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An Efficient Chemoenzymatic Synthesis of Coenzyme A and Its Disulfide

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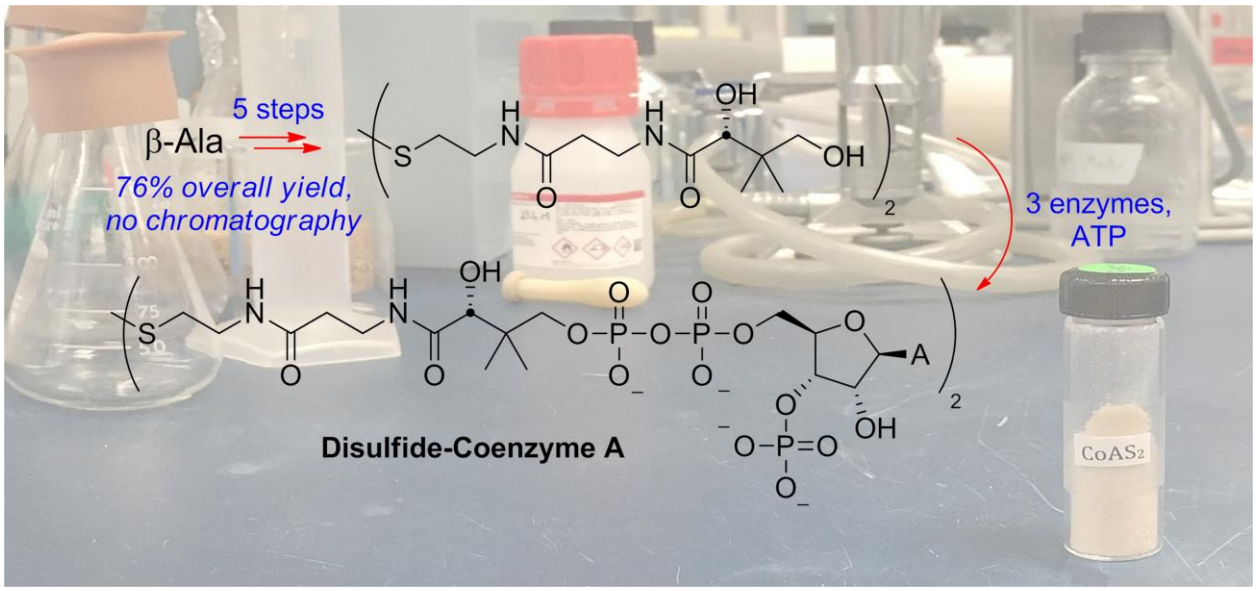


Abstract

We have developed a chemoenzymatic route to coenzyme A (CoASH) and its disulfide that is amenable to gram-scale synthesis using standard laboratory equipment. By synthesizing the symmetrical disulfide of pantetheine (pantethine), we avoided the need to mask the reactive sulfhydryl and also prevented sulfur oxidation by-products. No chromatography is required in our synthetic route to pantethine, which facilitates scale-up. Furthermore, we discovered that all three enzymes of the CoASH salvage pathway (pantetheine kinase, phosphopantetheine adenyltransferase and dephospho-coenzyme A kinase) accept the disulfide of the natural substrates and functionalize both ends of the molecules. This yields CoA disulfide as the product of the enzymatic cascade, a much more stable form of the cofactor. Free CoASH can be prepared by *in situ S-S* reduction.

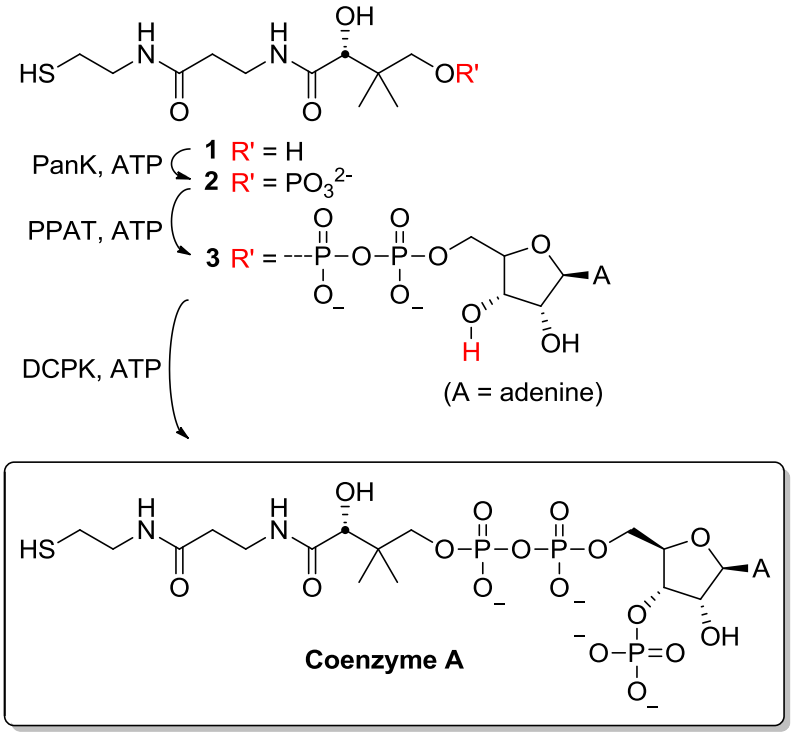
Keywords: Coenzyme A, chemo-enzymatic synthesis, disulfide, pantetheine, pantethine

Introduction

Coenzyme A (CoASH) is an essential acyl carrier and co-substrate for many biochemical processes. In addition to recognizing the acyl moiety of CoA derivatives, cognate enzymes also commonly interact with the cofactor portion and show little tolerance for structural perturbations (1). One must therefore supply native CoA intermediates in stoichiometric quantities for such enzymes. This is particularly relevant for enzyme-mediated aldol reactions and Claisen condensations, which are commonly used to form *C-C* bonds in many metabolic pathways. Such reactions might also be highly useful in chemical synthesis; unfortunately, enthusiasm for this approach must be tempered by the high cost of commercial CoASH ($2600 / g with >85% purity). To remove this obstacle, we have developed a simple chemo-enzymatic route to CoASH that can be carried out on gram scales and requires no chromatographic purification until the final target has been reached. Synthesis of the enzyme substrate requires only five steps and has a 76% overall yield from commercially available β-alanine.

Preparative quantities of CoASH have been isolated from microbial sources such as bakers' yeast (2) or *Brevibacterium ammoniagenes* IFO 12071 (3). Dried cells of wild-type or mutant *B. ammoniagenes* were subsequently used in chemo-enzymatic routes since they contained all five of the necessary CoASH biosynthetic enzymes (4). More recently, enzyme-assisted strategies have been developed by several groups for CoASH and its analogs (5) based on fundamental biochemical studies by Drueckhammer (6), Strauss and Begley (7), and Jackowski (8). Our route to CoASH was inspired by the work of Strauss and Begley and employs the three-enzyme cascade of the CoASH salvage pathway (Scheme 1). All three steps are ATP-dependent and the proteins from *Escherichia coli* have been cloned and overexpressed (6b, 8d, 9). Initial phosphorylation of **1** by pantetheine kinase (PanK) yields **2**, whose adenylation by phosphopantetheine adenyltransferase (PPAT) provides dephospho-CoA **3**. The final step installs the 2'-phosphate using dephospho-coenzyme A kinase (DCPK) to yield CoASH. In principle, all three enzymes could be present simultaneously; however, most PPAT enzymes (including

the *E. coli* form) are subject to feedback inhibition by CoASH (8c). This makes successive enzyme-catalyzed reactions more convenient for preparative purposes, an approach pioneered by Wright (5b). The three enzyme-catalyzed steps in CoASH production need little or no optimization; what discourages their large-scale use is the lack of an economical source for the substrate. One key observation is that the three enzymes tolerate significant structural alterations at the sulfhydryl moiety and this has allowed CoASH analogs to be synthesized by the same route (5). Based on this, we hypothesized that the three enzymes might therefore accept a disulfide dimer of pantetheine (known as pantethine) and simultaneously functionalize both ends, ultimately producing the disulfide of CoASH.

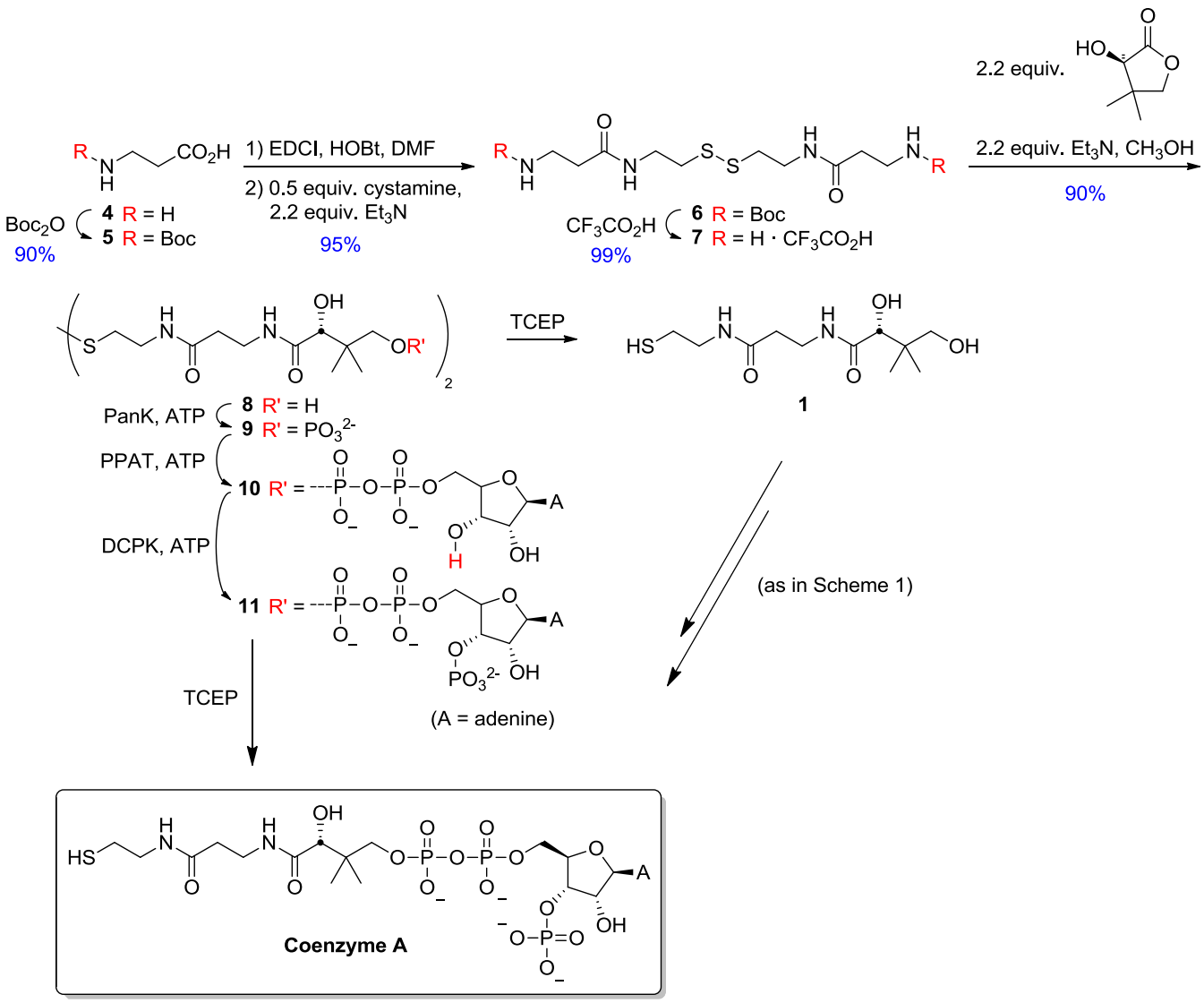


Scheme 1

We chose the disulfide derivative of **1** as the biocatalytic precursor for several reasons. First, a disulfide eliminates the two steps needed to install and later remove the sulfhydryl protecting group. In a sense, our "protecting group" is a second molecule of the target compound. This is an atom economical strategy that eliminates carrying extraneous mass through the sequence. A disulfide also

diminishes the chances of forming sulfur oxidation by-products during the synthetic operations. The dimeric intermediates were organic-soluble, which allowed the desired products to be isolated by solvent extraction with no need for chromatographic purification. This is a significant advantage when scaling up the chemical synthesis steps. Finally, the commercial price of oxidized CoASH – the product of our synthetic route – is almost 10-fold greater than that of CoASH itself (10).

In principle, the biocatalytic precursors (pantetheine **1** or pantethine **8**) could also be synthesized enzymatically or obtained commercially; however, we found it more convenient to prepare the building block by traditional organic methodologies (Scheme 2). While routes to **1**, **8** and analogs are known (5a, 11), our goals were to optimize each reaction and eliminate chromatographic separations that would hinder scale-up. We also sought a simple method to isolate the disulfide of CoASH on a preparative scale that avoided HPLC.



Scheme 2

Results and Discussion

To synthesize panthethine **8**, β-alanine **4** was reacted with a slight excess of Boc_2O at 0ºC to afford *N*-protected **5** in 90% yield (12),(13). The crude product, isolated by simple solvent extraction, was sufficiently pure to be used directly in the key coupling step with 0.50 equivalents of cysteamine disulfide (cystamine). Initial attempts using PPh_3, I_2 and imidazole in CH_2Cl_2 gave **6** in only 15 – 20% yield; in addition, the phosphine oxide by-product had nearly the same mobility on silica gel

chromatography as **6**. One solution was to synthesize a linear polystyrene with pendant phosphines (14), which was used in place of free PPh_3. Unfortunately, because both **6** and the polymeric reagent were soluble in CH_2Cl_2, post-reaction separation remained an issue (15). We achieved smooth coupling of **5** and cystamine by employing EDCI and HOBt (16). Several aspects of this process were optimized to facilitate scale-up. In addition to the originally-reported solvent (CH_2Cl_2), we also screened several other conditions. DMF at room temperature proved best along with dropwise addition of cystamine. Most importantly, we eliminated column chromatography by judicious extractions that removed both HOBt and the carbodiimide by-product and provided **6** in 95% yield with no need for further purification. Formation of a symmetrical dimer with no contamination by monoacylation product was confirmed by the observation of only 8 signals in the ^{13}C NMR spectrum of **6**.

The Boc group was removed quantitatively using TFA to yield ammonium salt **7** (17). This was coupled with D-pantolactone by heating an ethanolic solution at reflux in the presence of excess Et_3N as described by King *et al*. (11). Pantethine **8** was isolated after solvent extraction. The four non-enzymatic steps proceeded in 76% overall yield on gram scales and no column chromatography was required. We believe that this route is thus amenable to scale-up. Moreover, this process give an easy access to pantetheine **1** by TCEP reduction (or other reducing agent as 2-mercaptoethanol).

Base-catalyzed racemization of D-pantolactone, *e.g.*, when heated in DMF, has been reported (18). Because our conversion of **7** to **8** involved heating an ethanolic solution of D-pantolactone in the presence of Et_3N, we carried out a control reaction to determine whether racemization might have occurred prior to coupling. Optically pure D-(-)-pantolactone was heated at reflux in EtOH overnight in presence of 5 equivalents of Et_3N, which mimicked the reaction conditions employed in the coupling step. Chiral-phase GC analysis of the reaction mixture revealed only a single peak for pantolactone within the detection limits of the instrument (19). Based on these results, and the ^{13}C NMR spectrum of

8, we believe that our reaction product is diastereomerically pure and that no significant racemization took place.

Before attempting the enzymatic cascade with the disulfide, we carried out pilot experiments with the known substrate **1**. The disulfide bond of **8** was reduced quantitatively by a slight molar excess of *tris*(2-carboxyethyl)phosphine (TCEP) in the buffer used for the enzyme-catalyzed reactions (75 mM Tris-Cl, 25 mM KCl, 250 mM $MgCl_2$, pH 7.5). The crude product was used directly as a substrate for the three successive enzyme-catalyzed reactions using PanK, PPAT and DPCK in the presence of excess ATP to ensure that all conversions proceeded to completion (Scheme 1). This sequence yielded a sample of CoASH whose HPLC and MS data were identical to those of commercially-supplied material. These reactions were performed on a small scale and quantitative conversion was observed at each step according to HPLC analysis (Figure 1). As expected, the intermediates and final product were somewhat unstable in the presence of oxygen, likely because of thiol oxidation.

To avoid side-reactions involving the free thiol of **1** and its derivatives, we employed disulfide **8** as an alternate substrate for the three CoASH salvage enzymes. The limited solubility of pyrophosphate in the presence of Mg^{2+} caused precipitation during reactions with PPAT at high substrate concentrations, which interfered with the enzymatic reaction. Filtration to remove the salt followed by adding additional PPAT solved this issue. Quantitative conversions were observed for each step (as monitored by HPLC; Figure 2).

To ensure that all the reactions had proceeded to completion at both ends of the symmetrical substrates, samples were reduced after each step with TCEP and analyzed by HPLC. In each case, only a single product was observed, corresponding to the intermediates in the sequence that commenced with **1**.

We explored several strategies for purifying the disulfide of CoASH. Reversed-phase HPLC, while effective, had very low capacity that greatly limited its practical scalability. We initially used anion-

exchange resins developed for protein purification, *e.g.*, Mono-Q. These provided high product purities, but also suffered from limited capacities. Dowex anion exchange resin combined low material cost, high loading capacity and simple operation. After binding, increasing concentrations of LiCl were used to separate CoA disulfide **11** from by-product (20). A linear LiCl gradient yielded individual fractions containing AMP, ADP, ATP and **11**, which allowed recovery of the adenosine by-products and regeneration into ATP if desired. For preparative purposes, however, we normally employed a simplified step gradient in which all the nucleotide by-products eluted as an initial group and the desired product **11** was eluted by 600 mM LiCl. CoA disulfide was desalted by absorption on activated charcoal. Excess LiCl was removed by rinsing the activated charcoal with deionized water, then CoA disulfide was eluted with a 40% acetone solution containing 0.028% ammonia. The solution was concentrated under reduced pressure and the residual water was removed by lyophilization to yield the desired product (20).

The structure of synthetic CoA disulfide was verified by ^{1}H, ^{13}C and ^{31}P NMR along with HRMS. All data supported the assignment and no extraneous peaks were observed. As expected, the ^{31}P spectrum showed only three peaks (a singlet at 0.60 ppm and doublets at -10.27 and -10.81 ppm), corresponding to the three phosphate moieties of **11**. No other significant phosphorus-containing species were observed at the limits of ^{31}P NMR detection, further confirming that the reactions by all three CoASH salvage enzymes proceeded to completion at both ends of the molecule. The ^{31}P NMR data showed that anion exchange chromatography had successfully removed all nucleotide mono-, di- or triphosphosphate by-products from the enzyme-catalyzed steps. The chemical purity of CoA disulfide purity was assessed by UV absorbance (ε^{260} = 14,320) and found to be *ca*. 82%. For biochemical purposes, the cofactor can be used directly in this form (21). As HPLC and NMR analysis showed only CoA disulfide, we attributed the mass impurity to inorganic salts (most likely LiCl carried over from ion exchange purification). Consistent with this notion, the purity of CoA disulfide could be raised to 90%

by a second adsorption / desorption treatment with activated charcoal (22). Similar values for purity were found by both UV absorbance and elemental analysis. The latter suggested that **11** had been isolated as a *hexakis*-ammonium salt with two waters of hydration.

We found that solid CoA disulfide could be stored at room temperature in the presence of oxygen for at least one month with no evidence of by-product formation. Coinjecting purified CoA disulfide with a commercial sample of CoASH gave two peaks using reversed-phase HPLC, as expected. When a slight molar excess of TCEP was added to the sample of CoA disulfide prior to HPLC analysis (23), only one peak was observed at the t_r corresponding to free CoASH (11.47 min). This allows the cofactor to be stored as the stable disulfide, then reduced *in situ* as needed for biochemical applications.

Because of its high value, we also prepared the disulfide of 3'-dephospho-CoA using an analogous sequence of two enzyme-catalyzed steps (but leaving out the third, DCPK). While the synthetic effort itself was successful, the desired product co-eluted with ADP when the standard ion-exchange purification method was employed. Future efforts toward 3'-dephospho-CoA will require a revised purification method.

Using the optimized methods and standard laboratory equipment, we prepared 5 g batches of **8** in a single campaign. This was converted to CoA disulfide in lots of 1.89 g per reaction (each divided into two anion exchange runs) with a purity ≥90%. This has allowed us to generate multi-gram amounts of the cofactor in approximately two weeks. The yield of purified CoA disulfide from precursor **8** was 95%; the overall yield of the sequence starting from β-alanine was 72%.

Conclusion

A simple chemo-enzymatic route to CoASH has been developed that emphasizes high yield and scalability. No chromatographic purification steps are required until the final target has been reached. Because the CoASH salvage pathway enzymes (PanK, PPAT and DPCK) can all accommodate disulfide

dimer substrates, we could prepare an even higher-value CoASH derivative, eliminate thiol protection / deprotection steps and avoid the possibility of sulfur oxidation by-products. We hope that this chemo-enzymatic methodology can enable further progress in using CoA-dependent enzymes in chemical synthesis by making the substrates more easily obtainable. While we currently have no plans to increase the reaction scales further, none of the procedures would appear to be problematic should future needs dictate further scale-up.

Experimental Section

General. Moisture- and O_2-sensitive reactions were carried out in flame-dried glassware under Ar. Evaporations were conducted under reduced pressure at temperatures below 35°C unless otherwise noted. ^{1}H NMR spectra were recorded at 300 MHz at 25°C in the indicated solvent and referenced to residual protons ($CDCl_3$, 7.26 ppm; CD_3OD, 4.87 ppm; D_2O, 4.75 ppm). ^{13}C NMR spectra were recorded at 75 MHz at 25°C in the indicated solvent and referenced to solvent ($CDCl_3$, 77.2 ppm; CD_3OD, 49.2 ppm). ^{31}P NMR spectra were recorded at 121 MHz at 25°C in D_2O and referenced to the solvent deuterium lock frequency.

HPLC methods. Enzymatic reactions were monitored by reversed-phase HPLC using a 150 × 4.6 mm Synergi™ Hydro-RP 80Å column using 50 mM NaP_i pH 5.0 and 50 mM NaP_i, pH 5.0 / 20% acetonitrile as Solvents A and B, respectively at a flow rate of 1 mL / min. Initial conditions (3% B) were maintained for 2.5 min, then a linear increase to 18% B over 5 min was immediately followed by a linear increase to 28% B over 3.5 min, then a linear increase to 90% B over 10 min. After a 5 min hold at 90% B, a linear decrease to 3% B over 3 min was followed by a 5 min hold at the initial conditions (3% B). The eluant was monitored by UV absorbance simultaneously at both 220 and 260 nm (to detect peptide bonds and adenyl moieties, respectively). These conditions allowed baseline separation of all relevant analytes in this study (pantetheine **1,** t_r = 14.64 min; 4'-phospho-pantetheine **2**, t_r = 7.39 min;

dephospho CoASH **3**, t_r = 13.36 min; CoASH t_r = 11.49 min; pantethine **8**, t_r = 20.86 min; 4'-phospho-pantethine **9**, t_r = 14.67 min; dephospho-CoASH disulfide **10**, t_r = 16.27 min; CoA disulfide **11**, t_r = 14.18 min).

3-((*tert*-butoxycarbonyl)amino)propanoic acid 5. To a stirred solution of 3-amino-propanoic acid (5.00 g, 55.7 mmol) in 10% NaOH (50 mL) at 0°C was slowly added 1.2 equiv. of Boc anhydride (15 mL, 66.7 mmol). The resulting mixture was allowed to warm to room temperature. The reaction was monitored by TLC (10% MeOH/$CHCl_3$) and when complete (3 hr), the mixture was diluted with EtOAc (50 mL), brought to pH 2 with 1 M HCl, and the layers separated. The organic layer was washed with water (15 mL) and brine (15 mL), then dried over $MgSO_4$. The drying agent was rinsed with 20 mL of ice-cold hexanes and concentrated *in vacuo*. The resulting oil was dissolved in a minimum volume of CH_2Cl_2 and added slowly to a stirred solution of pentane (100 mL) cooled to 0ºC. The resulting white solid was collected by filtration (9.99 g, 90%). m.p. 76-77ºC, lit. m.p. 75-77ºC (24). ^{1}H and ^{13}C NMR data matched those reported by McCubbin *et al*. (25).

di-*tert*-Butyl(((disulfanediylbis(ethane-2,1-diyl))bis(azanediyl))bis(3-oxopropane-3,1-diyl))dicarbamate 6. To a stirred solution of **5** (4.00 g, 21.1 mmol) in DMF (40 mL) at rt was added HOBt (2.45 g, 21.1 mmol) followed by EDCI (3.71 mL, 21.1 mmol). The reaction was stirred for 30 min, then a solution containing 0.5 equiv. of cystamine (2.38 g, 10.6 mmol) and 2.2 equiv. of Et_3N (6.30 mL, 46.4 mmol) in DMF (10 mL) was added dropwise. The mixture was stirred overnight, then diluted 5-fold with water and extracted with Et_2O (3 × 200 mL). The combined organic layers were washed successively with 30 mL 1 M HCl, 10% $NaHCO_3$ and water, then dried over $MgSO_4$. The solution was concentrated *in vacuo* and the desired product **6** was obtained as a white solid (4.95 g, 95%). m.p. 102-103ºC. HRMS: m/z $[M + H]^+$ calcd for $C_{20}H_{38}O_6N_4S_2$: 495.2306; found: 495.2326. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.72 (s, 2H), 5.28 (s, 2H), 3.57 (q, J = 6 Hz, 4H), 3.41 (q, J = 6 Hz, 4H), 2.83 (t, J = 6

Hz, 4H), 2.45 (t, J = 6 Hz, 4H), 1.44 (s, 18H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) : δ 171.9, 155.9, 78.9, 38.2, 37.4, 36.6, 35.9, 28.1 ppm.

***N,N'*-(Disulfanediylbis(ethane-2,1-diyl))bis(3-aminopropanamide) · (CF_3CO_2H)$_2$ 7**. Excess CF_3CO_2H (37.3 mL, 487.19 mmol) was added to a solution of **6** (4.82 g, 9.74 mmol) in CH_2Cl_2 (100 mL) at rt. The reaction mixture was allowed to stir for 6 hr, then the solvent was removed *in vacuo* to afford the crude product as the *bis*-trifluoroacetate salt (5.08 g, 99%). HRMS: m/z $[M + H]^+$ calcd for $C_{10}H_{22}O_2N_4S_2$: 295.4320; found: 295.1248. ^{1}H NMR (300 MHz, CD_3OD): δ 8.31 (s, 6H), 8.01 (s, 2H), 3.46 (t, J = 6 Hz, 4H), 3.18 (t, J = 6 Hz, 4H), 2.79 (t, J = 6 Hz, 4H), 2.61 (t, J = 6 Hz, 4H) ppm. ^{13}C NMR (75 MHz, CD_3OD): δ 172.4, 159.0 (d, ^2J = 42 Hz), 116.0 (q, ^1J = 283 Hz), 39.6, 38.3, 37.2, 33.0 ppm.

(*S*)-*N*-((*R*)-17,19-Dihydroxy-18,18-dimethyl-3,12,16-trioxo-7,8-dithia-4,11,15-triazanonadecyl)-2,4-dihydroxy-3,3-dimethylbutanamide 8. Three equivalents of D-(-)-pantolactone (3.73 g, 28.7 mmol) and 2.5 equiv. of Et_3N (3.23 mL, 23.92 mmol) were added to a solution of **7** (5 g, 9.57 mmol) in EtOH (40 mL). The solution was allowed to stir overnight at reflux. The solvent was evaporated under reduced pressure and excess D-(-)-pantolactone was removed by triturating the residue with diethyl ether (2 × 50 mL). Solvent was removed *in vacuo* to afford **8** as an orange solid (5.04 g, 90%). mp = 63-65°C. HRMS: m/z $[M + Na]^+$ calcd for $C_{10}H_{23}O_2N_4S_2$: 577.1300; found: 577.2309. ^{1}H NMR (300 MHz, CD_3OD) : δ 3.95 (s, 2H), 3.49 (m, 12H), 2.82 (t, J = 6 Hz, 4H), 2.48 (t, J = 6 Hz, 4H), 0.89 (2s, 12H). ^{13}C NMR (75 MHz, D_2O): 174.9, 173.8, 75.6, 68.3, 38.5, 38.0, 36.4, 35.3, 35.2, 20.4, 19.1 ppm.

Overexpression and purification of PanK, PPAT, and DPCK. Individual plasmids that overexpressed *E. coli* PanK, PPAT, and DPCK (kindly provided by Prof. Steve Bruner, University of Florida; also available from Addgene) were used to transform *E. coli* BL21(DE3) cells with selection for kanamycin resistance (50 μg / mL) on LB plates. Single colonies of the appropriate strains were used to

inoculate 50 mL portions of liquid LB medium containing 50 μg / mL kanamycin and cultures were grown overnight at 37°C and 250 rpm. Aliquots (40 mL) were diluted into 4 L of the same medium in a New Brunswick M9 fermenter. Cultures were grown at 37ºC with stirring at 600 rpm and air flow at 6 L / min until reaching an optical density of 0.6 at 600 nm. Overexpression of PanK, PPAT, or DPCK was induced by adding sterile isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM, along with a solution of 16 g of dextrose in 100 mL of water. The culture was incubated for an additional 4 h at 30°C, then the cells were harvested by centrifugation (6000 × *g* at 4ºC for 15 min). The cell paste was resuspended in 20 mL of cold lysis buffer (50 mM Tris-HCl, pH 8.0) and disrupted by two passages through a French pressure cell at 18,000 psi. The lysate was clarified by centrifugation (18,000 × *g* for 20 min at 4ºC) and applied to a 5 mL Ni–NTA agarose column (GE Healthcare) previously equilibrated with 20 mL of binding buffer (20 mM NaP_i, 500 mM NaCl, 20 mM imidazole, pH 7.4). The column was washed with binding buffer until the A^{280} value returned to its baseline value, then the desired protein was eluted by washing with 20 mM NaP_i, 500 mM NaCl, 500 mM imidazole, pH 7.4. The protein solution was concentrated at 4ºC by ultrafiltration and typical yields of purified enzymes from 4 L cultures were 130 - 150 mg. Purified proteins were stored in 50% glycerol in 1 mL aliquots at -80°C.

CoASH synthesis. The reaction mixture contained 20 mM KCl, 10 mM $MgCl_2$, 5 mM ATP (5 μmol), 50 mM Tris–Cl, pH 7.5, and 2.5 mM pantethine **8** (2.5 μmol) in a final volume of 1 mL. Two equivalents of solid TCEP (3 μmol) were added to produce pantetheine **1** *in situ* (23). The initial phosphorylation reaction was initiated by adding 0.17 mg of purified PanK and incubating at rt for 2 hr. After this time, 0.16 mg of purified PPAT and an additional 5 μmol of ATP was added and incubation was continued (overnight at rt). Finally, 0.16 mg of purified DPCK along with an additional 5 μmol of ATP were added and incubation was continued overnight at rt. Samples were analyzed by reversed-phase HPLC to ensure that conversions had proceeded to completion before the next enzyme was added.

The purified product was characterized by MALDI MS: calcd. for $C_{21}H_{36}N_7O_{16}P_3S$ $[M + H]^+$ 768.1152; found 768.1242.

CoA disulfide synthesis. The reaction mixture contained 25 mM KCl, 250 mM $MgCl_2$, 30 mM ATP (3.0 mmol), 75 mM Tris–Cl, pH 7.5, and 13 mM pantethine **8** (1.3 mmol, 0.720 g) in a final volume of 100 mL. The reaction was initiated by adding 3 mg of purified PanK and incubating at rt for 2 hr. After this time, 3 mg of purified PPAT along with an additional 3.0 mmol of ATP was added and incubation was continued (overnight at rt). The $Mg{\cdot}PP_i$ complex that precipitated during the reaction was removed by filtration. Finally, 3 mg of purified DPCK and 3.0 mmol of ATP were added and incubation was continued (overnight at rt). Samples were analyzed by reversed-phase HPLC to ensure that conversions had proceeded to completion before the next enzyme was added.

CoA disulfide was purified by injecting half of the crude reaction mixture (50 mL, containing *ca*. 14 mM CoA disulfide) onto a 650 × 25 mm Dowex 1 × 2 anion exchange column that had been equilibrated with solvent A (10 mM HCl, 300 mM LiCl). The column was washed with solvent A for 180 min (to elute ATP, ADP and AMP), then the buffer was changed 100% solvent B (10 mM HCl, 600 mM LiCl) for 120 min to elute CoA disulfide. The flow rate was 10 mL / min throughout. Fractions containing CoA disulfide were then evaporated under vacuum. The material from both runs was combined. The resulting solid (33 g) was dissolved in a minimum volume of water (200 mL) and brought to pH ≈ 3, then activated charcoal (20 g) was added. The resulting suspension was stirred for 30 minutes at rt and then the solid was collected by vacuum filtration. The filter cake was washed with 1 L of deionized water (adjusted to pH ≈ 3) to remove unwanted inorganic salts, then CoA disulfide **11** was eluted with a 40% (v/v) aqueous acetone solution containing 0.028% ammonia. The combined solutions containing **11** were concentrated *in vacuo* to remove acetone, then water was removed by lyophilization to give the desired product **11** as a pale yellow solid (1.97 g). The adsorption onto activated charcoal was repeated a second time to provide analytically-pure **11** (1.89 g, 95%). Purity was assessed by

dissolving 5.0 mg of purified **11** in 100 mL of 1 M Tris-Cl, pH 7.5; this solution yielded A^{260} = 0.8013. Based on the extinction coefficient (ε^{260} = 14,320), the solution contained 4.5 mg of **11** in 100 mL. Elemental analysis calcd. for $C_{42}H_{70}N_{14}O_{32}P_6S_2 \cdot 6\ NH_4 \cdot 2\ H_2O$: %C 30.15, %H 5.66, %N 16.74; found %C 27.42, %H 5.40, %N 15.31. MALDI MS calcd. for $C_{42}H_{70}N_{14}O_{32}P_6S_2$ $[M + H]^+$ 1533.2159; found 1533.2234. ^{1}H NMR (300 MHz, D_2O) : δ 8.47 (s, 2H), 8.14 (s, 2H), 6.11 (d, *J* = 6 Hz, 2H), 4.67 (s, 4H), 4.55 (s, 2H), 4.21 (t, *J* = 3 Hz, 4H), 3.96 (s, 2H), 3.78 (m, 2H), 3.53 (m, 2H), 3.38 (m, 7H), 2.69 (q, *J* = 6 Hz, 4H), 2.41 (t, *J* = 6 Hz, 4H), 1.34,1.24,0.84,0.71 (4s, 12H). ^{31}P (121 MHz, D_2O): 0.60 (s), -10.27 (d, *J* = 22 Hz), -10.81 (d, *J* = 22 Hz). ^{13}C NMR (75 MHz, D_2O): 174.6, 173.7, 155.0, 152.4, 148.9, 139.6, 118.2, 86.5, 83.5, 74.1, 73.9, 71.8, 65.5, 58.0, 38.3, 36.5, 35.3, 35.2, 20.8, 18.2 ppm.

Acknowledgements

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Supporting Information

NMR, HRMS and HPLC data for all intermediates and CoA disulfide (17 pages).

References and Notes

(1) Polyketide synthases and non-ribosomal peptide synthases are exceptions to this rule and often accept acylated *N*-acetylcysteamines. Such synthases first catalyze transacylation to a covalently-linked thioester intermediate, which is the actual substrate for subsequent conversions.

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mixture was kept on ice with periodic mixing for 30 min. The reaction was quenched by adding 100 μL of 1 M $NaHCO_3$. HPLC indicated compete conversion of CoASH to succinyl-CoA

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Figure Legends

Figure 1. HPLC characterization of pantatheine conversions. A. Pantetheine **1**. **B**. Phosphopantetheine **2**. **C**. Dephospho-coenzyme A **3**. **D**. Coenzyme A.

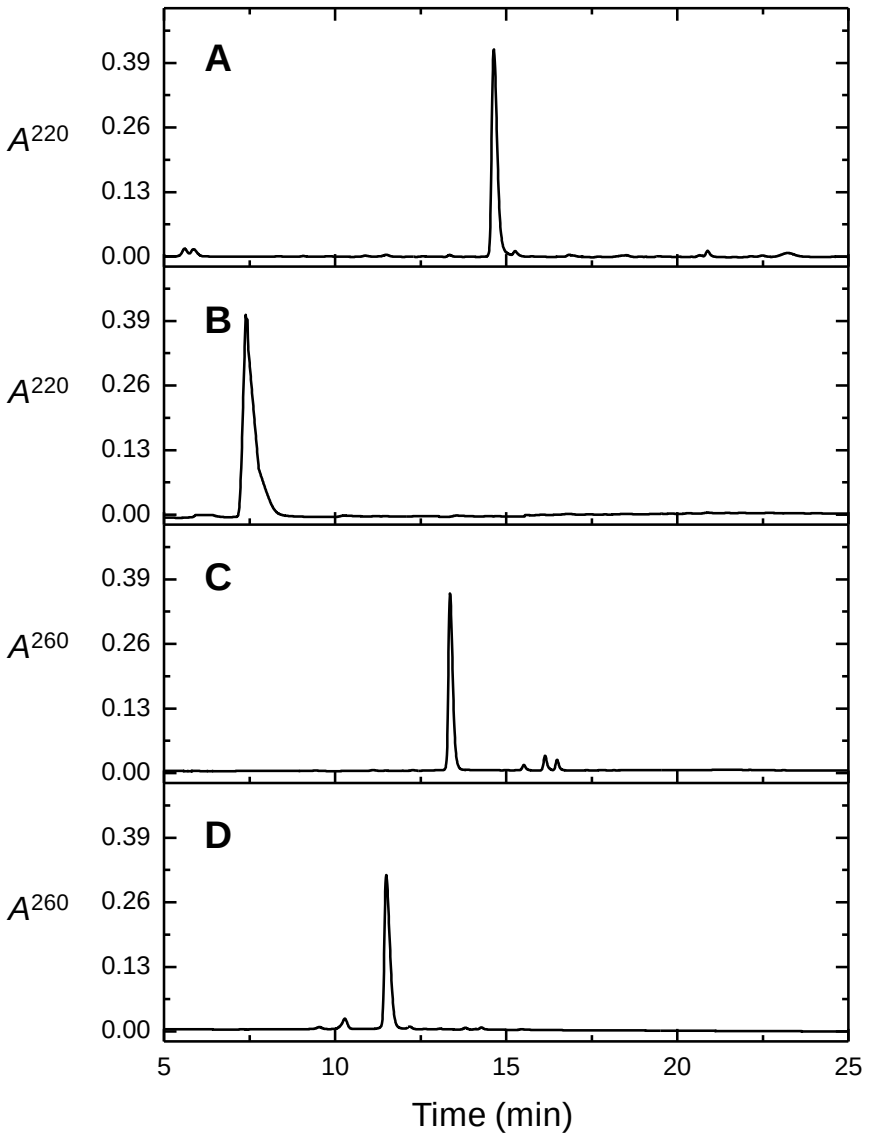
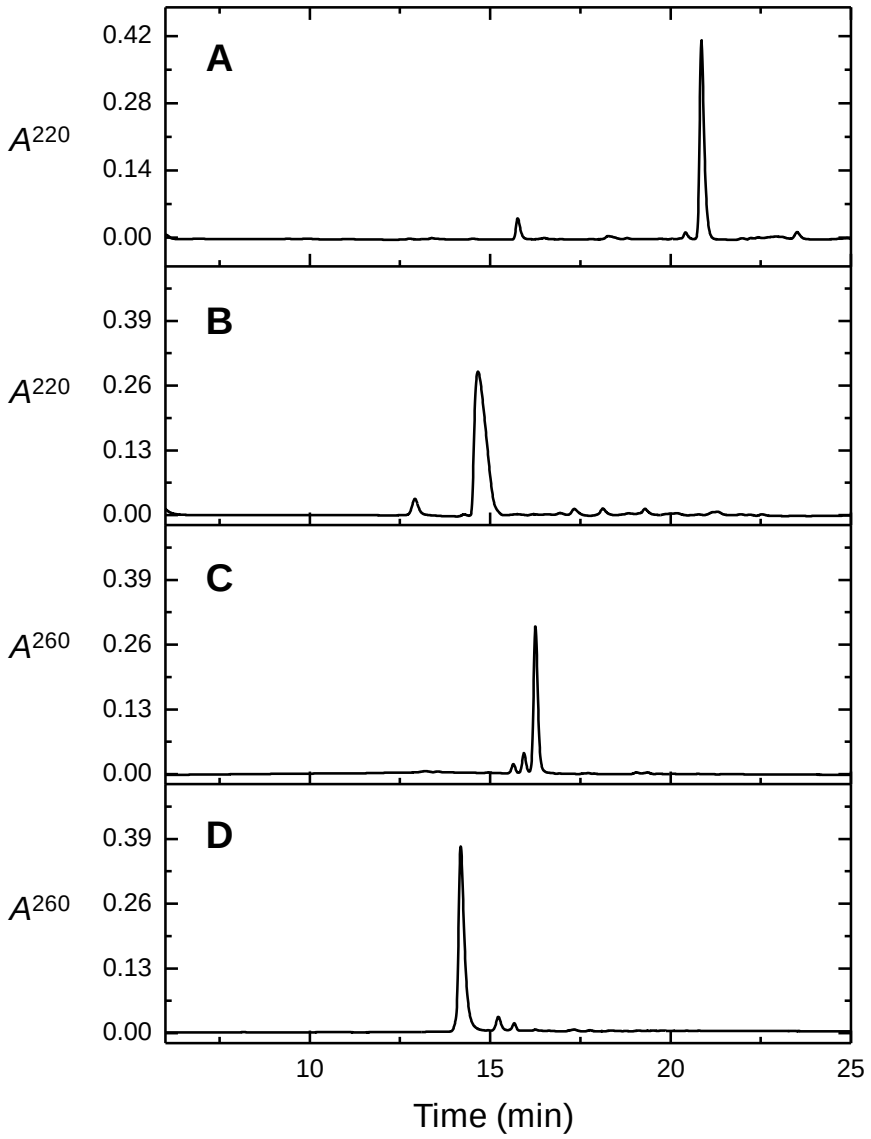
Figure 2. HPLC characterization of pantathine conversions. A. Pantethine **8**. **B**. Phosphopantethine **9**. **C**. Dephospho-coenzyme A disulfide **10**. **D**. Disulfide-coenzyme A **11**.