Citrate synthase activity was assayed by measuring the formation of CoA by monitoring the increase in absorbance at 412 nm upon reaction of CoA with DTNB. For determination of inhibition constants, rates were measured at four and five concentrations of **7** and **8**, respectively.

Inhibition Studies of Thiolase. To study the inactivation of thiolase by **1c**, a sample of thiolase (from *Alcaligenes eutrophus*) was incubated at 0 °C with solutions of **1c** (10.4 μM to 111 μM) in Tris buffer solution (20 mM, pH 8) containing mercaptoethanol (5 mM) and EDTA (1 mM). At set periods of time, aliquots of the enzyme incubation were added to a Tris buffer solution (65 mM, pH 8) containing acetoacetyl CoA (60 μM), CoA (60 μM), and $MgCl_2$ (5 mM) for a total volume of 1 mL and the rate of hydrolysis of acetoacetyl CoA at was monitored at 303 nm. To study the reactivation of thiolase after incubation with

1c, a sample of enzyme was incubated with **1c** (150 μM) in a Tris buffer solution (20 mM, pH 8.1) containing mercaptoethanol (5 mM) and EDTA (1 mM). At set periods of time, an aliquot of this incubation was diluted 50-fold into a Tris buffer solution (20 mM, pH 8.1) containing mercaptoethanol (5 mM) and EDTA (1 mM). The activity was then measured by immediately diluting a 20 μL aliquot of this solution into an assay mixture containing Tris buffer (65 mM, pH 8.0), acetoacetyl CoA (60 μM), CoA (60 μM) and $MgCl_2$ (5 mM) for a total volume of 1 mL and monitoring the rate of hydrolysis of acetoacetyl CoA at 303 nm. When the activity had decreased to approximately 50% of the initial value, reactivation of thiolase activity was monitored by assaying the activity of the diluted solution of enzyme at various times until the enzyme regained nearly all of the initial activity. The first-order rate constant for reactivation was determined from a plot of the log of the rate less the rate of the reaction before the enzyme was incubated with inhibitor versus time since dilution. In a separate experiment, the concentrated enzyme plus **1c** was diluted into a buffer solution that contained 106 μM NH_2OH, and reactivation of thiolase activity was monitored by assaying the activity of this solution as described above.

To show that CoA could competitively protect the enzyme from inactivation, thiolase was incubated with 117 μM **1c** in the presence of 0−400 μM CoA. After 90 min of incubation, a 10 μL aliquot of this incubation was added to an assay mixture containing Tris buffer (65 mM, pH 8.0), acetoacetyl CoA (60 μM), CoA (60 μM), and $MgCl_2$ (5 mM) for a total volume of 1 mL. Remaining enzyme activity was determined by monitoring the rate of hydrolysis of acetoacetyl CoA at 303 nm. Activity remaining was scaled to a control incubation containing enzyme alone.

Inhibition Studies of Carnitine Acetyltransferase. To probe for time-dependent inhibition of carnitine acetyltransferase with **1c** in the presence of carnitine or thiocarnitine, carnitine acetyltransferase (from pigeon breast muscle) was incubated with **1c** (300 μM) and l-carnitine (1 mM) or D,L-thiocarnitine (0.5 mM) and DTT (2.5 mM) in potassium phosphate buffer (25 mM, pH 7.5). Aliquots were removed over the course of an hour and assayed for activity by diluting into a cuvette containing potassium phosphate buffer (25 mM, pH 7.5), 4,4′ dithiopyridine (PDS, 0.2 mM), l-carnitine (0.2 mM), and acetyl CoA (100 μM) for a total volume of 1 mL. Carnitine acetyltransferase activity was determined by measuring the formation of CoA by monitoring the increase in absorbance at 324 nm upon reaction of CoA with PDS. For inhibition studies of carnitine acetyltransferase with the acetyl CoA analogue **1c** to determine the K_i for **1c**, assays were conducted in potassium phosphate buffer (25 mM, pH 7.5), containing PDS (0.2 mM), l-carnitine (0.2 mM), carnitine acetyltransferase (0.03 units, from pigeon breast) and acetyl CoA (10−100 μM). For determination of inhibition constants, rates were measured at three concentrations of **1c**.

Inhibition Studies of Chloramphenicol Acetyltransferase. To probe for time-dependent inhibition of chloramphenicol acetyltransferase with **1c** in the presence of chloramphenicol, chloramphenicol acetyltransferase (from *E. coli*) was incubated with **1c** (200 μM) and chloramphenicol (50 μM) in Tris buffer (10 mM, pH 7.8). Enzyme activity was measured over the course of 75 min by diluting aliquots of the incubation mixture into Tris buffer (10 mM, pH 7.8) containing chloramphenicol (50 μM), acetyl CoA (126 μM), and DTNB (1 mM). Enzyme activity was determined by measuring the formation of CoA by monitoring the increase in absorbance at 412 nm upon reaction of CoA with DTNB.

Inhibition Studies of Succinyl-CoA:3-Oxoacid CoA Transferase. To probe for time-dependent inhibition of succinyl-CoA:3-oxoacid CoA transferase with **1c** or **6**, succinyl-CoA:3-oxoacid CoA transferase (from pig heart) was incubated with **1c** or **6** (150 μM) in Tris buffer (67 mM, pH 8.1) that contained $MgSO_4$ (5 mM) and succinate (0 or 25 mM). Aliquots were removed over the course of an hour and diluted into an assay mixture containing Tris buffer (67 mM, pH 8.1), acetoacetyl CoA (33 μM), succinate (10 mM), and $MgSO_4$ (5 mM) for a final volume of 1 mL. Activity was determined by measuring the rate of disappearance of acetoacetyl CoA at 303 nm.

Inhibition Studies of Succinyl CoA Synthetase. For competitive inhibition studies of succinyl CoA synthetase with the succinyl CoA analogue **6**, assays were conducted in potassium phosphate buffer (50 mM, pH 7.4), containing GDP (0.1 mM), $MgCl_2$ (10 mM), succinyl CoA synthetase (0.005 units, from porcine heart), and succinyl CoA (5−30 μM). Succinyl CoA synthetase activity was assayed by measuring decrease in absorbance at 230 nm, corresponding to the conversion of succinyl CoA to CoA. For determination of inhibition constants, rates were measured at four concentrations of analogue **6**.

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