Coenzyme A Hemithioacetals as Easily Prepared Inhibitors of CoA Ester-Utilizing Enzymes

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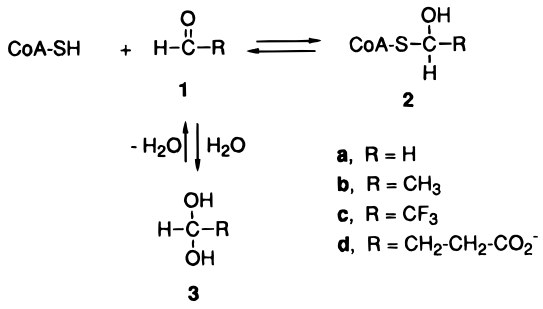
Hemithioacetals are formed by reactions of coenzyme A (CoA) with aldehydes in aqueous solution. Equilibria for hemithioacetal formation with four commercially available aldehydes and rate constants for hemithioacetal dissociation have been studied. The hemithioacetals are viewed as acyl-CoA analogs having a tetrahedral center in place of the planar trigonal thioester carbonyl carbon. These compounds may serve as mimics of the tetrahedral intermediate or transition state in the reactions of acyl-CoA dependent acyltransferase enzymes. The hemithioacetal generated by reaction of CoA with formaldehyde is a poor inhibitor of chloramphenicol acetyltransferase, with a K_i more than 6-fold higher than the K_m for the substrate acetyl-CoA. The hemithioacetals formed by reaction of CoA with acetaldehyde and trifluroacetaldehyde are substantially better inhibitors, with K_i values approximately 2.4-fold and 10-fold lower than the K_m values for acetyl-CoA, respectively. The hemithioacetal formed by reaction of CoA with succinic semialdehyde inhibits succinic thiokinase, with a K_i 4-fold lower than the K_m for the substrate succinyl-CoA. The CoA hemithioacetals provide a novel readily accessible new class of acyl-CoA analogs for use in mechanistic and structural studies of CoA ester-utilizing enzymes.

Thioesters of coenzyme A (CoA) serve as substrates in a wide variety of enzymatic processes.[1] Mechanistic and structural studies of CoA ester-utilizing enzymes have benefited from acyl-CoA analogs in which the thioester moiety is modified. CoA thioethers, prepared by alkylation of CoA, are potent inhibitors of several enzymes and have been utilized in mechanistic and structural studies of their target enzymes.[2] CoA dithioesters, prepared by acylation of CoA with a phenyl dithioester, have also been studied and though generally less stable than thioesters to nonenzymatic reactions, are resistant or sluggish toward enzyme-catalyzed reactions.[3,4] Nonhydrolyzable ketone isosteres, prepared by nonenzymatic[5,6] and more recently enzymatic[7] methods have found substantial utility in enzymology. Transition state analog inhibitors of several CoA ester-utilizing enzymes have been prepared. Some of these analogs can be made by simple alkylation of CoA[2] while other analogs require assembly of the CoA moiety from simpler starting materials.[7] Acyl-CoA analogs have been employed in studies of enzyme−inhibitor complexes by spectroscopic[8−10] and crystallographic[11−13] methods. These studies have demonstrated the value of acyl-CoA analogs in providing vital new insights into the roles of mechanism and structure in enzyme catalysis.

Scheme 1



We report here a novel and more easily prepared class of acyl-CoA analogs, the CoA hemithioacetals **2.** These analogs are generated in situ by reaction of CoA with aldehydes **1** in aqueous solution (Scheme 1). These compounds are unique among the known acyl-CoA analogs in that they may mimic the tetrahedral intermediate or transition state in enzyme-catalyzed acyl transfer reactions, such as the reactions of chloramphenicol acetyltransferase (Scheme 2) and succinic thiokinase (Scheme 3). CoA hemithioacetals have apparently not been utilized as enzyme inhibitors previously, though a hemithioacetal is believed to be an intermediate in the reaction of HMG-CoA reductase.[14] The CoA hemithioacetals are also somewhat similar to the hemithioacetal and acetal adducts formed by cysteine and serine proteases, respectively, in their inhibition by aldehyde and ketone derivatives of peptides.[15] This paper presents studies of the formation of the CoA hemithioacetals **2a**−**c** and the investigation of these compounds as inhibitors

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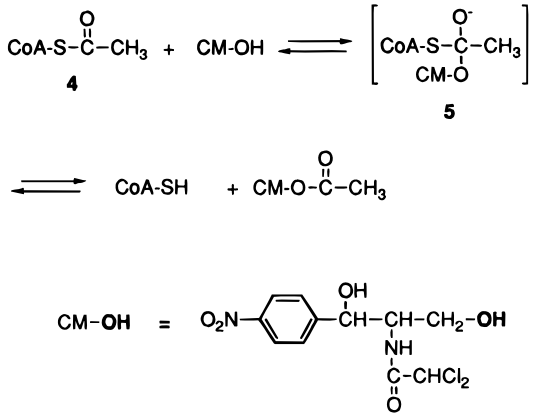
Scheme 2



Scheme 3

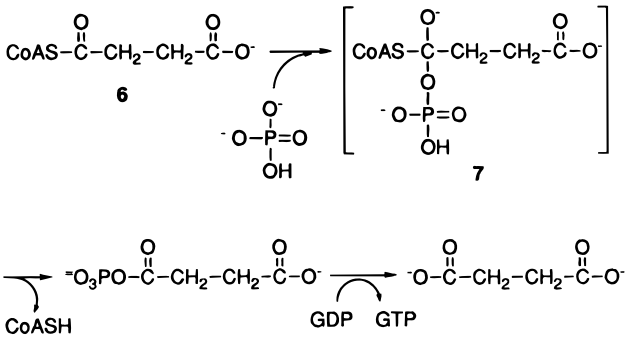


Table 1. Equilibrium and Dissociation Constants for CoA Hemithioacetals in H_2O

ald	K_{app}[a] (M^{-1})	K_{hyd}[b]	K_{eq}[c] (M^{-1})	k_{diss}[d] (s)
1a	350	2280	8.0×10^5	1.3×10^{-2}
1b	15	1.06	31	fast
1c	4	2.9×10^4	1.2×10^5	3.0×10^{-3}
1d	20	≥10	≥200	fast

[a] $K_{app} = [\mathbf{2}]/[\text{CoASH}]([\mathbf{1}] + [\mathbf{3}])$. [b] $K_{hyr} = [\mathbf{3}]/[\mathbf{1}]$ (data for **1a**−**c** from ref 16). [c] $K_{eq} = [\mathbf{2}]/[\text{CoASH}][\mathbf{1}]$. [d] At pH 7.0.

of chloramphenicol acetyltransferase. Also presented is the succinyl-CoA analog **2d** and its inhibition of the enzyme succinic thiokinase. The hemithioacetals show modest inhibition with K_i values severalfold lower than the K_m values for the respective substrates.

Results

Hemithioacetal Formation and Dissociation. Hemithioacetal **2** formation between CoA and aldehydes **1a**−**c** was studied in aqueous phosphate buffer (pH 7), the aldehydes existing in equilibrium with their hydrated forms **3** (Scheme 1). Apparent equilibrium constants were determined on the basis of the total amount of hydrated and unhydrated aldehyde and are shown in Table 1. Also shown are literature values for the equilibrium constants for aldehyde hydration[16] along with true equilibrium constants for hemithioacetal formation from free aldehyde, calculated from the observed equilibrium and hydration constants. Rate constants for hemithioacetal dissociation are also included. With formaldehyde (**1a**) and acetaldehyde (**1b**), equilibrium constants of 700 and 30 M^{-1}, respectively, were determined by ^1H NMR in D_2O and corrected for the previously determined solvent isotope effect to give the values shown in Table 1.[17,18] These correspond to true equilibrium constants of 8×10^5 and 31, respectively. The rate of the dissociation reaction of **2a** into CoA and **1a** was measured by coupling CoA formation with reaction with DTNB,[19] monitoring the increase in absorbance at 412 nm vs time after the initial burst. The dissociation reaction of **2b** was very rapid and not measureable. With trifluoroacetaldehyde (**1c**), an apparent equilibrium constant of 4 M^{-1} was determined by quantitation of free CoA of an equilibrated sample by reaction with DTNB, monitoring the initial burst in absorbance at 412 nm. Measurement of the rate of additional increase after the initial burst provided the rate of dissociation of the hemithioacetal. The apparent equilibrium constant was further verified by ^{19}F NMR using 20% D_2O in H_2O as solvent. The equilibrium constant given in Table 1 for hydration of **1c** is a value measured for trichloroacetaldehyde and proposed as a value for **1c**,[16] though more recent calculations suggest that the equilibrium constant for hydration of **1c** should be substantially larger than that for trichloroacetaldehyde and thus larger than the value given in Table 1.[20] This would translate to a correspondingly larger value for the actual equilibrium constant for hemithioacetal formation.

Table 2. Michaelis and Inhibition Constants for Chloramphenicol Acetyltransferase

	K_m or K_i (μM)		
compd	pH 7	pH 8	pH 9
4	13	40	81
1b	3.3×10^5		
1c	4.5×10^5		
2a	86		
2b	5.5 (2.4)[a]	16 (2.5)	
2c	1.2 (11)	2.4 (17)	2.3 (35)

[a] Values in parentheses are ratios of K_m for **4** to K_i for **2b** or **2c** at each pH.

The pH dependence on the equilibrium constant for formation of **2c** and the rate constant for dissociation of **2c** were also studied. No measurable change in the equilibrium constant was observed over the pH range of 5−10. The dissociation constant increased by a factor of 4.5 per unit increase in pH over the pH range of 5−8.

With succinic semialdehyde (**1d**), the equilibrium constant for hemithioacetal formation was determined in aqueous phosphate buffer (pH 7.4) by ^1H NMR using *N*-acetylcysteamine instead of CoA. NMR analysis was performed in mixtures of 10−95% D_2O in H_2O using water suppression. An isotope effect of 0.49 was observed, and extrapolation to pure H_2O provided the apparent equilibrium constant of 20 M^{-1}. No change in the position of equilibrium was observed after the initial spectrum. No free aldehyde was observeable in the ^1H NMR spectrum of **1d**, and this result was used to provide the lower limit for the hydration equilibrium of **1d** given in Table 1.

Enzyme Inhibition. Compounds **2a**−**c** were tested as inhibitors of chloramphenicol acetyltransferase (Scheme 2) using a standard enzyme assay, monitoring disappearance of the thioester absorbance at 240 nm.[21] Data are presented in Table 2 along with the K_m values for

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acetyl-CoA and K_i values for CoA and the aldehydes **1b** and **1c**. **1b** and **1c** were weak noncompetitive inhibitors while CoA was a fairly weak competitive inhibitor. For inhibition studies, **2a** and **2c** were preformed by incubation of a solution of **1a** or **1c** (31 mM) and CoA (12 mM) in phosphate buffer. Aliquots of the resulting hemithioacetal solution were added to enzyme assay solutions to determine inhibition. Inhibition by **2b** was studied by including 80 mM **1b** in the assay buffer, with the concentration of **2b** varied by varying the CoA concentration. Hemithioacetal concentrations were calculated from CoA and aldehyde concentrations using the measured equilibrium constants. Competitive inhibition was observed by **2a**, **2b**, and **2c**. The pH dependence on inhibition of chloramphenicol acetyltransferase by **2b** and **2c** was also studied (Table 2). The K_m for acetyl-CoA and the K_i for **2b** increased with increasing pH. The K_i for **2c** also increased but by a lesser amount.

The hemithioacetals **2b** and **2c** were also tested as inhibitors of the enzyme citrate synthase at pH 7.5. Citrate synthase assays were conducted by monitoring disappearance of the thioester absorbance at 240 nm. K_i values of 20 and 24 μM were determined, both higher than the K_m for acetyl-CoA of 16 μM. The hemithioacetal formed between CoA and glyoxylate was also tested as an inhibitor of citrate synthase, and a K_i of 50 μM was obtained.

Inhibition studies of succinic thiokinase (Scheme 3, the final step of the reaction involves a phosphohistidine intermediate not shown) were conducted using succinyl-CoA, phosphate, and GDP as substrates with the reaction monitored by coupling GTP formation to the reaction of phosphofructokinase as described in the Experimental Section.[22] Monitoring of thioester absorbance was not possible due to high absorbance by succinic semialdehyde at 235 nm. Substantial noncompetitive inhibition was exhibited by succinic semialdehyde (**1d**). Butyraldehyde and isovaleraldehyde showed levels of noncompetitive inhibition similar to that of **1d**. In separate experiments, no time-dependent inhibition by **1d** was observed. It was also found that succinic semialdehyde inhibited the coupling enzymes to an appreciable extent, necessitating their use in large quantities.

Inhibition of succinic thiokinase was analyzed by measuring rates at varying concentrations of succinyl-CoA in the presence of several inhibitory species as shown in Figure 1. CoA and dethia-CoA (which lacks the thiol group) exhibited modest and virtually identical inhibition at concentrations of 24 μM. Inhibition by hemithioacetal was determined with concentrations of 6 mM aldehyde and 24 μM CoA, giving a hemithioacetal concentration of 2.6 μM on the basis of the measured K_{eq} for hemithioacetal formation. For comparison, inhibition was determined with 6 mM aldehyde in the presence of 24 μM dethia-CoA. While no detectable increase in inhibition was seen in the mixture of dethia-CoA plus aldehyde relative to inhibition by aldehyde alone, substantially increased inhibition was observed when CoA was included with the aldehyde. From the increase in the slope of the reciprocal plot, K_i for the hemithioacetal was determined to be 4.3 μM. Inhibition constants determined for all species studied are shown in Table 3.

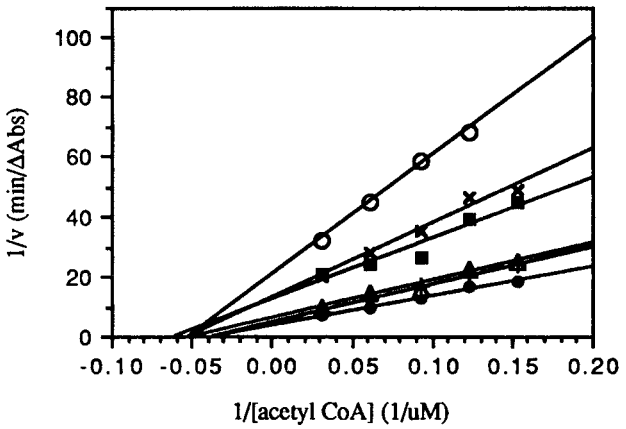

Figure 1. Lineweaver−Burk plot showing inhibition of succinic thiokinase: no inhibitor (●), 24 μM dethia-CoA (+), 24 μM CoA (△), 6 mM succinic semialdehyde (×), 24 μM dethia-CoA and 6 mM succinic semialdehyde (■), 24 mM CoA and 6 mM succinic semialdehyde (○).

Table 3. Inhibition of Succinic Thiokinase

inhibitor	K_i (μM)	inhibitor	K_i (μM)
6	18 (K_m)	dethia-CoA	125
1d	5000	**2d**	4.3
CoA	125		

Discussion

Previously studied acyl-CoA analogs have all required significant synthetic manipultions, ranging from acylation or alkylation of CoA to complete assembly of the CoA moiety. In contrast, the hemithioacetal analogs described in this paper are prepared simply by the mixing of CoA and an aldehyde in aqueous solution. This has been demonstrated with four commercially available aldehydes.

Equilibria and Rates for Hemithioacetal Formation and Dissociation. Equilibrium constants for hemithioacetal formation were determined in order to deduce concentrations of hemithioacetals from concentrations of added CoA and aldehyde in inhibition studies. A solvent isotope effect on hemithioacetal formation of 0.44 has been reported.[17,18] This value, along with a small correction for the isotope effect on hydrate formation, was applied to correct the equilibrium constants determined by ^{1}H NMR in D_2O to the values expected in H_2O. With trifluoroacetaldehyde (**1c**), the slow rate of hemithioacetal formation and dissociation permitted the equilibrium constant to be determined in H_2O by quantitation of free CoA of an equilibrated sample by reaction with DTNB. ^{19}F NMR experiments in aqueous phosphate buffer containing 20% D_2O for instrument locking provided further verification of the equilibrium constant. The lower apparent equilibrium constant compared with that of acetaldehyde is explained by the greater effect of the electron-withdrawing trifluoromethyl group on the equilibrium for hydrate formation than on the equilibrium for hemithioacetal formation.[23]

With succinic semialdehyde, the equilibrium for hemithioacetal formation was determined using *N*-acetylcysteamine instead of CoA. This provided simplified NMR spectra, permitted studies at higher concentration than those achieveable with CoA, and simplified pH control. Efficient control of pH was important since succinic

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semialdehyde has been reported to exist in a neutral cyclic hemiacetal form which is converted to an open carboxylate form upon hemithioacetal formation;[24,25] thus the equilibrium may be pH dependent. The upper limit of 10% free aldehyde observed by ^{1}H NMR and given in Table 1 could be due to cyclic hemiacetal formation rather than hydrate formation. However, it is expected that the cyclic hemiacetal form will not be favored at neutral pH and above because the cyclic form would be driven to the open free acid form by deprotonation of the acid. The solvent isotope effect on the equilibrium with succinic semialdehyde was also studied, since the equilibria are potentially more complex than those with acetaldehyde. The isotope effect was similar to that observed previously with acetaldehyde.

Hemithioacetal formation and dissociation are virtually instantaneous for aldehydes which exist largely in the free aldehyde form, as reported previously.[17] This is observed with acetaldehyde and succinic semialdehyde. The formation and dissociation reactions are much slower for formaldehyde and trifluoroacetaldehyde, which exist almost exclusively in hydrated form in aqueous solution. This is consistent with hemithioacetal formation and dissociation proceeding via the unhydrated aldehyde.[26] For inhibition studies with **2a** and **2c**, it was necessary to preincubate the aldehyde and CoA prior to assay to assure equilibration. On the basis of the rate constant for formation of **2c** of 1.2×10^{-2} M^{-1} s^{-1} (the product of the dissociation and equilibrium constants in Table 1) with 60 mM **1c** and a much lower concentration of CoA, formation of **2c** at pH 7 should reach about 50% of the equilibrium value in 16 min, neglecting effects of the dissociation reaction and the small decrease in concentration of **1c** as the reaction proceeds. This indicates that **1c** and CoA should be incubated for a few hours to assure equilibration. Formation of **2a** requires less equilibration time and the formation of **2b** and **2d** is complete almost instantaneously, requiring little or no preequilibration.

The slow dissociation rates for **2a** and **2c** permit preincubation at high concentration followed by dilution and rapid assay to determine inhibition under nonequilibrium conditions. This procedure allows studies of inhibition by the hemithioacetals in the presence of low, subequilibrium quantities of aldehyde and free CoA, if assays are conducted rapidly after dilution. This technique was used in inhibition studies of **2c** at pH 7. As the association and dissociation reactions are base catalyzed,[26] assays at higher pH were done only under equilibrium conditions.

CoA Hemithioacetals as Enzyme Inhibitors. The CoA hemithioacetals **2a**−**c** were tested as inhibitors of chloramphenicol acetyltransferase. Inhibition of chloramphenicol acetyltransferase by the formaldehyde hemithioacetal (**2a**) was quite modest, with a K_i higher than the K_m for acetyl-CoA. The acetaldehyde hemithioacetal (**2b**) was a somewhat better inhibitor, with a K_i 2.4-fold lower than the K_m for acetyl-CoA. This result is consistent with the hemithioacetal serving as a mimic of the tetrahedral intermediate or transition state in acyl transfer, resulting in enhanced binding relative to substrate. It is expected that the observed inhibition is due primarily to the isomer which matches the configuration of the tetrahedral intermediate. This is supported by recent results from this lab which found highly selective inhibition by one of two stereoisomers of secondary alcohols which differ from **2b** in replacement of the sulfur atom by a methylene group.[27] The K_i for the preferred isomer is thus probably about half the measured value.

The K_i for the trifluoracetaldehyde hemithioacetal (**2c**) is substantially lower than that for **2b**. Assuming inhibition by only one isomer, the K_i for **2c** is 22-fold lower than the K_m for acetyl-CoA. The greater inhibition by **2c** relative to **2b** could be due to inhibition by the deprotonated alkoxide form of **2c** vs the neutral hydroxy form of the less acidic **2b**. The alkoxide would be expected to better mimic of the oxyanionic tetrahedral intermediate or transition state and thus interact more strongly with the oxyanion binding site. The greater inhibition by **2c** could also be explained by the hydroxyl group of the hemithioacetal acting as a hydrogen bond donor in the enzyme−inhibitor complex, with **2c** being a better hydrogen bond donor due to its greater acidity. However, it appears more likely that the hydroxyl or alkoxide oxygen acts as a hydrogen bond acceptor to mimic the tetrahedral intermediate or transition state in the enyzme−inhibitor complex. The pK_a of **2c** is not known but may be near 10.2, the reported pK_a for trifluoroacetaldehyde hydrate.[28] The absence of any pH effect on the equilibrium for hemithioacetal formation up to pH 10 suggests that the pK_a for **2c** is not significantly lower than that of the thiol group of CoA which is estimated to be about 9.9, the reported pK_a for *N*-acetylcysteamine.[29] The pH dependence on binding of acetyl-CoA, **2b**, and **2c** was examined to further address this issue. The K_m for acetyl-CoA increases with increasing pH, and the K_i for **2b** increases similarly. In contrast, the K_i for **2c** shows a smaller increase on going from pH 7 to 8 and no further increase on going to pH 9. Ratios of K_m to K_i (in parentheses in Table 2) show that the relative affinity of **2c** vs acetyl-CoA increased slightly with increasing pH while the relative affinity of **2b** vs acetyl-CoA remained constant from pH 7 to 8. This could be explained by increased concentration of the alkoxide of **2c** compensating for the pH effects observed with **4** and **2b**. However, the effect is quite small, not nearly the 10-fold change in K_i per pH unit that would be expected. The results thus appear inconclusive in indicating the ionization state of the enzyme-bound inhibitor.

The hemithioacetals **2b** and **2c** were also viewed as possible mimics of the enol or enolate intermediate in the reaction of citrate synthase. However, both were poor inhibitors with K_i values greater than the K_m value for acetyl-CoA. This indicates that these analogs are not good mimics of any intermediate in the reaction of citrate synthase but are selective for enzymes which catalyze acyl transfer. This is consistent with the tetrahedral center of the hemithioacetals mimicking the tetrahedral intermediate in acyl transfer but not the planar trigonal center of an enol or enolate. The hemithioacetal with glyoxylate differs from the previously studied carboxymethyl-CoA[2,10] only by the presence of the hemithioacetal hydroxyl group. While carboxymethyl-CoA is a very potent inhibitor of citrate synthase, very poor inhibition was exhibited by the corresponding hemithioacetal. This

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suggests that there is no room in the active site for the extra hydroxyl group so that binding is impeded.

The hemithioacetal **2d** was expected to mimic the tetrahedral intermediate or transition state **7** in the reaction of succinic thiokinase. Studies of succinic thiokinase were complicated by much more potent inhibition by the aldehyde than that observed in the studies of chloramphenicol acetyltransferase. This inhibition appears to be nonspecific and probably not indicative of binding of succinic semialdehyde in the succinic acid binding site. This is supported by the noncompetitive nature of the inhibition and the fact that other aldehydes were also found to inhibit this enzyme. Inhibition by CoA was modest but significant. Inhibition by the combination of CoA and succinic semialdehyde was significantly greater than inhibition by either alone. However, since inhibition by both was significant, it was difficult to discount the possibility of the observed inhibition being due primarily to an additive effect of CoA and aldehyde rather than inhibition by the hemithioacetal. In order to address this issue, inhibition by dethia-CoA was studied. This compound has all of the functionality of CoA except for the thiol group and thus cannot form a hemithioacetal with an aldehyde. Inhibition of succinic thiokinase by dethia-CoA was indistinguishable from inhibition by CoA. However, the combined effect of dethia-CoA and aldehyde was almost identical to the effect of aldehyde alone and much less than the effect of CoA plus aldehyde. This verifies that the additional inhibition over that exhibited by aldehyde alone is due primarily to the hemithioacetal and not to free CoA.

Several acyl-CoA analogs have been prepared previously which mimic the enol or enolate intermediate in reactions of enzymes that catalyze deprotonation of the methyl group of acetyl-CoA as part of catalysis, most notably citrate synthase.[8−13] However, few acyl-CoA analogs have been prepared that posess a tetrahedral center in place of the thioester carbonyl carbon, which may mimic the tetrahedral intermediate or transition state in the reactions of enzymes that catalyze acyltransfer. Exceptions are β-hydroxyalkyl-CoA thioethers, differing from the hemithioacetals described here in that they contain an additional methylene group between the sulfur atom and the hydroxymethine carbon.[30,31]

Also recently reported have been sulfoxide and sulfone analogs[7] and secondary alcohol analogs, differing from the hemithioacetals by replacement of the sulfur atom with a methylene group.[27] Among these, only the secondary alcohol analogs have shown enhanced binding relative to the natural substrate for an enzyme catalyzing acyl transfer from a CoA ester.[27] The hemithioacetal analogs are unique both in their ready accessibility and in their potential to mimic the tetrahedral intermediate or transition state in enzyme-catalyzed acyl transfer. Indeed, the hemithioacetals are the best acyl-CoA analog inhibitors of these enzymes described thus far.

Acyl-CoA analogs have been very valuable for studies of the structures of enzyme−inhibitor complexes in part because the actual enzyme-CoA ester complexes are often unstable due to hydrolysis of the thioester. While the hemithioacetals are kinetically unstable, they are sufficiently stable thermodynamically that a high ratio of hemithioacetal to free CoA can be obtained. Still, formation and observation of an enzyme−hemithioacetal adduct may be complicated by enzyme binding of the CoA and the free aldehyde present in equilibrium with the hemithioacetal. The results of this work should help to define the proper conditions for observation of an enzyme−hemithioacetal adduct. While the inhibition constants are modest, the CoA hemithioacetals may be useful tools for studies of enzyme−inhibitor complexes and will be readily accessible in labs in which previously studied acyl-CoA analogs have not been available.

Experimental Section

Determination of Equilibrium and Dissociation Constants for Hemithioacetals. Equilibria for hemithioacetal formation of CoA with **1a** and **1b** were measured by 400 MHz ^{1}H NMR. NMR samples were prepared by lyophilization of an aqueous solution of potassium phosphate (pH 6.9) and dissolving the residue in D_2O to a final concentration of 5 mM. In 0.5 mL samples of this buffer were then dissolved 9.6 mg of CoA (12.5 μmol) and 1−1.5 equiv of the aldehyde. Equilibria were determined by integration of the methylene protons next to the thiol of CoA (2.58 ppm) relative to the same protons of **2a** (2.74 ppm) and **2b** (2.73 ppm). NMR spectra were obtained at 5, 15, and 30 min. The equilibrium for trifluoroacetaldehyde and CoA was determined by addition of 5 μL of an equilibrated (for 24 h) solution of CoA (12.5 μmol) and **1c** (30 μmol) in aqueous HEPES buffer (0.5 mL) to a solution of DTNB (0.4 mM) in 0.1 M HEPES buffer (pH 7.0) and monitoring the initial rapid increase in absorbance at 412 nm. The equilibrium was also determined by ^{19}F NMR in 20% D_2O in H_2O containing 5 mM phosphate (pH 6.9), 12.5 μmol of CoA, and 10 μmol of **1c**. The sample was equilibrated at room temperature for 5 h before analysis. The trifluoroacetaldehyde hydrate (**3c**) was observed at 75.9 ppm and the hemithioacetal **2c** at 83.8 ppm, relative to C_6F_6 at 0.0 ppm. The equilibrium for succinic semialdehyde and *N*-acetylcysteamine was studied by 500 MHz ^{1}H NMR with samples containing 50 mM potassium phosphate, pH or pD 7.4. Spectra were obtained using water suppression, and equilibria were determined by integration of both pairs of methylene protons of *N*-acetylcysteamine (3.33 and 2.62 ppm) and the hemithioacetal (3.37 and 2.68−2.88 ppm).

Dissociation constants for **2a** and **2c** were determined by addition of 5 μL of an equilibrated solution of CoA (12.5 μmol) and 30 μmol of **1a** or **1c** in aqueous HEPES buffer to a solution of DTNB (0.4 mM) in HEPES buffer (0.1 M, pH 7.0) and monitoring the rate of increase in absorbance at 412 nm after the initial burst.

Enzyme Inhibition Studies. For inhibition studies with chloramphenicol acetyltransferase, assays were conducted as described previously[21] by measuring the decrease in thioester absorbance at 240 nm where ϵ was measured to be 3.27×10^3 M^{-1} cm^{-1}. K_i values were calculated from double-reciprocal plots of $1/v$ vs 1/[acetyl-CoA] at three concentrations of inhibitor. Assays were run in 0.1 M HEPES, pH 7, containing 0.1 mM chloramphenicol, 0.015−0.120 mM acetyl-CoA, 0.01 unit of chloramphenicol acetyltransferase, and inhibitor. For **2a** and **2c**, an aliquot of a solution containing aldehyde (31.25 mM) and CoA (12.5 mM) in phosphate buffer (pH 7.0) equilibrated at room temperature (incubation time 30 min for **2a** and 5 h for **2c**) was used in the assay. For **1b**, the buffer in each assay contained 0.080 M acetaldehyde to which an aliquot of CoA was added. Concentrations of acetaldehyde and formaldehyde were quantified using yeast alcohol dehydrogenase.[32] Quantitation was repeated after performing the assays to insure that the aldehyde concentration did not change over the course of the assays. K_i values were calculated from double-reciprocal plots of $1/v$ vs 1/[acetyl-CoA] at four concentrations of each inhibitor.

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For inhibition studies of citrate synthase, assays were conducted as described previously[33] in 0.1 M HEPES, pH 7.5, containing 0.4 mM oxaloacetate, 0.005−0.09 mM acetyl-CoA, and 0.037 unit of citrate synthase (from porcine heart). The reactions were monitored at 240 nm using $\epsilon_{240} = 3.27 \times 10^3$ M^{-1} cm^{-1} for the thioester. Inhibitors were included as described above for chloramphenicol acetyltransferase. Inhibition studies with the glyoxylate hemithioacetal were conducted with 10 mM glyoxylate.

Inhibition of succinic thiokinase (succinyl-CoA synthetase from porcine heart) was studied using an assay mixture consisting of GDP (200 μM), Mg^{2+} (10 mM), fructose 6-phosphate (1 mM), NADH (240 μM), aldolase (5 units), phosphofructokinase (5 units), triosephosphate isomerase (55 units), and glycerophosphate dehydrogenase (5 units), which were added to 50 mM potassium phosphate buffer, pH 7.4, and equilibrated for 5 min. Inhibitor(s), succinic thiokinase (0.02 unit), and **6** (6.5−32 μM) were then added such that the total volume was 1 mL. The decrease in absorbance at 340 nM vs time was recorded. Rates were measured in the absence of any inhibitors and in the presence of one of the following: 6 mM **1d**, 24 μM CoA, 24 μM dethia-CoA, 6 mM **1d** plus 24 μM CoA, and 6 mM **1d** plus 24 μM dethia-CoA. Concentrations of **1d** were quantified by enzymatic assay with Gabase.[34]

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