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The importance of the amide bond nearest the thiol group in enzymatic reactions of coenzyme A

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Abstract

Analogues of coenzyme A (CoA) and of CoA thioesters have been prepared in which the amide bond nearest the thiol group has been modified. An analogue of acetyl-CoA in which this amide bond is replaced with an ester linkage was a good substrate for the enzymes carnitine acetyltransferase, chloramphenicol acetyltransferase, and citrate synthase, with K_m values 2- to 8-fold higher than those of acetyl-CoA and V_{max} values from 14 to >80% those of the natural substrate. An analogue in which an extra methylene group was inserted between the amide bond and the thiol group showed less than 4-fold diminished binding to the three enzymes but exhibited less than 1% activity relative to acetyl-CoA with carnitine acetyltransferase and no measurable activity with the other two enzymes. Analogues of several CoA thioesters in which the amide bond was replaced with a hemithioacetal linkage exhibited no measurable activity with the appropriate enzymes. The results indicate that some aspects of the amide bond and proper distance between this amide and the thiol/thioester moiety are critical for activity of CoA ester-utilizing enzymes.

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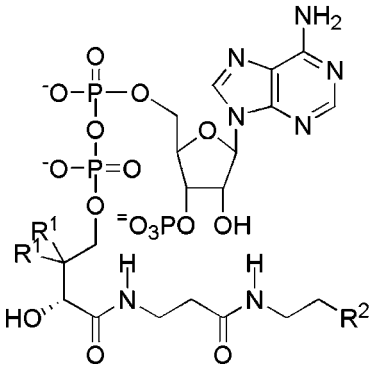
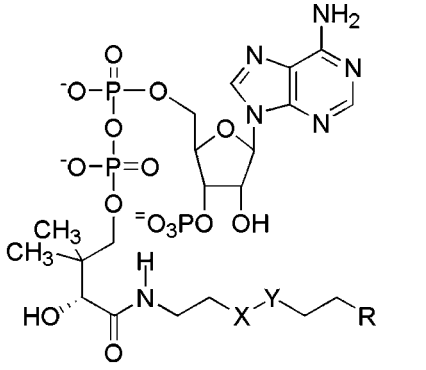
1. Introduction

A number of analogues of coenzyme A (CoA) **1** and of acetyl-CoA **2** have been previously reported from this group (see Fig. 1) [1–8]. For example, the carboxylate analogue **3**, which was designed to mimic the enolate form of acetyl-CoA has been shown to be a potent inhibitor of citrate synthase, with a K_i value 1000-fold lower than the K_m for acetyl-CoA [1,9]. In more recent work to begin to address the role of functionality remote from the thiol or thioester moiety, the didemethyl analogue of CoA **4** was prepared [10]. NMR analysis showed that the methyl groups have only a moderate effect on the conformation of the part of the molecule in the vicinity of the geminal methyl groups. The corresponding acetyl thioester **5** was a fairly good substrate for acetyl-CoA utilizing enzymes with modestly increased K_m values and moderately decreased k_{cat} values relative to the natural substrate acetyl-CoA.

The work described in this manuscript addresses the importance of the amide bond nearest the thiol group and the ethylene group linking this amide to the thiol moiety. In previous work from this group, a modified form **6** of the carboxylate analogue **3** was prepared in which the amide bond was replaced with a pair of methylene



1, $R^1 = CH_3$, $R^2 = SH$
2, $R^1 = CH_3$, $R^2 = S\text{-}C(=O)\text{-}CH_3$
3, $R^1 = CH_3$, $R^2 = CH_2\text{-}CO_2^-$
4, $R^1 = H$, $R^2 = SH$
5, $R^1 = H$, $R^2 = S\text{-}C(=O)\text{-}CH_3$



6, X-Y = $CH_2\text{-}CH_2$, R = $CH_2\text{-}CO_2^-$
7, X-Y = C(=O)-O, R = SH
8, X-Y = C(=O)-O, R = S-C(=O)-CH_3
9, X-Y = C(=O)-NH-CH_2, R = SH
10, X-Y = C(=O)-NH-CH_2,
R = S-C(=O)-CH_3
11, X-Y = CH(OH)-S, R = S-C(=O)-R'

Fig. 1. Structures of coenzyme A and of analogues and derivatives used in this work.

groups [7]. This substitution was shown to diminish binding to citrate synthase more than 10,000-fold. Reported here is an oxoester analogue of CoA **7**, in which the amide bond nearest the thiol group is replaced with an ester linkage, along with studies of its acetate thioester **8** as a substrate for acetyl-CoA utilizing enzymes. Studies of the homocysteamine analogue **9** and the corresponding acetyl thioester **10**, in which an extra methylene group is present between the amide bond and thiol group are also reported. Finally, this manuscript describes studies of CoA ester analogues **11** in which the amide bond is replaced with a reversibly formed hemithioacetal linkage. The results indicate that the carbonyl group of the amide bond nearest the thiol or thioester and proper distance between this amide and the thiol or thioester group are critical for the activity of CoA-utilizing enzymes.

2. Materials and methods

2.1. General methods

Reagents and enzymes were obtained from Sigma–Aldrich and used as supplied. Wild type [1,11] or recombinant [12,13] phosphopantetheine adenylyltransferase and dephosphocoenzyme A kinase and recombinant pantothenate kinase [14] were prepared as described previously. Analytical HPLC was performed using a Rainin Microsorb C-18 column (4.6 × 250 mm, 5 μm) with monitoring at 215 and 260 nm with a photodiode array detector and a flow rate of 1 mL/min. Solvent conditions for analytical HPLC were 5% methanol in aq. monopotassium phosphate (50 mM) for 2 min, followed by a linear gradient to 60% methanol over 12 min and maintenance at 60% methanol for at least 5 min. Preparative HPLC was performed on a Rainin Microsorb C-18 column (21.4 mm × 25 cm) with monitoring at 215 and 280 nm and a flow rate of 10 mL/min with solvent gradients as described for individual compounds. The concentrations of CoA analog solutions were determined using $\varepsilon_{260} = 15{,}400\ M^{-1}\ cm^{-1}$. Computational studies were conducted using Gaussian03, with aqueous solvation described by Tomasi's polarized continuum model [15].

2.2. Oxoester CoA 7

To a solution of **12** [1] (20 mg, 26 μmol) in water (1 mL) was added β-mercaptoethanol (140 μL, 2 mmol) and the pH was adjusted to 7.6 with aq. NaOH. After 75 min, analytical HPLC showed predominantly the thioester product at retention time 12.7 min, with minor oxoester **7** at 14.2 min. The reaction mixture was diluted to 50 mL with water, the pH was adjusted to 2.5, and the β-mercaptoethanol was removed via ion exchange chromatography on DEAE–Sepharose (20 × 2.5 cm, 0–0.2 M NaCl, pH 2.8, product eluted at 0.15 M NaCl). The fractions containing thioester and oxoester **7** were pooled and allowed to stand for 1 month at room temperature. Analytical HPLC showed near complete conversion of the thioester to **7**. The product was lyophilized and purified by HPLC (5 min at 5% methanol

in 10 mM potassium phosphate, pH 4.5, followed by a linear gradient to 35% methanol over 45 min). The fractions eluted at 30–35 min were combined and lyophilized to give **7** (3.1 mg, 4.0 μmol, and 15% yield). $\lambda_{max} = 260$ nm. Four hundred megahertz ^{1}NMR (D_2O) δ 0.68 (s, 3H), 0.80 (s, 3H), 2.55 (t, 2H, $J = 6.4$ Hz), 2.67 (t, 2H, $J = 6.4$ Hz), 3.04 (t, 2H, $J = 5.4$ Hz), 3.48 (dd, 1H, $J = 4.8$, 10.0 Hz), 3.75 (dd, 1H, $J = 4.8$, 10.0 Hz), 3.93 (s, 1H), 4.10 (t, 2H, $J = 6.4$ Hz), 4.16 (br s, 2H), 4.52 (s, 1H), 6.12 (d, 1H, $J = 6.0$ Hz), 8.27 (s, 1H), 8.52 (s, 1H). HRMS (FAB): [M − H] calcd for $C_{21}H_{34}N_6O_{17}P_3S$ *m/z* 767.091. Found 767.095.

2.3. *S-Acetyl-oxoester-CoA* ***8***

To a solution of **7** (2.7 mg, 3.5 μmol) in Hepes buffer (1 mL, 25 mM, pH 8.2) was added *S*-thiophenyl acetate (0.1 mL, 0.75 mmol) in acetonitrile (0.9 mL). The turbid solution was capped and stirred vigorously at room temperature. The reaction was monitored by HPLC (product $t_r = 14.8$ min) and was complete in 1 h. The aqueous layer was extracted with ether (5×4 mL), the pH was adjusted to 4.5 with aq. HCl, and the product was purified by HPLC (10 min 5% methanol in 0.01 M sodium phosphate, pH 4.5, followed by a linear gradient to 40% methanol over 40 min). The product eluted at 39–46 min, the fractions containing product were combined and lyophilized to give **8** (1.2 mg, 1.5 μmol, and 42% yield). $\lambda_{max} = 260$ nm. Four hundred megahertz ^{1}NMR (D_2O) δ 0.70 (s, 3H), 0.83 (s, 3H), 2.29 (s, 3H), 2.53 (t, 2H, $J = 6.4$ Hz), 3.06 (t, 2H, $J = 6.2$ Hz), 3.35–3.45 (m, 2H), 3.46–3.52 (m, 1H), 3.75–3.82 (m, 1H), 3.96 (s, 1H), 4.13 (t, 2H, $J = 6.2$ Hz), 4.19 (br s, 2H), 4.55 (br s, 1H), 6.14 (d, 1H, $J = 6.4$ Hz), 8.28 (s, 1H), 8.54 (s, 1H).

2.4. *Homocysteamine CoA* ***9***

A suspension of homocysteamine hydrochloride (4.4 g, 10 mmol) in water (5 mL) was added to a solution of **12** (100 mg, 130 μmol) in water (5 mL) and the pH was adjusted to 10 with 6 M aq. NaOH. The reaction was stirred at room temperature under nitrogen and monitored by HPLC. Complete disappearance starting material ($t_r = 17.3$ min) and appearance of product ($t_r = 13.7$ min) was observed after 4 h. The reaction mixture was filtered, the pH was adjusted to 4.5 with aq. HCl, and the product was partially purified by HPLC (5 min 5% methanol in 0.01 M potassium phosphate, pH 4.5, followed by a linear gradient to 45% methanol over 40 min). The product, eluted at 25–32 min, was further purified by ion exchange chromatography on DEAE–Sepharose (20×2.5 cm, 0–0.2 M NaCl, pH 2.8). The product eluted at 0.13 M NaCl. Fractions containing product were combined and lyophilized to give **9** (45 mg, 58 μmol, and 45% yield). $\lambda_{max} = 260$ nm. Four hundred megahertz ^{1}NMR (D_2O) δ 0.72 (s, 3H), 0.85 (s, 3H), 1.65–1.72 (m, 2H), 2.39 (t, 2H, $J = 6.4$ Hz), 2.45 (t, 2H, $J = 7.0$ Hz), 3.17 (t, 2H, $J = 6.8$ Hz), 3.41 (t, 2H, $J = 6.2$ Hz), 3.52 (dd, 1H, $J = 4.8$, 10.0 Hz), 3.79 (dd, 1H, $J = 4.8$, 10.0 Hz), 3.96 (s, 1H), 4.20 (br s, 2H), 4.55 (br s, 1H), 6.16 (d, 1H, $J = 6.0$ Hz), 8.31 (s, 1H), 8.56 (s, 1H). HRMS (FAB): [M − H] calcd for $C_{22}H_{38}N_7O_{16}P_3S$ *m/z* 780.191. Found 780.126.

2.5. 3-Acetyl-homocysteamine-CoA **10**

A solution of **9** (40 mg, 51 μmol) in water (1.5 mL) adjusted to pH 8.2 with aq. NaOH was added to a solution of *S*-phenyl thioacetate (0.2 mL, 1.5 mmol) in acetonitrile (2 mL). The resulting biphasic mixture was stirred vigorously at room temperature and the reaction was monitored by HPLC. The reaction was complete in 1 h, at which time stirring was stopped and the acetonitrile layer was discarded. The aqueous layer was extracted with ether (5×4 mL), the pH was adjusted to 4.5 with aq. HCl, and the product was purified by HPLC (5 min 5% methanol in 0.02 M sodium phosphate, pH 4.5, followed by a linear gradient to 45% methanol over 45 min). The product eluted at 30–38 min. Fractions containing product were combined and lyophilized to give **10** (23 mg, 28 μmol, and 55% yield). $\lambda_{max} = 260$ nm. Four hundred megahertz ^{1}NMR (D_2O) δ 0.72 (s, 3H), 0.85 (s, 3H), 1.63–1.70 (m, 2H), 2.29 (s, 3H), 2.41 (t, 2H, $J = 6.4$ Hz), 2.78 (t, 2H, $J = 7.2$ Hz), 3.13 (t, 2H, $J = 6.8$ Hz), 3.43 (t, 2H, $J = 6.0$ Hz), 3.53 (dd, 1H, $J = 4.8$, 10.0 Hz), 3.80 (dd, 1H, $J = 4.8$, 10.0 Hz), 3.98 (s, 1H), 4.22 (br s, 2H), 4.56 (br s, 1H), 6.15 (d, 1H, $J = 6.0$ Hz), 8.26 (s, 1H), 8.54 (s, 1H). HRMS (FAB): [M − H] calcd for $C_{24}H_{39}N_7O_{17}P_3S$ *m/z* 822.134. Found 822.138.

2.6. 3-Aminopropionaldehyde diethyl acetal **16**

A solution of **15** (9.96 g, 60 mmol) and sodium azide (4.9 g, 75 mmol) in dimethyl sulfoxide (60 mL) was stirred for 16 h at 50 °C. The mixture was poured into ice water (50 mL) and extracted with ethyl acetate (3×30 mL). The combined organic layers were washed with sat. aq. sodium chloride (30 mL), dried over sodium sulfate, and concentrated under reduced pressure to give 3-azidopropionaldehyde diethyl acetal as a pale yellow oil which was used in the next step without further purification (9.9 g, 57 mmol, and 95% yield). Two hundred megahertz ^{1}H NMR ($CDCl_3$) δ 1.19 (t, 6H, $J = 5.5$ Hz), 1.86 (m, 2H), 3.35 (m, 2H), 3.47 (m, 2H), 3.65 (m, 2H), 4.59 (m, 1H). To a solution of 3-azidopropionaldehyde diethyl acetal (9.9 g, 57 mmol) in methanol (100 mL) was added 5% Pd/C (0.5 g). The apparatus was charged with hydrogen (50 psi) and shaken for 2 h at room temperature. The solution was filtered through celite and the solvent was removed under reduced pressure to give **16** as a yellow oil (8.2 g, 56 mmol, and 98% yield). Three hundred megahertz ^{1}H NMR ($CDCl_3$) δ 1.19 (t, 6H, $J = 5.3$ Hz), 1.79 (q, 2H, $J = 5.0$ Hz), 2.75 (t, 2H, $J = 5.3$ Hz), 3.49 (m, 2H), 3.64 (m, 2H), 4.56 (t, 1H, $J = 5.2$ Hz).

2.7. (*R*)-*N*-(3,3-Diethoxypropyl)-3,3-dimethyl-2,4-dihydroxybutanamide

To a solution of **16** (2 g, 13.6 mmol) in toluene (10 mL) was added (*R*)-pantolactone **17** (1.3 g, 10 mmol) and the mixture was heated at reflux for 16 h. The solvent was removed under reduced pressure and the product was purified by chromatography on silica gel (9:1 ethyl acetate/hexane) to give the title compound as a viscous yellow oil (2.38 g, 8.6 mmol, and 86% yield). Three hundred megahertz ^{1}H NMR (D_2O) δ 0.79 (s, 3H), 0.82 (s, 3H), 1.09 (t, 6H, $J = 4.7$ Hz), 1.72 (m, 2H), 3.19 (t,

2H, $J = 3.8$ Hz), 3.31 (m, 2H), 3.51 (m, 2H), 3.62 (m, 2H), 3.86 (s, 1H), 4.56 (t, 1H, $J = 3.9$ Hz). ^{13}C NMR (D_2O) δ 9.85, 14.71, 16.01, 28.22, 30.19, 34.15, 58.36, 63.86, 71.25, 97.10.

2.8. (*R*)-*N*-(3-Oxopropyl)-3,3-dimethyl-2,4-dihydroxybutanamide **18**

A solution of (*R*)-*N*-(3,3-diethoxypropyl)-3,3-dimethyl-2,4-dihydroxybutanamide (0.41 g, 1.5 mmol) in aq. HCl (10 mL, 0.1 M) was stirred at room temperature for 16 h. The solvent was removed under reduced pressure to give **18** as a pale yellow oil which was used in the next step without further purification (0.26 g, 1.3 mmol, and 87%). Three hundred megahertz ^1H NMR (D_2O) δ 1.01 (s, 6H), 1.89 (m, 2H), 3.39–3.60 (m, 4H), 3.92 (s, 1H).

2.9. P1-Adenosine-5′-diphosphate-P2-[3(*R*)-hydroxy-4-(2- formylethylamino)-2, 2-dimethyl-4-oxobutyl] ester **13**

To a solution of **18** (0.20 g, 1.0 mmol) in water (10 mL) was added phosphoenolpyruvate (1.04 mmol), ATP (0.20 mmol), $MgCl_2$ (0.80 mmol), KCl (0.40 mmol), and triethanolamine hydrochloride (18 mg, 0.10 mmol). The pH was adjusted to 7.8 and the volume was adjusted to 20 mL by addition of water. Pantothenate kinase (20 U), pyruvate kinase (20 U), and sodium azide (5 mg) were added and the mixture was allowed to stand at room temperature. HPLC analysis and enzymatic determination of phosphoenolpyruvate showed the reaction to be complete after 16 h. ATP (1.0 mmol) was added and the pH was adjusted to 7.0. Phosphopantetheine adenylyltransferase (20 U) and inorganic pyrophosphatase (20 U) were added and the mixture was left at room temperature. HPLC analysis and enzymatic determination of ATP showed the reaction to be complete after 96 h. The solution was centrifuged to remove insoluble matter and the pH was adjusted to 4.0 with 2 N aq. HCl. The solution was loaded onto a DEAE–Sepharose column (2.5×18 cm), which had been equilibrated with 3 mM aq. HCl. The column was washed with 100 mL of 3 mM HCl followed by a linear gradient of LiCl (0–0.2 M) in 3 mM HCl (total volume 450 mL). Compound **13** eluted at 0.11–0.13 M LiCl, as determined by HPLC analysis. Fractions containing product were combined, adjusted to pH 4.0, and lyophilized to give **13** (465 mg, 0.67 mmol, and 67% yield). Three hundred megahertz ^1H NMR (D_2O, approximately 1:1 aldehyde and hydrate) δ 0.81 (s, 3H), 0.92 (s, 3H), 1.87 (m, 1H), 2.80 (t, 1H, $J = 11.9$ Hz), 3.29 (m, 1H), 3.61 (m, 2H), 3.85 (m, 1H), 4.03 (s, 1H), 4.29 (m, 3H), 4.42 (s, 1H), 4.58 (s, 1H), 5.1 (t, 0.5 H, $J = 11.0$ Hz), 6.12 (d, 1H, $J = 10.2$ Hz), 8.16 (s, 1H), 8.45 (s, 1H), 9.71 (s, 0.5H). MS: [M − H] calcd for $C_{23}H_{35}N_7O_{14}P_2$ *m/z* 696.1798. Found 696.1.

2.10. S-(2-Mercaptoethyl)thioacetate **14a**

To a solution of 1,2-ethanedithiol (40 mmol) in pyridine (10 mL) and dichloromethane (10 mL) was added acetic anhydride (40 mmol). The reaction was stirred at room temperature for 16 h. The solvent was removed under reduced pressure and

the product was purified by chromatography on silica gel using dichloromethane/hexanes 1:1 to give **14a** as a light yellow oil (4.13 g, 30.4 mmol, and 76% yield). Three hundred megahertz ^{1}H NMR ($CDCl_3$) δ 1.59 (t, 1H, $J = 10.2$ Hz), 2.30 (s, 3H), 2.63 (q, 2H, $J = 7.9$ Hz), 3.03 (m, 2H).

2.11. S-(2-Mercaptoethyl)-3-oxothiobutanoate *14b*

To a solution of ethanedithiol (1.9 mL, 2.1 g, and 22 mmol) in tetrahydrofuran (10 mL) was added diketene (1.6 mL, 1.7 g, and 20 mmol) and dimethylaminopyridine (2 mmol). The solution was stirred for 18 h at room temperature. Diethyl ether (70 mL) was added and the resulting solution was washed with aq. sodium bisulfate (5%, 2×10 mL), sat. aq. sodium bicarbonate (2×10 mL), and sat. aq. sodium chloride (2×10 mL). The organic layer was dried over magnesium sulfate, concentrated under reduced pressure, and purified by chromatography on silica gel (ethyl acetate/hexanes/acetic acid 2:8:0.5) to give **14b** as a yellow oil (2.88 g, 16.2 mmol, and 81% yield). Three hundred megahertz ^{1}H NMR ($CDCl_3$) δ 1.69 (m, 1H), 2.32 (s, 3H), 2.79 (m, 2H), 3.20 (m, 2H), 3.76 (s, 2H).

2.12. (2-Mercaptoethyl)-3-oxobutanoate *14c*

To a solution of 2-mercaptoethanol (1.5 mL, 1.7 g, and 22 mmol) in tetrahydrofuran (10 mL) was added diketene (1.6 mL, 1.7 g, and 20 mmol) and dimethylaminopyridine (0.24 g, 2.0 mmol). The solution was stirred for 18 h at room temperature. Diethyl ether (70 mL) was added and the resulting solution was washed with aq. sodium bisulfate (5%, 2×10 mL), sat. aq. sodium bicarbonate (2×10 mL), and sat. aq. sodium chloride (2×10 mL). The organic layer was dried over magnesium sulfate, concentrated under reduced pressure, and purified by chromatography on silica gel (ethyl acetate/hexanes 2:3) to give **14c** as a yellow oil (2.01 g, 12.4 mmol, and 62% yield). Three hundred megahertz ^{1}H NMR ($CDCl_3$) δ 1.59 (t, 1H, $J = 7.9$ Hz), 2.37 (s, 3H), 2.80 (m, 2H), 3.53 (s, 2H), 4.29 (t, 2H, $J = 6.2$ Hz).

2.13. N-(S-2-Mercaptoethyl thio)methyl phenylacetamide *26*

A solution of **25** [16] (0.87 g, 5.3 mmol) in trifluoroacetic acid (10 mL) was added dropwise to a solution of ethanedithiol (1 g, 10.2 mmol) in trifluoroacetic acid (15 mL) over 10 min. The reaction was stirred at room temperature for 1.5 h. Trifluoroacetic acid was removed under reduced pressure. The residue was dissolved in 50 mL methylene chloride and washed with 0.2 M sodium bicarbonate solution (3×20 mL) and brine 920 mL). The organic layer was dried over magnesium sulfate. The solvent was removed by rotary evaporation. The crude product was dissolved in 2 mL methylene chloride, and purified by chromatography using 1:1 hexane/ethyl acetate as the eluent. The solvent was removed to give **26** as a colorless liquid (0.56 g, 2.3 mmol, and 43%). The product was stored under N_2. ^{1}H NMR ($CDCl_3$, 300 MHz) 7.2–7.4 (m, 5H) 5.7–5.8 (s, broad, 1H), 4.36 (d, 2H), 3.61 (s, 2H), 2.73 (m, 4H), 1.61 (t, 1H).

2.14. Compound **28**

To a solution of **26** (0.56 g, 2.3 mmol) and triethylamine (0.25 g, 0.35 mL, and 2.3 mmol) in anhydrous methylene chloride (20 mL) at 0 °C, a solution of γ-methoxycrotonyl chloride [17] **27** (0.31 g, 2.3 mmol) in methylene chloride (10 mL) was added dropwise. The ice bath was then removed, the brown reaction mixture was stirred at room temperature for 25 min and washed with 0.1 M potassium acetate/acetic acid buffer (pH 4, 2×20 mL) and brine (20 mL). The organic layer was dried over magnesium sulfate and the solvent was evaporated. The product was purified by chromatography on silica gel with 2:1 hexanes/ethyl acetate followed by 1:2 hexanes/ethyl acetate. The solvent was removed to give **28** as an orange liquid (0.40 g, 1.2 mmol, and 51% yield). ^{1}H NMR ($CDCl_3$, 250 MHz) 7.2–7.4 (m, 5H), 6.85 (dt, 1H), 6.35 (dt, 1H), 6.3–6.5 (br s, 1H, triplet at high concentration), 4.41 (d, 2H), 4.08 (q, 2H), 3.60 (s, 2H), 3.41 (s, 3H), 3.03 (t, 2H), 2.67 (t, 2H). ^{13}C NMR ($CDCl_3$) 189.35, 170.73, 140.75, 134.52, 128.99, 128.51, 127.22, 126.87, 60.02, 43.20, 40.82, 30.56, 28.60.

2.15. Compound **14d**

To a solution of **28** (0.10 g, 0.29 mmol) in methanol (10 mL) was added aq. Tris buffer (0.1 M, pH 7.5) until a precipitate began to form. Penicillin amidohydrolase (50 U) was added, the pH was readjusted to 7.5 by addition of 0.1 M aq. HCl, and the solution was stirred at room temperature. The reaction was monitored for thiol formation by addition of a 20 μL sample to Tris buffer (1 mL, pH 8) containing DTNB (0.01 mM) and measuring the absorbance at 412 nM. Thiol concentration was calculated using $\varepsilon_{420} = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$. A maximum thiol concentration of about 0.22 mmol was observed after 3 h. The resulting solution was frozen and aliquots were used directly in assays for crotonase activity.

2.16. Enzyme assays

Chloramphenicol acetyltransferase activity was determined as previously described [1] in Tris buffer (1 mL, 0.1 M, pH 7.0) containing chloramphenicol (0.1 mM), DTNB (0.2 mM), acetyl-CoA (0.01–0.08 mM) or analogue **8** (0.02–0.15 mM), and chloramphenicol acetyltransferase from *Escherichia coli* (0.01 U). The reactions were monitored at 412 nm using $\varepsilon_{412} = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$. The K_i value for **10** was determined from assays at 0, 50, 100, and 200 μM concentrations of **10**. Activity with **11a** was determined using 30% ethanol with the Tris buffer, with 0.2 U enzyme and **14a** (50 mM), and was compared to activity with natural substrate under the same solvent conditions. The assay was initiated by addition of **13** (0.1 mM) and monitored for disappearance of the thioester absorbance at 233 nm. Assays with natural substrate in the presence of **13** (0.1 mM) were performed to test for inhibition.

Carnitine acetyltransferase activity was determined as previously described [18] in potassium phosphate buffer (1.0 mL, 25 mM, pH 7.5) containing carnitine acetyl-

transferase from pigeon breast muscle (0.026 U with acetyl-CoA and **8**, 3.4 U with **10**), L-carnitine (2.0 mM), DTNB (0.2 mM), and acetyl-CoA (0.01–0.1 mM) or analogue **8** (0.007–0.050 mM) or **10** (0.014–0.125 mM). The reactions were monitored at 412 nm using $\varepsilon_{412} = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$.

Citrate synthase activity was determined as previously described [1] in Tris buffer (1 mL, 0.1 M, pH 8.0) containing citrate synthase from Porcine heart (0.01 U), oxaloacetate (0.5 mM), DTNB (0.1 mM), acetyl-CoA (3–30 μM) or analogue **8** (0.02–0.15 mM). The reactions were monitored at 412 nm using $\varepsilon_{412} = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$. The K_i value for **10** was determined from assays at 0, 20, and 40 μM concentrations of **10**. Activity with **11a** was determined using 30% ethanol in the Tris buffer, with L-malate (3 mM), NAD (0.22 mM), citrate synthase (0.2 U), malate dehydrogenase (12 U), and **14a** (50 mM). The assay was initiated by addition of **13** (0.1 mM) and monitored for appearance of NADH at 340 nm. For comparison, activity with natural substrate under the same solvent conditions was determined as was activity with natural substrate in the presence of the same concentration of **13**.

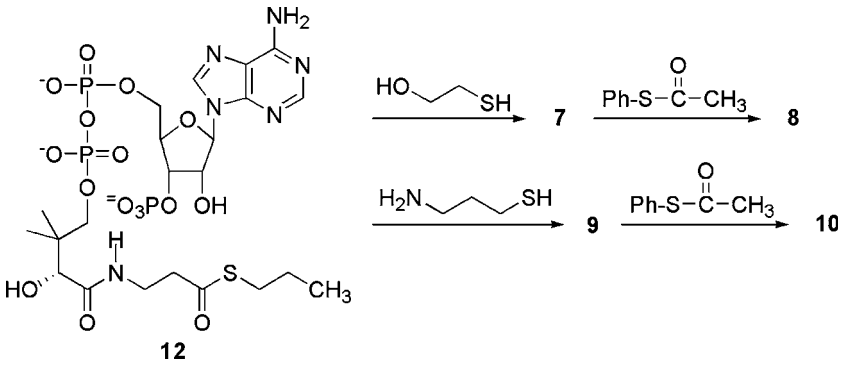
Phosphotransacetylase activity was determined in Tris buffer (1.0 mL, 0.1 M, pH 7.4) containing acetyl phosphate (7.4 mM), ammonium sulfate (19 mM), coenzyme A (28–86 μM) or homocysteamine-CoA **9** (28–160 μM), and phosphotransacetylase from *Bacillus stearothermophilus* (0.03 U). Initial rates were measured from the increase in absorbance due to the thioester bond at 233 nm ($\varepsilon_{233} = 4400\ M^{-1}\ cm^{-1}$).

Assays of hemithioacetal substrate analogue **11a** with citrate synthase and chloramphenicol acetyltransferase were performed as described above. Assays of 3-hydroxyacyl-CoA dehydrogenase were done in Tris buffer (1.0 mL, 50 mM, pH 7.3) containing 30% ethanol and NADH (0.3 mM), 3-hydroxyacyl-CoA dehydrogenase from Porcine heart (1.0 U) and **14b** (0.17–5 mM) or **14c** (50 mM). The baseline was recorded and the assay was initiated by addition of **13** (0.1 or 0.5 mM). The reaction was monitored at 340 nm using $\varepsilon_{340} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$. Comparative assays with natural substrate contained acetoacetyl-CoA (0.1 mM) in place of **13** and **14b** or **14c** under the same solvent conditions and used only 0.02 U enzyme.

Assays of crotonase were done in Tris buffer (1.0 mL, 50 mM, pH 7.5) containing crotonase from Bovine liver (1.0 U) and **14d** (0.5 mM). The baseline was recorded and the assay was initiated by addition of **13** (0.2 mM). The reaction was monitored at 280 nm using $\varepsilon_{280} = 3.6 \times 10^3\ M^{-1}\ cm^{-1}$ [19]. Comparative assays with natural substrate contained crotonyl-CoA (0.1 mM) in place of **13** and **14 d** and used only 0.02 U enzyme.

3. Results

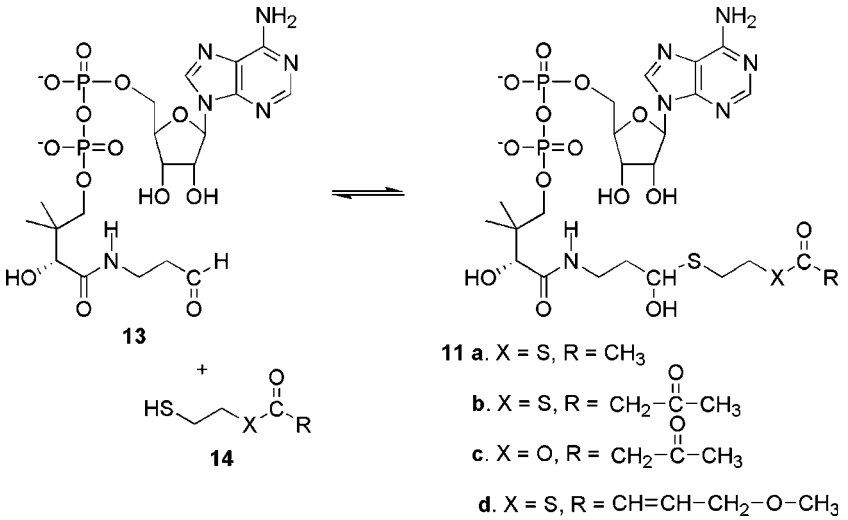
The ester analogue of CoA **7** and the corresponding acetate thioester **8** were prepared as shown in Scheme 1. Reaction of the CoA analogue synthon **12** with mercaptoethanol formed **7** by initial formation of the thioester followed by rearrangement to the oxoester. **12** was prepared using the enzymes that catalyze the final steps in CoA biosynthesis as previously reported [1]. **7** was converted to the acetyl thioester



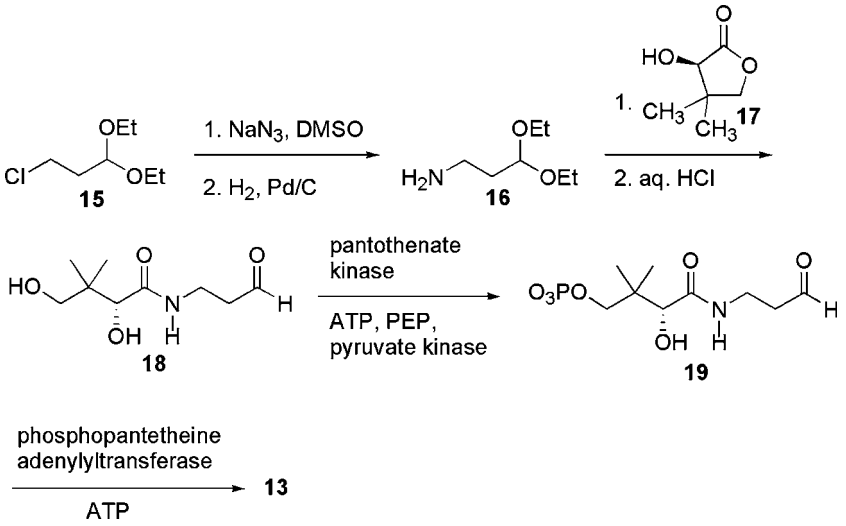
Scheme 1. Synthesis of ester and homocysteine analogues of acetyl-CoA.

8 by reaction with *S*-phenyl thioacetate. The previously reported homocysteamine analogue **9** [5] was prepared by reaction of 3-aminopropanethiol with **12**. **9** was also converted to the acetyl thioester **10** by reaction with *S*-phenyl thioacetate.

Hemithioacetal analogues **11** were generated in situ from the truncated aldehyde analogue of CoA **13** and the appropriate thiol **14** (Scheme 2). **13** was prepared as shown in Scheme 3. Chloropropionaldehyde diethyl acetal **15** was converted to the azide, which was reduced to the amine **16**. Reaction of **16** with pantolactone **17** followed by acid-catalyzed hydrolysis of the acetal formed the truncated aldehyde analogue of pantetheine **18**. **18** was phosphorylated enzymatically to form the phosphopantetheine analogue **19** catalyzed by pantothenate kinase, using ATP along with the ATP regeneration system of phosphoenolpyruvate and pyruvate kinase [20].



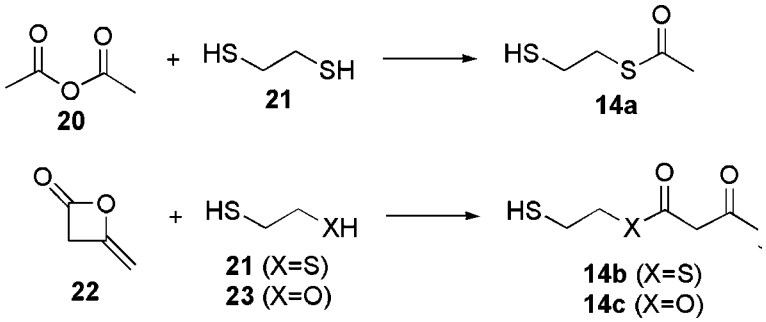
Scheme 2. In situ generation of hemithioacetal analogues of CoA esters.



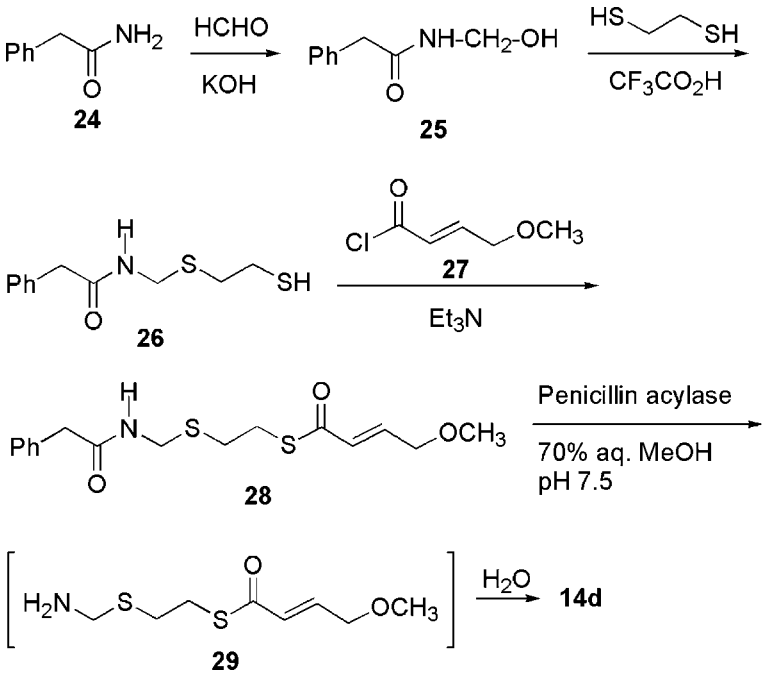
Scheme 3. Synthesis of a truncated aldehyde analogue of CoA.

19 was coupled with ATP catalyzed by phosphopantetheine adenylyltransferase to form the dephospho-CoA analogue **13**. Inorganic pyrophosphatase was included in this final step to hydrolyze the pyrophosphate produced, thus making the reaction irreversible. Attempts to phosphorylate **13** using dephospho-CoA kinase were unsuccessful.

Thioesters were prepared as shown in Scheme 4 for formation of hemithioacetal analogues of CoA esters upon reaction with **13**. The acetyl thioester **14a** was prepared by reaction of excess ethanedithiol **21** with acetic anhydride **20**. The acetoacetyl thioester **14b** was prepared by reaction of ethanedithiol **21** with diketene **22** [21]. The corresponding oxoester **14c** was prepared by reaction of mercaptoethanol **23** with diketene. The γ-methoxycrotonyl thioester **14d** was prepared as shown in Scheme 5. Reaction of phenylacetamide **24** with formaldehyde and base formed the *N*-(hydoxymethyl) amide **25** [16]. Reaction of **25** with ethanedithiol and trifluo-



Scheme 4. Synthesis of acyl derivatives of ethanedithiol and mercaptoethanol.



Scheme 5. Synthesis of a methoxycrotonyl thioester of ethanedithiol.

roacetic acid formed **26** [16,22]. **26** was reacted with γ-methoxycrotonyl chloride **27**, which was prepared from ethyl crotonate as previously described [17], to form **28**. The thiol was deprotected enzymatically using penicillin acylase to initially form the intermediate **29**, which spontaneously hydrolyzed to the thiol **14d** [16]. The thiol was not characterized due to its instability, but was used directly in an enzymatic assay.

The CoA ester analogues were tested as substrates for some commercially available CoA ester utilizing enzymes. Table 1 shows results for the acetyl-CoA oxoester analogue **8** and the homocysteine analog **10** along with activities for natural acetyl-

Table 1
Kinetic data for acetyl-CoA analogues as substrates and inhibitors of acetyl-CoA utilizing enzymes.

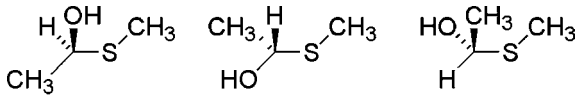
Substrate	Carnitine acetyltransferase		Chloramphenicol acetyltransferase		Citrate synthase	
	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}
2[a]	60	1	32	1	7	1
8	35	0.83	67	0.14	54	0.46
10	116	.006	116 (K_i)	$<10^{-5}$	20 (K_i)	$<10^{-5}$

[a] Relative V_{max} values of 1 correspond to 38 μmol/min/mg enzyme for carnitine acetyltransferase, 45 μmol/min/mg enzyme for chloramphenicol acetyltransferase, and 32 μmol/min/mg enzyme for citrate synthase.

CoA **2** for three enzymes. The oxoester **8** was a fairly good substrate for chloramphenicol acetyltransferase, carnitine acetyltransferase, and citrate synthase with K_m values slightly lower to 8-fold higher than those of acetyl-CoA and V_{max} values from 14 to >80% those of the natural substrate. The homocysteamine analogue **10** exhibited less than 1% the activity of the natural substrate with carnitine acetyltransferase and gave no measurable activity with chloramphenicol acetyltransferase or citrate synthase. K_m or K_i values were about 2- to 3.5-fold higher than the K_m for acetyl-CoA. The homocysteine CoA analog **9** was also tested as a substrate for phosphotransacetylase. The V_{max} for **9** was just less that 0.1% the activity with CoA **1**, while the K_m for **9** was 53 μM, compared to 83 μM for CoA.

Hemithioacetal formation with the truncated aldehyde analog was studied by NMR, using the phosphopantetheine analog **19** and mercaptoethanol **23** as a model. At 0.1 M mercaptoethanol and less than 10 mM aldehyde, 90% of the aldehyde was converted to the hemithioacetal, with the free aldehyde and hydrate accounting for 10%. Efforts to use hemithioacetals of ethanedithiol **21** as substrates for CoA-utilizing enzymes were not successful due to the insolubility of ethanedithiol, even in the presence of organic cosolvents. The acetyl thioester **11a** was generated from **14a** and **13** in 30% aq. ethanol to achieve solubility of **14a**, and using at least 50 mM **14a** to insure conversion of most of **13** to the hemithioacetal **11a**. No measurable activity was observed with the acetyl-CoA utilizing enzymes chloramphenicol acetyltransferase and citrate synthase under conditions in which less than 0.1% of the activity of the natural substrate under the same solvent conditions would have been detectable. No inhibition of either enzyme by **13** was observed at the concentration of **13** used in the assays. The acetoacetyl thioester **11b** is the hemithioacetal analogue of the natural substrate for β-hydroxybutyryl-CoA dehydrogenase. Again, 30% aqueous ethanol was necessary to achieve sufficient solubility of the thiol **14b**. The more water-soluble oxoester **14c** was also prepared and tested as a substrate for β-hydroxybutyryl-CoA dehydrogenase at a concentration of 50 mM in the presence of 0.1 mM **13**. However, no measurable activity was observed with **14b** or **14c**, indicating less than 0.1% of the activity of the natural substrate. **14d** was designed as a water-soluble compound for which the hemithioacetal **11d** was viewed as a potential substrate for crotonase. However, no crotonase activity was observed with **14d** in the presence of **13**, again indicating less than 0.1% of the activity of the natural substrate.

Computational studies were conducted on the simple hemithioacetal **30** to model the structure and conformation of the hemithioacetal linkage (Fig. 2). Energy minima were found for the three staggered conformations, which were optimized at the B3LYP/6-311+Gd,p level with an aqueous solvation model. Conformer **30a**, in which the two methyl groups are anti was determined to be about 0.4 kcal/mol lower



30a **30b** **30c**

Fig. 2. Three staggered conformations of a simple model hemithioacetal.

in energy than **30b** in which the *S*-methyl group is anti to the hydroxyl group but about 0.5 kcal/mol higher in energy than **30c** in which the *S*-methyl group is anti to hydrogen. The same order but somewhat larger energy differences were observed in MP2 calculations using the same basis set and in calculations without the solvation model. The dihedral angle between methyl groups in conformer **30a** is 171°, the methyl being skewed 9° away from the OH and toward H. The C–S bond lengths are about 1.8 Å and the C–S–C angle is about 100° in all three structures.

4. Discussion

Syntheses of the oxoester **7** and homocysteamine **9** analogues of CoA were based on the previously reported general CoA analogue synthon **12** [1]. In this synthon, the amide bond nearest the thiol group is replaced with a thioester and for synthetic convenience the thiol group is replaced with a methyl group, or in more recent work by a hydrogen atom. Reaction of **12** with mercaptoethanol resulted initially in formation of the thioester. The characteristic thioester absorbance at 230–235 nm was observed as a shoulder on the adenine absorbance peak centered at 260 nm. Rearrangement to the oxoester was observed by monitoring the disappearance of this shoulder in the UV spectrum. The equilibrium between *S*-acetyl thiopropanol and *O*-acetyl thiopropanol is reported to favor the oxoester by a factor of 56 [23], and the equilibrium constant between **7** and the thioester isomer is expected to be similar. The yield of **7** was only about 15%, apparently due to substantial hydrolysis of the thioester and/or oxoester over the course of the rearrangement reaction. This rearrangement was conducted under acidic conditions to avoid base-catalyzed hydrolysis and thiol oxidation. The homocysteamine analogue **9** was prepared by the more standard aminolysis reaction employed previously in the synthesis of several other CoA analogues. The yield in the conversion of **7** to the *S*-acetyl derivative of only 42% was also apparently due largely to partial hydrolysis of the ester under the basic conditions of the acetylation reaction. The synthesis of the truncated aldehyde analogue of dephospho CoA **13** followed standard reactions for the synthesis of the aldehyde analogue of pantothenic acid **18**. **18** was efficiently phosphorylated by pantothenate kinase and the resulting phosphopantothenic acid analogue **19** was efficiently converted to the dephospho-CoA analogue **13**. However, attempts to phosphorylate **13** catalyzed by dephospho-CoA kinase were not successful. Addition of mercaptoethanol or ethanolamine to convert the aldehyde to a hemithioacetal or imine, respectively, bearing the hydroxyethyl group as a mercaptoethyl mimic also failed to facilitate phosphorylation of **13**. The synthesis of acetyl and acetoacetyl derivatives of ethanedithiol and mercaptoethanol were straightforward as shown in Scheme 2. Synthesis of the methoxycrotonyl thioester **14** was more complicated. Reaction of the acid chloride **27** with one equivalent or greater of ethanedithiol resulted in thiol addition to the double bond in addition to reaction with the acid chloride. Reaction with less than one equivalent of ethanedithiol resulted in formation of a mixture of products including unacylated, monoacylated, and diacylated dithiol, and attempts to isolate the desired monoacylated product were unsuccessful.

Mono-*t*-BOC protected ethanedithiol was prepared and reacted with the acid chloride **27** to form the thioester. However, removal of the *t*-BOC protecting group under acidic conditions was accompanied by conjugate addition of the thiol group and other unidentified side reactions. Finally, an enzymatically cleaved thiol protecting group was employed as shown in Scheme 5. The thioester **28** was purified and characterized. Penicillin acylase was then used to generate the thiol **14d**, which was not isolated or characterized [22].

Previous observation of a more than 10,000-fold decrease in affinity of a carboxylate analogue of acetyl-CoA to citrate synthase upon substitution of the outermost amide bond with a pair of methylene groups indicated the potential importance of this amide bond in the function of CoA [7]. The oxoester is perhaps the most conservative substitution for this amide bond. Relative to an amide, the oxoester is less conformationally rigid, lacks the hydrogen bond donating amide hydrogen, and the carbonyl oxygen should be less basic and thereby a poorer hydrogen bond acceptor [24]. The results of Table 1 indicate that at least with the three enzymes studied, the effect of the amide to ester substitution is quite modest. Only with citrate synthase is the effect on substrate binding more than about 2-fold and only with chloramphenicol acetyltransferase is there more than about a 2-fold decrease in V_{max}.

The homocysteamine analogue **9** was reported previously and was shown to serve as a substrate for the holo-acyl carrier protein synthase from *E. coli* and as an inhibitor of the phosphotransacetylases from *E. coli* and from *C. kluyveri* [5,25]. The *S*-acetyl derivative **10** has apparently not been previously reported. In studies of acetoacetyl-CoA thiolase, an acetoacetyl derivative of a homopantetheine analogue was previously shown to exhibit a less than 2-fold decreased V_{max} and an approximately 3-fold increased K_m relative to the normal acetoacetyl pantetheine derivative [26]. The activity of **9** with phosphotransacetylase observed in this work is in contrast to the absence of activity observed in previous work [25]. However, this may simply reflect a greater sensitivity of our assays by use of larger quantities of enzyme. The similar K_m value for **9** relative to CoA is in agreement with the similar K_i value previously reported for the *E. coli* enzyme, though a much higher K_i value was reported for the phosphotransacetylase from *C. kluyveri* [25]. The kinetic results with **9** and **10** indicate that the extra methylene group has little effect on enzyme binding. However, the observation of little or no enzyme activity indicates that precise positioning of the thiol or acetylthio group in an enzyme active site is essential for activity, and this positioning is perturbed by the extra methylene group. These observations contrast the previous observations with acetoacetyl-CoA thiolase, where the more complex acetoacetyl group perhaps facilitates its own proper positioning in the active site despite the extra methylene group in the cysteamine moiety.

It was envisioned that if the hemithioacetal analogues **11** were accepted as substrates for CoA thioester utilizing enzymes, the truncated CoA aldehyde **13** could be used to activate simple thioesters as substrates for CoA thioester utilizing enzymes. As hemithioacetal formation is reversible, only a catalytic amount of **13** could be sufficient for quantitative enzymatic processing of the simple thioester. However, this potential strategy failed as none of the substrate analogues exhibited activity in

assays with their target enzymes. The generally limited aqueous solubility of the simple thioesters also proved problematic.

Calculations of the simple model hemithioacetal **30** indicate that the most stable conformation is represented by **30c** rather than **30a**, the conformation that best mimics the *trans* geometry of the amide bond. The higher energy of **30b** may be attributed to the loss of the stereoelectronic effect between the sulfur lone pair and the anti-bonding orbital of the C–O bond. The initially surprising greater stability of **30c** relative to **30a** is supported by the greater stability of the gauche relative to the anti-conformation of 2-thiobutane (CH_3–S–CH_2–CH_3) in the gas phase and similar observations in the solid state [27]. This may be due to a favorable van der Waal distance between methyl groups in the gauche conformation, imparted by the greater length of the C–S bonds relative to a C–C bond.

Hydrogen bonding between the N–H of the amide bond nearest the thiol group and the protein is consistently observed in enzyme–CoA complexes, including those of enzymes studied in this work. This N–H forms a hydrogen bond to the backbone carbonyl oxygens of Leu 273 in citrate synthase [28], Ala149 in chloramphenicol acetyltransferase [29], Gln555 in carnitine acetyltransferase [30], and Ala96 in crotonase [31]. Hydrogen bonding of the amide carbonyl oxygen of CoA is less consistently apparent but is observed with the side chain of Asn373 and the backbone amide N–H of Gly317 in citrate synthase [28] and a bound water molecule in chloramphenicol acetyltransferase [29]. Though hydrogen bonding to the amide N–H is always observed, a striking result of the studies with the oxoester analogue is that this hydrogen bond is not critical for substrate binding or catalysis. Replacement of the amide bond with the hemithioacetal linkage introduces much greater changes in structure. The bond angles at both the sulfur atom and the hemithioacetal carbon are substantially smaller than the angles expected for the amide, and the carbon-sulfur bonds are about 0.3 Å longer than the corresponding bonds of the amide. The anti-conformer that best mimics the amide bond is not the most stable conformation of the hemithioacetal and thus the concentration of the potentially active conformer is only a fraction of the total concentration. Hemithioacetal formation also introduces a chiral center, which further diminishes the concentration of any single structure, though either stereoisomer could potentially be accepted as a substrate. The hydroxyl oxygen of the hemithioacetal could potentially hydrogen bond to groups that form hydrogen bonds to the carbonyl oxygen of the natural amide functionality at this position. However, the hemithioacetal oxygen is certainly positioned differently due to the change in carbon hybridization and thus it is uncertain how effective such hydrogen bonding may be. Some combination of these factors result in a complete absence of observable activity with the hemithioacetal-linked substrate analogues.

5. Conclusion

A previously reported CoA ester analogue having the amide bond nearest the thiol/thioester group replaced with a pair of methylene groups and newly reported

analogues having a hemithioacetal linkage in place of this amide bond or an extra methylene group between the amide and thioester all exhibited greatly diminished binding and/or activity with the appropriate CoA ester utilizing enzymes. In contrast, an acetyl-CoA analogue having an ester linkage in place of the amide nearest the thioester was a very good substrate for the three acetyl-CoA utilizing enzymes tested. These results suggest that the carbonyl group and/or the double bond character and planarity of the amide or ester bond and proper distance between this bond and the thioester are necessary and sufficient for efficient substrate recognition and catalysis by CoA ester utilizing enzymes.

Acknowledgments

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