Development of a Second Generation Coenzyme A Analogue Synthon

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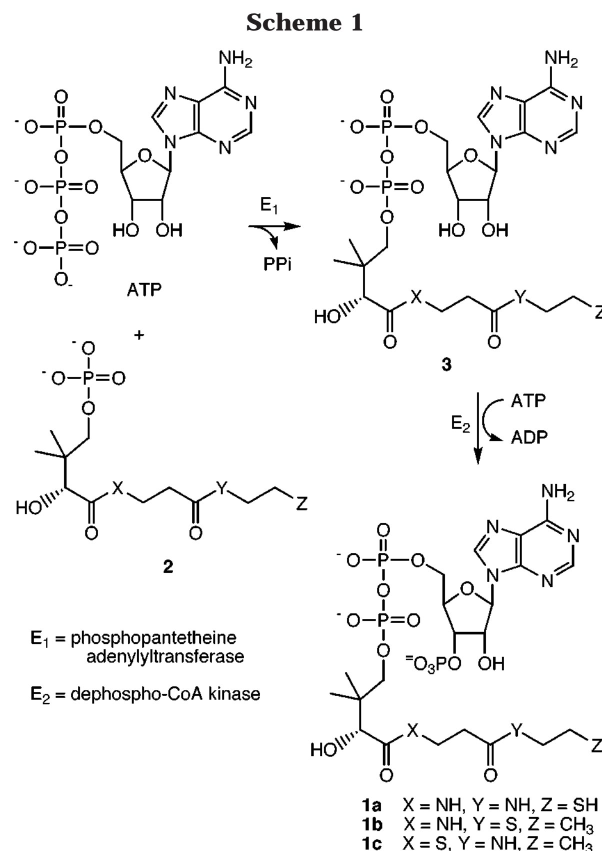
We have previously reported a general synthetic approach to analogues of coenzyme A (CoA) which involves enzymatic synthesis of a general CoA analogue synthon having a thioester linkage in place of the amide bond nearest the thiol group (Martin et al. *J. Am. Chem. Soc.* **1994**, *116*, 4660). We report here the synthesis of a second CoA analogue synthon **1c** which has the amide bond more distant from the thiol group replaced with a thioester. This analogue was prepared by nonenzymatic synthesis of a racemic phosphopantetheine analogue followed by enzymatic conversion to the corresponding CoA analogue. Stereochemical analysis showed that the natural enantiomer of the phosphopantetheine analogue was selectively converted to product by the enzyme phosphopantetheine adenylyltansferase, yielding a product that possessed the desired stereoconfiguration. Reaction of the new synthon **1c** with a primary amine results in amide bond formation to form the CoA analogue of interest. This new methodology provides access to an even broader array of CoA analogues modified in the β-alanylcysteamine moiety. This has been demonstrated in the synthesis of an analogue having an extra methylene group in the β-alanine moiety and two analogues in which the amide bond nearest the thiol group is replaced with a pair of methylene groups.

We have previously reported a general synthetic approach to analogues of coenzyme A (CoA) **1a** and CoA esters using a combination of enzymatic and nonenzymatic reactions.[1,2] This method involves enzymatic synthesis of a general CoA analogue synthon **1b** having a thioester linkage in place of the amide bond nearest the thiol group of CoA (Scheme 1). For synthetic convenience, the thiol group is replaced with a methyl group. An aminolysis reaction is then performed to reform the amide bond present in CoA and to introduce the functionality of interest in place of the thiol group (Scheme 2). This methodology has been used to prepare mimics of the enol or enolate intermediate in the reactions of enzymes which catalyze deprotonation of acetyl-CoA[2−4] and of the tetrahedral intermediate in the reactions of acetyl-CoA-dependent acetyltransferases.[5] More recently this methodology has been employed in the synthesis of an isomer of propionyl-CoA in which the orientation of the thioester is reversed relative to that of a natural CoA thioester.[6] This compound was shown to be a time-dependent inactivator of thiolase and served as a synthon for certain other acyl-CoA analogues.

Despite its success as a facile route to a wide variety of CoA analogues, the previously described synthetic methodology permits modification only in the terminal acyl-thioethyl moiety. Reported here is the synthesis of a new thioester analogue of CoA **1c**, in which the more central amide bond of CoA is replaced by a thioester, and again the terminal thiol group is replaced by a methyl group. Analogue **1c** was prepared by enzymatic synthesis from a newly prepared phosphopantetheine analogue **2c** (Scheme 1). While **2c** was prepared in racemic form, the

Scheme 1



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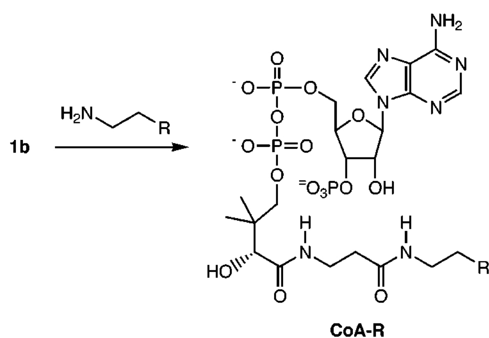
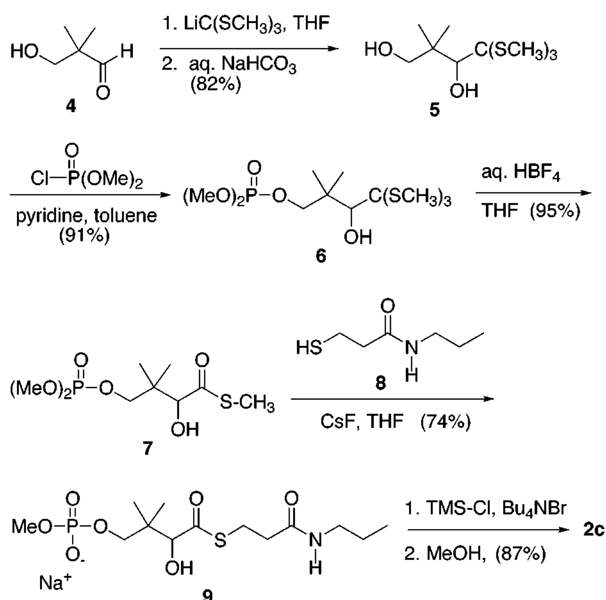
Scheme 2



Scheme 3



natural (R) isomer was selectively converted to product as indicated by stereochemical analysis of **3c**. Aminolysis reactions of **1c** have been used to prepare CoA analogues with modifications more distant from the terminal thiol than are available through the previously described synthon **1b**. This new CoA analogue synthon is expected to be useful in the preparation of a variety of analogues not available from synthon **1b**.

Results

Synthesis of CoA Analogue Synthon 1c. The synthesis of CoA analogue **1c** was performed using the enzymes catalyzing the final two steps of CoA biosynthesis as reported previously for the synthesis of **1b** (Scheme 1).[2] The phosphopantetheine analogue **2c** was prepared as shown in Scheme 3. Reaction of the aldehyde **4** with the lithium salt of tris(methylthio)methane followed by workup with aqueous $NaHCO_3$ formed the racemic pantoic acid derivative **5**, having the acid group protected as a trithioortho ester.[7] Conversion to the dimethyl phosphate ester **6** was accomplished by reaction with dimethyl chlorophosphate[8] as described previously for similar compounds.[2] This was followed by hydrolysis of the trithioortho ester with fluoboric acid to give the

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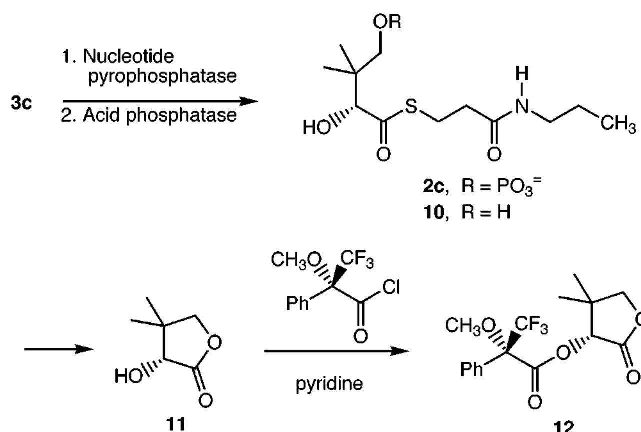
Scheme 4



thioester **7**.[9] Reaction with *N*-propyl-β-mercaptopropionamide **8** and cesium fluoride resulted in thioester exchange and removal of one methyl group of the phosphate triester to form **9**. Further deprotection of the phosphate with trimethylsilyl chloride and bromide ion gave **2c** in racemic form.[2,10] Enzymatic conversion of **2c** to **1c** was accomplished using the enzymes that catalyze the final two steps of CoA biosynthesis (Scheme 1) immobilized in polyacrylamide gel,[11] as in the previously reported synthesis of **1b**.[2,12] Phosphoenolpyruvate and pyruvate kinase were added to regenerate the equivalent of ATP used in the kinase step.[13] The reaction was complete in 7 days, and the product was isolated by reverse-phase HPLC.

The initial product **3c** formed in the phosphopantetheine adenylyltransferase catalyzed reaction of **2c** with ATP was isolated and subjected to stereochemical analysis as shown in Scheme 4. Reaction with nucleotide pyrophosphatase[14] converted **3c** back into the phosphopantetheine analogue **2c**. Reaction of the resulting sample of **2c** with acid phosphatase liberated the pantetheine analogue **10**, which spontaneously lactonized to form pantolactone **11**. This lactone was converted to the Mosher ester derivative **12**,[15] and the ^{1}H NMR spectrum of **12** was compared with the spectra of the Mosher ester derivatives of authentic racemic and natural (R)-pantolactone. The spectrum of **12** derived from **3c** showed only the natural (R)-isomer (90% ee), with no detectable amount of the (S)-isomer.

Synthesis of CoA Analogues by Aminolysis Reactions of 1c. The analogue **1c** was used to prepare three analogues not accessible via previously described methodology. These include the CoA analogue **14**, in which an extra methylene group is inserted into the β-alanine moiety (Scheme 5). For the preparation of **14**, 4-aminobutyrylcysteamine **13** was prepared as shown in Scheme 6. Reaction of cystamine dihydrochloride **16** with 4-bromobutyryl chloride **15** in a biphasic mixture of chloroform

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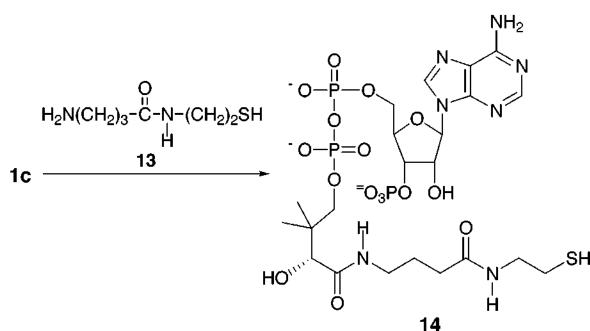
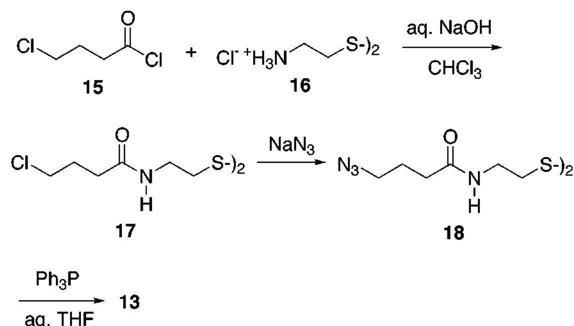
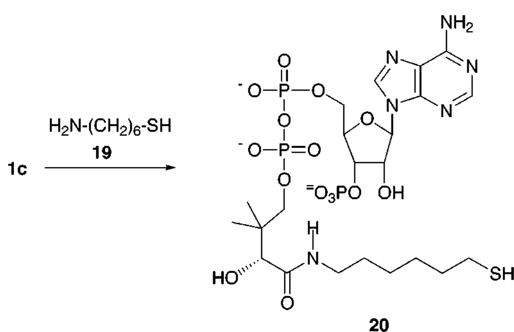
Scheme 5



Scheme 6



Scheme 7



and aqueous sodium hydroxide formed the amide **17**. Displacement of the bromide with azide to form **18** was followed by reduction of the azido and disulfide groups using triphenylphosphine in aq THF to form **13**.[16,17] Also prepared was CoA, analogue **20**, in which the chain length is unchanged relative to that of CoA but the amide bond between the cysteamine and β-alanine moieties is replaced with a pair of methylene groups (Scheme 7). For the preparation of **20**, 6-aminohexanethiol **19** was prepared as shown in Scheme 8. 6-Bromohexanenitrile **21** was converted to the thiol **22** by displacement of the bromide with thiophosphate followed by acid hydrolysis of the resulting thiophosphate ester.[18] Reduction of the nitrile with the borane–dimethyl sulfide complex formed the amine **19**.[19] The third analogue prepared was the deamide carboxylate analogue **24**. This is an analogue of acetyl-CoA in which the amide bond nearest the thioester is replaced with a pair of methylene groups (as in **20**) and the acetylthio moiety is replaced with a

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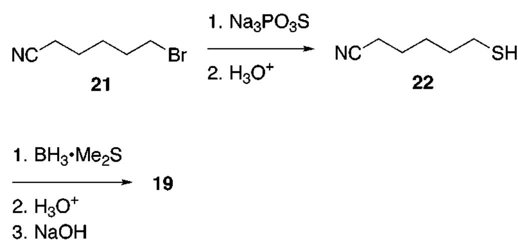
Scheme 8



Scheme 9

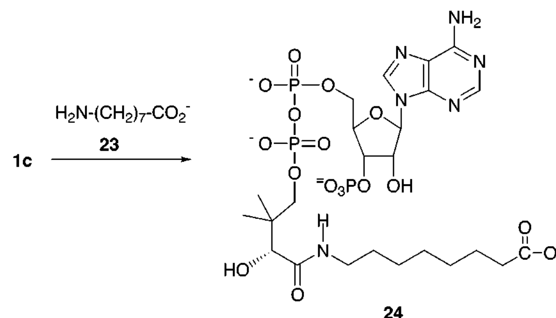


Table 1. Inhibition of Citrate Synthase by Carboxylate Analogues of Acetyl-CoA

	K_m or K_i
24	260 μM (K_i)
25	16 nM (K_i)
26	16 μM (K_m)

carboxymethylene group. Analogue **24** was prepared by reaction of **1c** with 8-aminooctanoic acid **23** as shown in Scheme 9. The aminolysis reactions of Schemes 5 and 7 were performed at room temperature in acetonitrile containing a minimum amount of water for solubilizing **1c**. The reactions were monitored by analytical HPLC and were complete in about 7 days. The reaction of Scheme 9 was carried out in aqueous solution and was complete in 4 days. The products were purified by reverse-phase HPLC.

Analogue **24** was tested as an inhibitor of citrate synthase, and the results are shown in Table 1. Also shown in Table 1 are the K_m for acetyl-CoA **26** and the K_i for the related inhibitor **25** (Figure 1). These results show that **24** is a very poor inhibitor of citrate synthase, with a K_i more than 10-fold higher than the K_m for acetyl-CoA and more than 10 000-fold higher than the K_i for **25**.

Discussion

The extension of previous methodology for CoA analogue synthesis as described here allows for the facile, routine synthesis of CoA analogues not previously available. Synthesis of the analogue **1c** required the development of a method for synthesis of the corresponding phosphopantetheine analogue **2c**. In previous work, generation of the unphosphorylated pantetheine analogue **10** (shown in Scheme 4) from a hydroxyl-protected derivative resulted in rapid cyclization to the lactone **11**, even when **10** was generated in the presence of reactive phosphorylating agents.[20] This cyclization is facilitated by the geminal dimethyl groups, which are expected to

(20) Bibart, R. T.; Drueckhammer, D. G. Unpublished results.

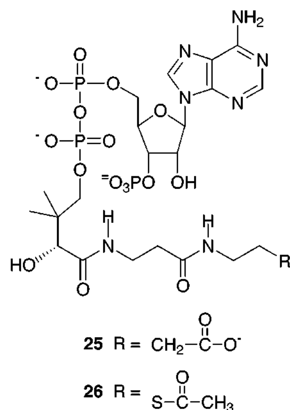


Figure 1. Structures of acetyl-CoA **26** and a carboxylate analogue **25**.

induce a conformation favorable for lactonization.[21,22] It was thus concluded that the phosphate group in some form must be introduced prior to formation of the thioester. Use was made of a trithioortho ester as a protected thioester that is resistant to reaction with nucleophiles. The tris(methylthio)ortho ester of pantoic acid **5** was prepared in racemic form by addition of the tris(methylthio)methane anion to the aldehyde **4** following general procedures for similar addition to other aldehydes.[7] This allowed for introduction of the phosphate group as the dimethyl ester prior to release of the thioester by acid hydrolysis. The thioester exchange reaction was driven by removal of volatile methanethiol and was accompanied by nucleophilic reaction at a phosphate methyl group. Two equivalents of the thiol were thus required, with one equivalent being converted to the methyl thioether. Phosphate deprotection was accomplished by previously described methods, consisting of conversion to the bis(trimethylsilyl) ester which was converted to the phosphate monoester upon addition of methanol.[2,10]

The phosphopantetheine analogue **2c** was accepted as a substrate by phosphopantetheine adenylyltransferase, and the resulting dephospho-CoA analogue **3c** was subsequently accepted by dephospho-CoA kinase, as indicated by monitoring of product formation by analytical HPLC and subsequent isolation and characterization of the products **3c** and **1c**. Since the phosphopantetheine analogue **2c** prepared as in Scheme 3 was racemic, it was necessary to address the stereochemistry of the product of the enzyme-catalyzed conversion to the CoA analogue **1c**. The degradation sequence of Scheme 4 was devised to convert the product to the known compound pantolactone, for which stereochemical analysis could be performed by standard procedures. This sequence involved hydrolysis of **3c** back into the phosphopantetheine analogue **2c** catalyzed by nucleotide pyrophosphatase followed by further conversion to the known product pantolactone **11**. Nucleotide pyrophosphatase has been previously shown to accept several dinucleotides as substrates.[14] The analysis was performed on the dephospho-CoA analogue **3c** rather than the CoA analogue **1c**, since preliminary studies showed that dephospho-CoA was a good substate for the enzyme nucleotide pyrophosphatase while CoA was a very poor substrate. Fortunately, the dephospho-CoA analogue **3c** resulting from enzymatic coupling of **2c** with ATP was found to have the proper configuration as determined by the procedure of Scheme 4, with no detectable amount of the (*S*)-isomer. This demonstrates that the phosphopantetheine adenylyltransferase is highly selective for the enantiomer of **2c** having the natural (*R*)-configuration. Some precedence for this enantioselectivity is provided by the almost 20-fold selectivity for the proper configuration at this position of pantetheine pivalate thioesters by thiolase.[23] It is likely that the dephospho-CoA kinase may also selectively utilize the (*R*)-isomer of **3c** over any trace of (*S*)-isomer that may be formed in the previous step, providing further enantiomeric enrichment of **1c**.

The utility of **1c** as a versatile CoA analogue synthon was demonstrated by the synthesis of the homo-β-alanyl **14** (Scheme 5) and "deamide" **20** (Scheme 7) analogues of CoA, and the deamide carboxylate analogue **24**. This illustrates the utility of this methodology in manipulating the structure of CoA at positions more distant from the terminal thiol group. **14** is an isomer of a previously prepared analogue having an extra methylene group in the cysteamine portion of the molecule.[24] These analogues may be useful in elucidating details of the effects of the β-alanine and amide moieties in orienting the thiol group of CoA in the proper position in an enzyme active site for enzyme catalysis to proceed. Initial insight into the importance of these functionalities is provided by the inhibition studies with **24**. This analogue differs from the previously studied carboxylate analogue of acetyl-CoA **25** only in the replacement of the amide bond functionality with a pair of methylene groups (Figure 1). Such a substitution is expected to have a minimal effect on the position of the terminal carboxylate. This replacement results in a 10^4-fold decrease in affinity of the inhibitor for citrate synthase. As **25** is viewed as a mimic of the enol or enolate intermediate, the greatly diminished inhibition upon deletion of the amide functionality indicates strong binding of the amide functionality by citrate synthase in stabilization of the enol or enolate complex.

The aminolysis reactions of **1c** in the synthesis of **14** and **20** were carried out in an aqueous acetonitrile mixed solvent system due to the insolubility of the amines **13** and **19** in aqueous solution and the insolubility of **1c** in purely organic solvent. The aminolysis reactions required 7 days at room temperature for complete disappearance of starting material, being much slower than similar reactions of **1b** which proceed to completion in 12−24 h. This decreased reactivity was not predicted but may be attributable to steric effects, which more than compensate for any activating effect of the electronegative α-hydroxyl group. As the amine **23** is water soluble, the aminolysis reaction to form **24** was performed in aqueous solution. The reaction appeared somewhat faster with complete disappearance of starting material observed in 4 days, suggesting that the solvent conditions may be partially responsible for the slower aminolysis rates with **13** and **19**. However, the yield was lower than in the slower reactions with **13** and **19** in mixed solvent and the reaction was accompanied by the formation of several

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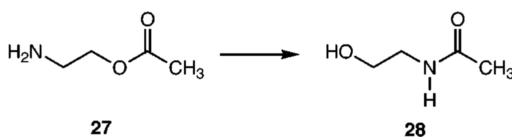
Scheme 10



byproducts, one of which is likely the product resulting from hydrolysis of the thioester.

Another limitation in the synthesis of CoA analogues using the first generation method of Scheme 2 is illustrated by an oxoester analogue of a CoA ester. Synthesis of such an analogue from **1b** would require the aminolysis reaction to be performed with an ethanolamine ester **27**. However, this compound would undergo intramolecular acyl transfer to form the amide **28** rather than the desired reaction with the thioester of **1b**, as shown in Scheme 10. This problem has also been encountered in efforts to prepare other acyl CoA analogues having electrophilic functionality in place of the thioester. Availability of the synthon **1c** should permit aminolysis reactions to be performed with a β-alanylamide derivative of **27**, in which the oxoester and nucleophilic amine groups are sufficiently separated such that intramolecular acyl transfer will not occur. Compound **1c** is thus expected to be valuable in the preparation of various CoA ester analogues not accessible from **1b**.

The work described here has solved most of the limitations in the previously described method for CoA analogue synthesis. Analogues are now accessible having a wide range of functionality in place of the β-alanylcysteamine moiety including previously inaccessible analogues. This methodology should facilitate further mechanistic and structural studies of CoA ester-utilizing enzymes using synthetic CoA analogues as probes.

Experimental Section

General Experimental. Reagents were obtained from Aldrich, Sigma, or Mallinckrodt and used as supplied while commercial enzymes were from Sigma. Phosphopantethein adenylytransferase and dephospho CoA kinase were isolated and immobilized as described previously.[2] Methylene chloride (CH_2Cl_2), pyridine, toluene, and acetonitrile (CH_3CN) were distilled from calcium hydride, and tetrahydrofuran (THF) was distilled from sodium. Analytical HPLC was performed using a Microsorb C-18 column (4.6 mm × 25 cm) with monitoring at 215 and 260 nm. Solvents used were aqueous potassium phosphate (solvent A: 50 mM, pH 4.5) and methanol (solvent B). Compounds were eluted at a flow rate of 1 mL/min with 5% solvent B for 2 min, followed by a linear gradient to 60% solvent B over 12 min, and then maintenance at 60% solvent B. Preparative HPLC was done on a Microsorb C-18 column (21.4 cm × 25 cm) with monitoring at 215 and 280 nm and a flow rate of 10 mL/min. Solvents were 0.2% trifluoroacetic acid in water (solvent A) and 0.2% trifluoroacetic acid in methanol (solvent B). Compounds were eluted with 5% B for 5 min followed by a linear gradient to 80% B over 45 min. Product CoA analogues eluted between 33 and 42 min. Mass spectral analysis was performed at the University of Riverside Mass Spectrometry Facility, Riverside, CA. K_i values were determined by computer fitting of data to the equation $v = (V_{max}[S])/(K_m(1 + [I]/K_i) + [S])$.[25] The concentrations of CoA analogue solutions were determined using $\epsilon_{260} = 15400\ M^{-1}\ cm^{-1}$.

4,4,4-Tris(methylthio)-2,2-dimethyl-1,3-butanediol (5). To a solution of tris(methylthio)methane (23.1 g, 150 mmol) in dry THF (500 mL) at −78 °C was added *n*-butyllithium (10 mL, 10M solution in hexanes, 100 mmol) slowly over 1 h to keep the temperature below −75 °C. The solution was stirred for an additional 1 h at −78 °C, and a solution of 2,2-dimethyl-3-hydroxypropionaldehyde **4** (5.00 g, 32.9 mmol, 70% pure) in dry THF (20 mL) was added. The solution was allowed to slowly warm to room temperature and was stirred at room-temperature overnight. Aqueous $NaHCO_3$ (100 mL, 1 M) was added followed by ether (100 mL), and the layers were separated. The ether layer was washed with aq $NaHSO_4$ (50 mL, 5%), $NaHCO_3$ (50 mL, 1 M), and saturated aqueous NaCl (50 mL), dried over $MgSO_4$, and evaporated. The residue was purified by chromatography on silica gel (2:1 hexane/ethyl acetate) to give **5** (R_f = 0.40) as a colorless oil (6.09 g, 26.9 mmol, 82% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.89 (bs, 1H), 3.70 (s, 1H), 3.68 (d, J = 11.2 Hz, 1H), 3.51 (d, J = 11.2 Hz, 1H), 2.95 (bs, 1H), 2.27 (s, 9H), 1.28 (s, 3H), 1.21 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 80.8, 74.7, 72.9, 41.2, 24.0, 20.7, 14.2.

(*R*,*S*)-4,4,4-Tris(methylthio)-2,2-dimethyl-1,3-butanediol, 1-Dimethyl Phosphate (6). To a solution of dimethyl phosphite (7.40 mL, 80.7 mmol) in dry toluene (150 mL) was added *N*-chlorosuccinimide (10.8 g, 80.7 mmol) and the solution stirred at 0 °C for 1 h followed by 1 h at room temperature. The resulting solution of dimethyl phosphorochloridate was transferred (leaving behind precipitated succinimide) into a solution of **5** (6.09 g, 26.9 mmol) in dry CH_2Cl_2 (200 mL) and dry pyridine (100 mL) at −40 °C. The reaction was allowed to warm to room temperature and stirred overnight, after which water (100 mL) was added and stirring continued for an additional 1 h. The solution was evaporated under reduced pressure and the residue dissolved in ethyl acetate (100 mL). The resulting solution was washed with water (50 mL), aqueous H_2SO_4 (50 mL, 1 M), aqueous $NaHCO_3$ (50 mL, 1 M), and saturated aqueous NaCl (50 mL), dried over $MgSO_4$, and evaporated. The residue was purified by chromatography on silica gel (ethyl acetate) to give **6** (R_f = 0.42) as a colorless oil (8.93 g, 24.5 mmol, 91% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.22 (dd, J = 9.3 Hz, 5.3 Hz, 1H), 3.79 (dd, J = 9.4 Hz, 5.2 Hz, 1H), 3.78 (d, J = 11.1 Hz, 6H), 3.77 (bs, 1H), 3.74 (s, 1H), 2.25 (s, 9H), 1.32 (s, 3H), 1.28 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 77.2, 77.6 (d, J = 6.2 Hz), 74.7, 54.1 (d, J = 5.9 Hz, 2C), 41.6 (d, J = 6.9 Hz), 22.9, 19.8, 14.4.

(*R*,*S*)-Pantothioic Acid *S*-Methyl Ester, 4-Dimethyl Phosphate (7). To a solution of **6** (8.93 g, 24.5 mmol) in dry THF (50 mL) at 0 °C was added 48% HBF_4 (3.52 mL, 27.0 mmol). The solution was allowed to warm to room temperature with stirring and was stirred at room temperature for 2 h. Ether (100 mL) was added, and the solution was washed with aqueous $NaHSO_4$ (50 mL, 5%), aqueous $NaHCO_3$ (50 mL, 1 M), and saturated aqueous NaCl (50 mL), dried over $MgSO_4$, and evaporated. The residue was purified by chromatography on silica gel (25:1 CH_2Cl_2/methanol) to give **7** (R_f = 0.40) (6.64 g, 24.2 mmol, 95% yield) as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.42 (d, J = 6.4 Hz, 1H), 4.20 (dd, J = 10.2 Hz, 7.3 Hz, 1H), 4.16 (d, J = 6.2 Hz, 1H), 3.80 (dd, J = 10.1 Hz, 7.7 Hz, 1H), 2.26 (s, 3H), 1.15 (s, 3H), 0.90 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 203.9, 79.1, 73.5 (d, J = 6.0 Hz), 54.4 (d, J = 5.9 Hz, 2C), 39.6 (d, J = 6.5 Hz), 20.8, 18.6, 11.1.

***N*-(3-Mercaptopropanoyl)propylamine (8).** To a solution of acryloyl chloride (20.0 mL, 246 mmol) in dry CH_2Cl_2 (300 mL) at 0 °C was added dropwise a solution of propylamine (21.8 mL, 270 mmol) in triethylamine (41.8 mL, 300 mmol), and the resulting solution was stirred at room-temperature overnight. The solution was washed with water (100 mL), H_2SO_4 (100 mL, 1 M), $NaHCO_3$ (100 mL, 1 M), and sat. aq NaCl (100 mL), and dried with Na_2SO_4 and the solvent evaporated to give *N*-acryloylpropylamine (24.3 g, 215 mmol, 87% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.95 (bs, 1H), 6.26 (s, 1H), 6.23 (d, J = 2.2 Hz, 1H), 5.60 (dd, J = 7.1 Hz, 4.9 Hz, 1H), 3.27 (q, J = 6.8 Hz, 2H), 1.45−1.65 (m, 2H), 0.93 (t, J = 7.4 Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 166.1, 131.2, 125.7, 41.0, 22.3, 11.0. The product (24.3 g, 215 mmol) was dissolved in ether (500 mL), thiolacetic acid (17.2 mL, 240 mmol) was added, and the reaction was stirred at room-temperature overnight. The reaction was then washed with water (150 mL), aqueous H_2SO_4 (150 mL, 1 M), aqueous $NaHCO_3$ (150 mL, 1 M), and saturated aqueous NaCl (50 mL), dried with $MgSO_4$,

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and evaporated. The product was recrystallized from ether/pentane to give 3-(acetylthio)propanoyl *n*-propylamine (39.4 g, 208 mmol) as white flakes in 97% yield. ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.83 (bs, 1H), 3.22 (q, $J = 6.9$ Hz, 2H), 3.14 (t, $J = 6.7$ Hz, 2H), 2.48 (t, $J = 6.7$ Hz, 2H), 2.34 (s, 3H), 1.41−1.62 (m, 2H), 0.93 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 196.7, 170.8, 41.1, 36.1, 30.4, 24.8, 22.5, 11.1. The product (10.0 g, 52.8 mmol) was dissolved in sodium methoxide (116 mL, 0.5 M in methanol, 58.0 mmol) and stirred at room temperature for 1 h. The solution was acidified with 1 M H_2SO_4 and extracted with ether (100 mL, 50 mL). The organic layers were combined, dried with $MgSO_4$, and evaporated to give **8** (7.34 g, 49.8 mmol) as a colorless oil in 94% yield with no further purification required. ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.88 (bs, 1H), 3.24 (q, $J = 6.9$ Hz, 2H), 2.75−2.90 (m, 2H), 2.49 (t, $J = 6.7$ Hz, 2H), 1.62 (t, $J = 8.3$ Hz, 1H), 1.45−1.64 (m, 2H), 0.94 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.6, 41.2, 40.4, 22.8, 20.5, 11.3.

(*R*,*S*)-3-Hydroxy-4-[[3-(propylamino)-3-oxopropyl]thio]-2,2-dimethyl-4-oxobutan-1-ol, 1-Methyl Phosphate Ester (9). To a solution of **8** (3.33 g, 22.6 mmol) and **7** (6.64 g, 24.2 mmol) in dry THF (20 mL) was added CsF (1.97 g, 12.9 mmol), and the solution was stirred under a steady stream of nitrogen vented to a 1 M aqueous NaOH bubbler to remove methanethiol. After 1 day, an additional 1.5 g (9.87 mmol) of CsF was added. After another day, the solvent was removed and the residue dissolved in water (50 mL). The resulting solution was extracted with ethyl acetate (25 mL). The aqueous solution was lyophilized and purified by HPLC. This provided **9** (2.15 g, 5.79 mmol, 51% yield) and **2c** (0.96 g, 2.69 mmol, 23% yield). Analytical HPLC: retention time = 16.8 min, $\lambda_{max} = 240$ nm. ^{1}H NMR (400 MHz, D_2O) δ 4.18 (s, 1H), 3.78 (dd, $J = 9.5$ Hz, 4.0 Hz, 1H), 3.58 (d, $J = 10.7$ Hz, 3H), 3.57 (dd, $J = 9.5$ Hz, 4.0 Hz, 1H), 3.05−3.14 (m, 4H), 2.54 (t, $J = 6.7$ Hz, 2H), 1.44−1.53 (m, 2H), 1.01 (s, 3H), 0.93 (s, 3H), 0.88 (t, $J = 7.3$ Hz, 3H). ^{13}C NMR (100 MHz, D_2O) δ 208.6, 175.6, 82.1, 73.7, 55.4, 43.8, 41.5 (d, $J = 7.1$ Hz), 37.8, 26.8, 24.7, 23.4, 21.7, 13.6.

(*R*,*S*)-3-Hydroxy-4-[[3-(propylamino)-3-oxopropyl]thio]-2,2-dimethyl-4-oxobutan-1-ol (2c). To a solution of **9** (2.15 g, 5.79 mmol) in dry CH_3CN (30 mL) were added trimethylsilyl chloride (6.35 mL, 50.0 mmol) and tetrabutylammonium bromide (1.92 g, 5.95 mmol), and the resulting solution was stirred at 40 °C for 15 h. The solvent was evaporated, the residue dissolved in methanol, and the methanol evaporated. Purification by HPLC provided **2c** (1.81 g, 5.06 mmol, 87% yield). Analytical HPLC: retention time = 15.5 min, $\lambda_{max} = 240$ nm. ^{1}H NMR (400 MHz, D_2O) δ 3.85 (s, 1H), 3.45 (dd, $J = 9.6$ Hz, 4.5 Hz, 1H), 3.23 (dd, $J = 8.8$ Hz, 4.6 Hz, 1H), 2.70−2.81 (m, 4H), 2.20 (t, $J = 6.5$ Hz, 2H), 1.10−1.17 (m, 2H), 0.65 (s, 3H), 0.56 (s, 3H), 0.52 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (100 MHz, D_2O) δ 208.3, 175.0, 81.9, 73.2, 43.5, 41.2 (d, $J = 7.5$ Hz), 37.4, 26.6, 24.2, 22.9, 21.2, 13.2.

Adenosine 5′-(Trihydrogen diphosphate) 3′-(Dihydrogen phosphate) 5′-[(*R*)-3-Hydroxy-4-[[3-(propylamino)-3-oxopropyl]thio]-2,2-dimethyl-4-oxobutyl] Ester (1c). To a solution of racemic **2c** (0.90 g, 2.52 mmol) in HEPES buffer (0.1 M, 16 mL, pH 7.5) were added phosphopantetheine adenylyltransferase and dephosphocoenzyme A kinase coimmobilized in polyacrylamide as described previously.[2] ATP (3.00 mmol, dissolved in 10 mL of water, pH adjusted to 7 with KOH), $MgCl_2$ (0.54 mmol), and inorganic pyrophosphatase (10 units) were added, and the reaction mixture was gently swirled in a rotary shaker at room temperature. Pyruvate kinase (22 units) and phospho(enol) pyruvate (0.5 mmol, as the potassium salt, pH adjusted to 7 with KOH) were added daily. The progress of the reaction was monitored by HPLC. The reaction was stopped after 7 days, at which time HPLC showed unreacted **2c** (retention time = 15.5 min) along with new peaks at 14.6 min (**1c**) and 16.4 min (**3c**). The reaction mixture was centrifuged, the supernatant was decanted, and the immobilized enzymes were washed twice with HEPES buffer (0.1 M, 5 mL). The combined washes and supernatant were filtered through a 0.45 μm nylon filter and were lyophilized. One-fourth of the crude material was dissolved in 3 mM HCl (80 mL), and the solution was adjusted to pH 3.5 with 1 M phosphoric acid. At 4 °C, the solution was loaded onto a DEAE cellulose column (2.5 × 17 cm), which was previously equilibrated with 3 mM HCl. The column was washed with 100 mL of 3 mM HCl and then with a linear gradient (1 L) of lithium choride (0−0.2 M) in 3 mM HCl. Individual fractions of 22 mL were collected and analyzed by HPLC. Compound **1c** eluted between 0.10 and 0.12 M lithium chloride. Fractions containing **1c** were adjusted to pH 4 with 1 M KH_2PO_4 and were lyophilized and further purified by HPLC to give pure **1c** as a lyophilized powder (60 mg, 78 μmol, 25% yield based on (*R*)-**2c**). Analytical HPLC: retention time = 14.6 min, $\lambda_{max} = 260$ nm. ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.56 (s, 1H), 8.33 (s, 1H), 6.10 (d, $J = 5.3$ Hz, 2H), 4.75 (bs, 1H), 4.52 (bs, 1H), 4.15−4.25 (m, 2H), 4.05 (s, 1H), 3.78 (dd, $J = 9.4$ Hz, 4.6 Hz, 1H), 3.54 (dd, $J = 9.4$ Hz, 4.3 Hz, 1H), 2.90−3.02 (m, 4H), 2.40 (t, $J = 6.7$ Hz, 2H), 1.30−1.40 (m, 2H), 0.84 (s, 3H), 0.75 (t, $J = 7.6$ Hz, 3H), 0.74 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 208.6, 176.2, 152.7, 150.9, 148.1, 144.6, 120.9, 89.8, 85.9 (d, $J = 8.7$ Hz), 81.7, 76.5, 76.5, 74.2 (d, $J = 5.7$ Hz), 67.6 (d, $J = 5.1$ Hz), 43.7, 41.3 (d, $J = 8.3$ Hz), 37.5, 26.6, 24.2, 22.9, 20.9, 13.1. HRMS (FAB): $[M - H]^-$ calcd for $C_{22}H_{36}N_6O_{16}P_3S$ *m*/*z* 765.1124, found 765.109. Compound **3c** eluted between 0.08 and 0.10 M lithium chloride from the DEAE column. Fractions containing **3c** were adjusted to pH 4 with 1 M KH_2PO_4 and were lyophilized and further purified by HPLC to give **3c** as a lyophilized powder (120 mg, 175 μmol, 56% yield based on (*R*)-**2c**). Analytical HPLC: retention time = 16.4 min, $\lambda_{max} = 260$ nm. ^{1}H NMR (400 MHz, D_2O) δ 8.51 (s, 1H), 8.32 (s, 1H), 6.03 (d, $J = 4.7$ Hz, 1H) 4.60 (t, $J = 5.0$ Hz, 1H) 4.44 (t, $J = 3.7$ Hz, 1H), 4.31 (bs, 1H), 4.20 (bs, 2H), 4.03 (s, 1H), 3.78 (dd, $J = 9.5$ Hz, 4.8 Hz, 1H) 3.54 (dd, $J = 9.2$ Hz, 2.8 Hz, 1H) 2.88−3.01 (m, 4H), 2.39 (t, $J = 6.9$ Hz, 2H) 1.32−1.39 (m, 2H), 0.84 (s, 3H), 0.74 (s, 3H), 0.74 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (100 MHz, D_2O) δ 208.5, 176.1, 152.5, 150.6, 147.9, 144.6, 120.7, 90.5, 86.4 (d, $J = 8.3$ Hz), 81.7, 77.2, 74.2 (d, $J = 5.7$ Hz), 72.5, 67.5, 43.7, 41.3 (d, $J = 7.7$ Hz), 37.5, 26.6, 24.3, 22.9, 20.9, 13.2. HRMS (FAB): $[M - H]^-$ calcd for $C_{22}H_{35}N_6O_{13}P_2$ S *m*/*z* 685.1461, found 685.1488.

Stereochemical Analysis of 3c. A 10 mg (15 μmol) amount of **3c** was dissolved in 800 μL of 0.1 M potassium phosphate buffer, pH 7.4, and 4.5 units of nucleotide pyrophosphatase (EC 3.6.1.9, *Crotalus atrox* venom from Sigma) was added. The reaction was monitored by analytical HPLC and complete disappearance of **3c** (retention time = 16.4 min, $\lambda_{max} = 260$ nm) and appearance of products (AMP, retention time = 8.71 min, $\lambda_{max} = 260$ nm, and **2c**, retention time = 16.48 min, $\lambda_{max} = 240$ nm) was observed in 4 h. **2c** was purified, and excess phosphate salts were removed by preparative HPLC (5 min at 5% methanol in 0.2% aqueous TFA followed by a linear gradient to 80% methanol in 0.2% aqueous TFA over 45 min, **2c** eluted between 30 and 38 min). Fractions containing **2c** were pooled and lyophilized and redissolved in 2.5 mL 0.1 M acetate, pH 5.0. Acid phosphatase (7.8 units) (EC 3.1.3.2, potato from Sigma) was added, and the reaction was monitored by analytical HPLC. After 6 h, an additional 7 units of phosphatase was added, and complete disappearance of starting material was observed in 21 h. The reaction was concentrated to approximately 500 μL under a stream of nitrogen and extracted five times with 3 mL of ether. The combined ether layers were concentrated by rotary evaporation and purified via flash chromatography on a 20 mL silica gel column (1:5 hexane/ethyl acetate, $R_f = 0.60$). The residue containing **11** was converted to the MTPA ester **12** using standard procedures.[15] Authentic MTPA esters of (*R*)- and (*S*)-pantoyllactone were prepared separately. The NMR spectrum of the MTPA ester of the sample of **12** derived from **3c** showed only the MTPA ester of (*R*)-pantoyllactone. (*S*)-MTPA-(*R*)-pantoyllactone: ^{1}H NMR (400 MHz, D_2O): δ 0.956 (s, 3H), 1.179 (s, 3H), 3.634 (s, 3H), 4.066 (s, 2H), 5.596 (s, 1H), 7.42−7.67 (m, 5H). (*S*)-MTPA-(*S*)-pantoyllactone: ^{1}H NMR (400 MHz, D_2O): δ 1.154 (s, 3H), 1.275 (s, 3H), 3.551 (s, 3H), 4.101 (s, 2H), 5.560 (s, 1H), 7.42−7.67 (m, 5H).

***N*,*N*-Bis(4-chlorobutyryl)cystamine (17).** To a solution of cystamine dihydrochloride **16** (6.76 g, 30.0 mmol) in aqueous

NaOH (200 mL, 2 M) was added chloroform (200 mL). 4-Chlorobutyryl chloride **15** (10.0 mL, 89.2 mmol) was slowly added to the gently stirred biphasic system, and stirring was continued overnight at 40 °C. The layers were then separated, and the organic layer was washed with aqueous H_2SO_4 (100 mL, 1 M), aqueous $NaHCO_3$ (100 mL, 1 M), and saturated aqueous NaCl (50 mL), dried with Na_2SO_4, and evaporated. The product was recrystallized from ether/pentane to give *N,N*-bis(4-chlorobutyryl)cystamine **17** (8.53 g, 23.6 mmol, 79% yield) as a colorless solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.48 (bs, 2H), 3.62 (t, J = 6.2 Hz, 4H), 3.59 (q, J = 6.3 Hz, 4H), 2.84 (t, J = 6.4 Hz, 4H), 2.42 (t, J = 7.4 Hz, 4H), 2.05−2.20 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.3, 44.4, 38.4, 37.6, 33.0, 28.1.

***N,N*-Bis(4-azidobutyryl)cystamine (18).** To a solution of **17** (4.25 g, 11.8 mmol) in DMSO (50 mL) was added sodium azide (1.95 g, 30.0 mmol), and the solution was stirred at room-temperature overnight. Saturated aq NaCl (50 mL) was added, and the solution was extracted with ether (3 × 25 mL). The combined ether layers were washed with water (2 × 25 mL), dried with $MgSO_4$, and evaporated to give *N,N*-bis(4-azidobutyryl)cystamine **18** (4.27 g, 11.4 mmol, 97% yield) as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.56 (bs, 1H), 3.58 (q, J = 6.3 Hz, 2H), 3.37 (t, J = 6.6 Hz, 2H), 2.83 (t, J = 6.5 Hz, 2H), 2.34 (t, J = 7.2 Hz, 2H), 1.85−2.01 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.3, 50.7, 38.4, 37.6, 32.9, 24.7.

***N,N*-Bis(4-aminobutyryl)cysteamine (13).** To a solution of **18** (2.20 g, 5.87 mmol) in THF (50 mL) were added water (0.72 mL, 40 mmol) and triphenylphosphine (5.11 g, 19.5 mmol), and the solution was stirred overnight at 40 °C. The solvent was evaporated, and the residue was dissolved in water (40 mL) and extracted with ethyl acetate (20 mL). The aqueous layer was lyophilized to give **13** (1.75 g, 10.8 mmol, 92% yield) as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.49 (t, J = 6.2 Hz, 2H), 2.98 (t, J = 7.7 Hz, 2H), 2.82 (t, J = 6.2 Hz, 2H), 2.35 (t, J = 7.4 Hz, 2H), 1.82−2.00 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 177.5, 41.6, 40.8, 39.4, 35.3, 25.8.

Adenosine 5′-(Trihydrogen diphosphate) 3′-(Dihydrogen phosphate) 5′-[(*R*)-3-Hydroxy-4-[[3-[2-(mercaptoethyl)amino]-3-oxobutyl]amino]-2,2-dimethyl-4-oxobutyl] Ester (14). A solution of **1c** (10 mg, 13 μmol) and **13** (0.32 g, 2 mmol) in acetonitrile (1 mL) was stirred at room temperature. The reaction was checked daily by HPLC for disappearance of starting material. Water (100 μmol) was added daily and the pH adjusted to 10.5. The reaction was complete in 7 days. Water (20 mL) was added and the solution extracted with EtOAc (3 × 10 mL). The aqueous layer was adjusted to pH 4.5, lyophilized, and purified by HPLC to give **14** (8 mg, 10 μmol) as a lyophilized powder in 80% yield. Analytical HPLC: retention time = 13.1 min, λ_{max} = 260 nm. ^{1}H NMR (400 MHz, D_2O) δ 8.66 (s, 1H), 8.41 (s, 1H), 6.20 (d, J = 5.8 Hz, 1H), 4.58 (bs, 1H), 4.23 (bs, 2H), 4.02 (s, 1H), 3.84 (bs, 1H), 3.57 (bs, 1H), 3.31 (t, J = 6.5 Hz, 2H), 3.19 (t, J = 6.7 Hz, 2H), 2.60 (t, J = 6.4 Hz, 2H), 2.24 (t, J = 7.4 Hz, 2H), 1.70−1.80 (m, 2H), 0.92 (s, 3H), 0.81 (s, 3H). MS (FAB): [M − H] calcd for $C_{22}H_{37}N_7O_{13}P_3S$ *m/z* 780, found 780.

6-Mercaptohexanenitrile (22). To a solution of sodium thiophosphate dodecahydrate (12 g, 30.4 mmol) dissolved in H_2O (80 mL) was added 6-bromohexanenitrile **21** (2 mL, 15.2 mmol) in DMF (10 mL) and the resulting suspension stirred overnight. The next morning H_2O was added (15 mL), the pH lowered to 4 with 10% HCl, and the solution stirred an additional 4 h. The product was extracted with pentane (2 × 80 mL), and the combined extracts were washed with brine (20 mL) and dried over $MgSO_4$. Solvent was removed via rotary evaporation to yield 1.7 g (84%) of 6-mercaptohexanenitrile **22**. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.37 (t, 1H, J = 7.9 Hz), 1.57 (m, 6H), 2.37 (t, 3H, J = 6.9 Hz), 2.48 (m, 2H).

6-Aminohexanethiol (19). 6-mercaptohexanenitrile **22** (1.7 g, 13 mmol) was dissolved in dry THF (20 mL) and heated to a gentle reflux under N_2. $BH_3{\cdot}Me_2S$ (2.8 mL, 28 mmol) was slowly added, and after several minutes a gel formed. The reaction was cooled to 0 °C, and 2 M HCl in CH_3OH (15 mL) was added slowly. After the gel had dissolved, the reaction was heated and the majority of the solvent removed by distillation. The reaction was filtered, the solid rinsed with CH_3OH, and the filtrate concentrated under rotary evaporation. The resulting oil was dissolved in H_2O (10 mL), and impurities were removed by extracting with CH_2Cl_2 (3 × 20 mL). The aqueous solution was adjusted to pH 10 with 1 N NaOH and extracted with CH_2Cl_2 (3 × 20 mL), the combined organic extracts were dried over $MgSO_4$, and the solvent was removed by rotary evaporation to yield 0.67 g (38%) of 6-aminohexanethiol **19** as a white solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.35 (m, 10H), 1.60 (m, 2H), 2.55 (t, 2H, J = 6.8 Hz), 2.70 (t, 2H, J = 6.8 Hz). ^{13}C NMR (50 MHz, $CDCl_3$): δ 24.52, 26.32, 28.17, 33.60, 33.92, 42.11.

Adenosine 5′-(Trihydrogen diphosphate) 3′-(Dihydrogen phosphate) 5′-[(*R*)-3-Hydroxy-4-[(6-mercaptohexyl)-amino]-2,2-dimethyl-4-oxobutyl] Ester (20). A solution of **1c** (10 mg, 13 μmol) and **19** (0.27 g, 2 mmol) in CH_3CN (1 mL) was stirred at room temperature. Water (100 μL) was added and the pH adjusted to 10.5 daily. The reaction was complete in 7 days as determined by HPLC. Water (20 mL) was added and the solution extracted with EtOAc (3 × 10 mL). The aqueous phase was adjusted to pH 4.5 and lyophilized and purified by HPLC to give **20** (8 mg, 10 μmol) as a lyophilized powder in 80% yield. Analytical HPLC: retention time = 15.5 min, λ_{max} = 260 nm. ^{1}H NMR (400 MHz, D_2O) δ 8.67 (s, 1H), 8.41 (s, 1H), 6.20 (d, J = 5.8 Hz, 1H) 4.58 (bs, 1H), 4.24 (bs, 2H), 4.13 (s, 1H), 3.84 (dd, J = 9.5 Hz, 4.4 Hz, 1H), 3.60 (dd, J = 9.1 Hz, 3.8 Hz, 1H), 2.94 (t, J = 7.4 Hz, 2H), 1.61 (t, J = 6.5 Hz, 2H), 1.50 (t, J = 6.1 Hz, 2H), 1.33 (bs, 4H), 0.91 (s, 3H), 0.84 (s, 3H). MS (FAB): $[M - H]^-$ calcd for $C_{22}H_{38}N_6O_{15}P_3S$ *m/z* 751, found 751.

Adenosine 5′-(Trihydrogen diphosphate) 3′-(Dihydrogen phosphate) 5′-[(*R*)-3-Hydroxy-4-[(6-carboxyhexyl)-amino]-2,2-dimethyl-4-oxobutyl] Ester (24). A 2 mL volume of a saturated solution of ω-aminocaprylic acid **19**, pH adjusted to 10.0 with sodium hydroxide (the solution was about 2 M in the amino acid), was added to **1c** (10 mg, 13 μmol). The reaction was monitored by HPLC, and disappearance of starting material **1c** and appearance of product **24** (retention time = 16.6 min) were complete in 4 days. Several other unidentified products were observed in the analytical HPLC traces. The solution was adjusted to pH 4.5 with aqueous HCl and purified by preparative reverse-phase HPLC to give 2.03 mg of **24** (20%). λ_{max} = 260 nm. ^{1}H NMR (400 MHz, D_2O): δ 0.72 (s, 3H), 0.84 (s, 3H), 0.87 (t, 2H, J = 7.4 Hz), 1.15 (m, 6H), 2.20 (m, 4H), 3.10 (m, 2H), 3.50 (m, 1H), 3.80 (m, 1H), 3.95 (s, 1H), 4.18 (br s, 2H), 4.53 (s, 1H), 6.13 (d, 1H, J = 5.6 Hz), 8.29 (s, 1H), 8.55 (s, 1H). HRMS (FAB): $[M - H]^-$ calcd for $C_{24}H_{40}N_6O_{17}P_3$ *m/z* 777.166, found 777.169.

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Supporting Information Available: ^{1}H NMR spectra of compounds **1c**, **2c**, **3c**, **5**−**9**, **12**−**14**, **17**−**20**, **22**, and **24**. This material is available free of charge via the Internet at http://pubs.acs.org.

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