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# Chemoenzymatic Synthesis of $^{36}S$ Isotopologues of Methionine and *S*-Adenosyl-L-methionine

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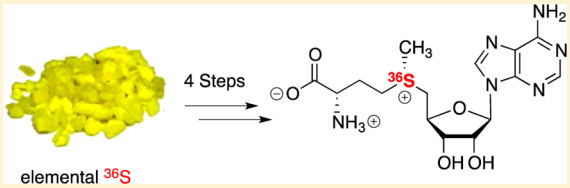
Supporting Information

**ABSTRACT:** Substrates containing isotope labels at specific atoms are required for transition-state analysis based on the measurement of multiple kinetic isotope effects. $^{36}S$-labeled L-methionine and *S*-adenosyl-L-methionine were synthesized from elemental sulfur using a chemoenzymatic approach with >98% $^{36}S$ enrichment. This method provides access to previously inaccessible sulfur isotope-labeled substrates for sulfur kinetic isotope effect studies.




The *S*-adenosyl-L-methionine (SAM, **1**, Figure 1) dependent enzymes are involved in a number of essential biological processes including protein and DNA methylation,[1−3] polyamine biosynthesis,[4] and a variety of single electron-transfer reactions catalyzed by members of the radical SAM enzyme superfamily.[5,6] In biological systems SAM is synthesized from adenosine triphosphate (ATP) and methionine (Met, **2**) by *S*-adenosyl-L-methionine synthetase (SAMsyn, or methionine adenosyltransferase MAT) enzymes.[7−9] This reaction generates a cationic sulfonium center that facilitates the variety of chemical transformations performed by SAM-dependent enzymes. The electronegative sulfur increases the electrophilicity of the attached methyl group carbon or aminopropyl group making them more reactive for methyl-transfer and aminopropyl-transfer reactions, respectively. The sulfur atom can also serve as a single electron acceptor in radical SAM reactions to generate 5′-deoxyadenosyl radicals.[5]

The measurement of multiple kinetic isotope effects (KIEs) is a powerful tool to study enzyme mechanism and enables the elucidation of enzyme transition states.[10,11] Carbon and hydrogen KIEs have been measured for methylation reactions catalyzed by catechol *O*-methyltransferase (COMPT)[12,13] and cyclopropane fatty acid synthase;[14] however, there are no measured sulfur atom KIEs for SAM-dependent enzymes despite the key role played by the sulfonium center in these systems. The absence of experimental sulfur KIEs is due, at least in part, to the difficulty accurately measuring isotope effects resulting from the relatively small mass difference ($\Delta m$) between the most abundant $^{32}S$ and $^{34}S$ isotopes of sulfur. This could be partially overcome by substituting the less abundant $^{36}S$ isotope in place of $^{34}S$, to give a larger $\Delta m$ comparable to that for $^{16}O$ and $^{18}O$ isotopes, for which KIEs have been experimentally measured for multiple enzymes.[15,16] This, however, requires new methods for the synthesis of sulfur isotope-labeled SAM.

Here we report a four-step chemoenzymatic synthesis of $^{36}S$-labeled Met and SAM starting from elemental $^{36}S$-sulfur. This method employs the enzymes *O*-acetylhomoserine sulfhydralase 2 (OAH2), betaine homocysteine methyltransferase (BHMT), and SAMsyn to convert inorganic sulfide ($S^{2-}$) to L-homocysteine (HCys **4**), Met **2**, and ultimately SAM **1** (Scheme 1).

An enzymatic approach to prepare $^{36}S$-labeled Met and AdoMet requires the synthesis of $Na_2{}^{36}S$ from milligram quantities of labeled sulfur. Naphthalene promoted reduction of gram quantities of elemental sulfur has previously been used in the preparation of hexamethyldisilathiane, however, the $Na_2S$ was generated *in situ* and never isolated.[17] This approach was scaled to allow for the reduction of 3−10 mg samples of elemental sulfur using 10 mol % naphthalene. Agitation in an ultrasonic water bath was used to promote sulfur reduction. The reaction mixture gradually turned from yellow to brown to dark green to white over the course of the reaction. After removing any remaining sodium metal and evaporating off the solvent, the resulting crude $Na_2{}^{36}S$ was obtained as an off-white solid, which was used directly as a substrate for OAH2 without purification.

OAH2 catalyzes the displacement of acetate from **3** by inorganic $S^{2-}$ to give **4** and acetate as products.[18] Crude $Na_2{}^{36}S$ was used directly as a substrate for OAH2, along with varying quantities of freshly prepared *O*-acetyl-L-homoserine **3**. After 16 h, the reaction products were analyzed using NMR spectroscopy (Table 1). With equal concentrations of $Na_2S$ and **3** we observed the formation of **4** along with a significant amount of the side product **5**. Compound **5** is produced by OAH2, where the product **4** reacts with **3** in the place of sulfide. In the presence of excess **3** we observed the side products almost exclusively. In the absence of OAH2 we observed no reaction between **3** and **4**. Using a 2-fold excess of $Na_2S$ to **3** prevented the formation of **5**. Under these conditions, HCys **4** was obtained as the exclusive product in nearly quantitative yield, as

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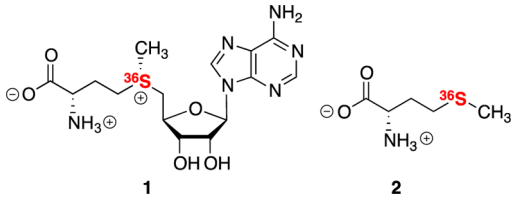


**Figure 1.** Structure of SAM **1** and Met **2**.

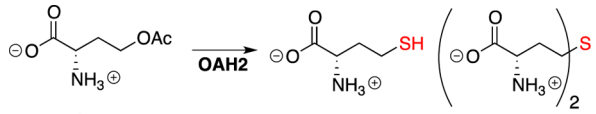
observed by NMR spectroscopy. After removing the enzyme, the product **4** was used directly as a substrate for the BHMT enzyme-catalyzed reaction.

BHMT catalyzes the transfer of a methyl group from betaine to **4** generating methionine and *N*,*N*-dimethylglycine ($Me_2Gly$).[19] Incubating **4** and 4 equiv of betaine with BHMT in the presence of 1 mM DTT gave a near quantitative yield of **2**. However, in the absence of DTT no products were observed, suggesting reduced compound **4** is required for efficient turnover by BHMT. $^{36}$S-Met **2** was isolated in a 28% overall yield from the starting elemental $^{36}$S-sulfur, after HPLC purification.

The reaction catalyzed by SAMsyn has been exploited on numerous occasions to synthesize both isotope labeled and unlabeled SAM *in vitro*.[20−22] Here we employ a variation of this approach using SAMsyn from *Escherichia coli* to convert **2** into $^{36}$S-labeled SAM **1**. The sulfonium center of biologically produced SAM exists exclusively as the (*S*)-isomer, which over time isomerizes to give equal amounts of both (*S*)- and (*R*)-stereoisomers. Compared to chemical methods to synthesize SAM, which produce equal amounts of both methyl group diastereomers, enzymatic synthesis gives access directly to pure (*S*)-SAM.

To minimize isomerization during the synthesis of **1**, the reaction progress was monitored by HPLC using conditions capable of separating both the (*S*) and (*R*)-SAM isomers. Reactions were stopped after 2−3 h before any of the (*R*)-isomer was observed. Allowing the reaction to proceed for longer gave a higher yield of **1**, but resulted in partial isomerization of the product (Table 2). Under these conditions we produced $^{36}$S-labeled SAM **1** as a single diastereomer in 40% yield (12% overall from labeled sulfur). The extent of $^{36}$S incorporation was determined by mass spectrometry for both labeled SAM **1** and Met **2** by comparing the 403/399 or 154/150 peak areas, respectively. Both compound **1** and **2** were obtained with ≥98.3% and ≥98.6% isotopic enrichment of $^{36}$S, respectively. This method provides access to isotopically and isomerically pure $^{36}$S-SAM from elemental sulfur in four steps, involving the chemical reduction of sulfur and three enzymatic transformations. $^{36}$S-labeled SAM prepared using this chemoenzymatic approach can now enable the measurement of sulfur KIEs for multiple SAM-dependent enzymes.

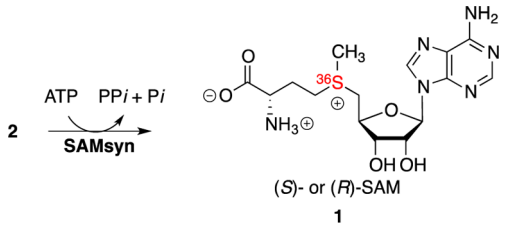
**Table 1. Optimization of OAH2 Reaction**



| equiv **3** | equiv $Na_2S$[a] | [%] **4**[b] | [%] **5**[b] |
|---|---|---|---|
| 2.00 | 1.00 | 20 | 80 |
| 1.00 | 1.00 | 33 | 67 |
| 1.00 | 2.00 | >95 | <5 |

[a]Based on the starting mass of sulfur. [b]NMR yield.

**Table 2. Isomerization during the Synthesis of (*S*)-SAM 1**



| time (h) | (*S*):(*R*)-SAM[a] |
|---|---|
| 2 | >19:1 |
| 3 | >19:1 |
| 5 | 9:1 |
| 8 | 6:1 |

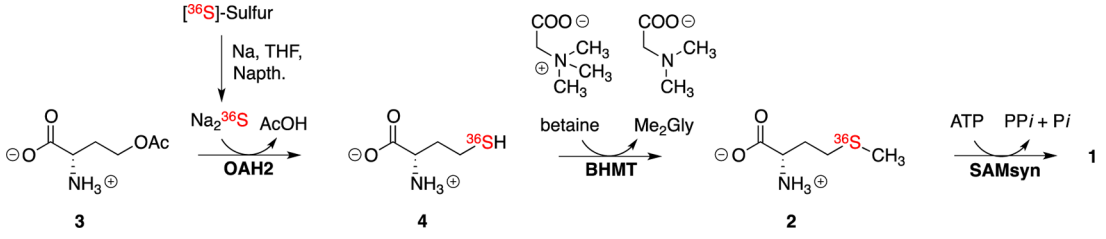
[a]By integration of HPLC peak area.

## EXPERIMENTAL SECTION

**General Procedures.** Chemicals and reagents were obtained from commercial sources and used without further purification. Isotopically pure elemental $^{32}$S (99.99% enrichment) and $^{36}$S (99.24% enrichment) were purchased from a commercial source. $^1$H ($^{13}$C) NMR spectra were recorded on a 300 or 600 MHz (75 or 150 MHz) NMR spectrometer. Mass spectra were recorded using ESI mode. HPLC purifications used a reversed phase C-18 column (4.6 × 250 mm), where buffer A is 0.1% formic acid in water, buffer B is 0.1% formic acid in 50% acetonitrile, and buffer C is 50 mM ammonium formate (pH 4.0). Two HPLC methods were used. For method 1, 100% buffer A was used at 1 mL/min for 4 min followed by a linear gradient of 0−90% buffer B over 6 min and holding at 90% buffer B for 10 min before re-equilibrating in 100% buffer A. For method 2, 100% buffer C at 1 mL/min was used for 4 min followed by a linear gradient of 0−60% buffer B over 8 min and holding at 60% buffer B for 10 min before re-equilibrating in 100% buffer C.

**Preparation of Enzymes for SAM Synthesis.** Recombinant ecMAT was expressed and purified from *E. coli* DM22pK300 as previously described.[8] The gene for wild-type human BHMT was synthesized and codon optimized for expression in *E. coli* DNA 2.0 (Menlo Park, CA). Recombinant BHMT was overexpressed in the pJ414 plasmid (DNA 2.0) in BL21(DE3) competent cells, and expression was induced by the addition of 200 μM IPTG for 18 h at 28

**Scheme 1. Four Step Chemoenzymatic Synthesis of [$^{36}$S]-SAM from Elemental Sulfur**





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°C. Cells were collected by centrifugation at 6000 ×g for 30 min. The cells were resuspended in 30 mM potassium phosphate (pH 7.6), 5 mM 2-mercaptoethanol, and 2 mM EDTA and lysed by sonication. Untagged BHMT was purified as previously described.[19]

The gene for wild-type *oah2* from *Thermus thermophilus* was synthesized and codon optimized by DNA 2.0 (Menlo Park, CA) according to the published protein sequence.[18] The gene was PCR amplified and cloned into pET28 vector (chemical supplier) between NdeI and *Hin*dIII site to introduce an N-terminal His×6 tag followed by a thrombin cleavage site. The cloned plasmid was confirmed by DNA sequencing. Overexpression of OAH2 in BL21(DE3) was carried out in 2 L of LB kanamycin medium and expression was induced with 0.75 mM IPTG overnight at 18 °C. Cells were collected by centrifugation at 6000 ×g for 30 min, and resuspended in lysis buffer (50 mM potassium phosphate, pH 7.8, 250 mM NaCl, and 0.1 mM pyridoxal 5′-phosphate (PLP)) supplemented by lysozyme, protease inhibitor cocktail (chemical supplier), and 10 mM imidazole. The cells were lysed by sonication, followed by centrifugation at 12,000 ×g for 30 min to remove insoluble cell debris. The supernatant was loaded onto a 5 mL Ni-NTA column equilibrated in the lysis buffer containing 10 mM imidazole. Impurities were removed by running step gradient of imidazole up to 100 mM. OAH2 was eluted using a linear gradient of lysis buffer containing 100 mM to 250 mM imidazole. Pure fractions of the enzyme were combined, and dialyzed in 50 mM potassium phosphate (pH 7.8), 1 mM EDTA, and 0.1 mM PLP. Final purity was >95% by SDS PAGE. The protein was concentrated to 6.6 mg/mL and stored at −80 °C.

**Synthesis of *O*-Acetyl-L-homoserine (3).** *O*-Acetyl-L-homoserine (**3**) was freshly prepared from L-homoserine (200 mg, 1.68 mmol) according to the literature protocol.[23] The product **3** was obtained as a white powder (70 mg, 25% yield). $^1$H NMR (300 MHz, $D_2O$) $\delta$ 4.17 (t, $J$ = 6.0 Hz, 2H), 3.77 (dd, $J$ = 7.0, 5.4 Hz, 1H), 2.29−2.06 (m, 2H), 2.03 (s, 3H); $^{13}$C{$^1$H} NMR (75 MHz, $D_2O$) $\delta$ 174.2, 173.8, 61.5, 52.6, 29.2, 20.4.

**General Procedure for Reduction of Elemental Sulfur by Sodium Metal.** Elemental sulfur ($^{32}$S, $^{36}$S, or natural abundance, 3 mg, 83−93 $\mu$mol), and naphthalene (1.5 mg, 12 $\mu$mol) were dissolved in 3 mL anhydrous tetrahydrofuran in a 5 mL glass vial. Approximately 100 mg of freshly cut sodium metal was added, and the vial was stoppered and sealed under dry argon. The mixture was agitated vigorously in an ultrasonic water bath for 16−24 h according to the previously published procedure.[17] The color of the mixture gradually turned from yellow to brown to dark green to white during the course of the reaction. When the reaction mixture had completely turned white, the THF suspension containing the sodium sulfide product was decanted leaving any remaining sodium metal. The THF fraction was evaporated under vacuum to give crude sodium sulfide as a yellow/brown solid. Dried sodium sulfide was stored as a solid at −20 °C.

The product from a 10 mg natural-abundance sulfur reduction was dissolved in 1 mL of water, and organic impurities were removed by extraction with hexanes. The aqueous solution was then mixed with 1 mL of 0.5 M $Ni(NO_3)_2$ solution to sediment all the $S^{2-}$ ion. The black precipitate was washed thoroughly with water several times and then dried in a 105 °C heating oven to give NiS as a black solid (15.7 mg, 55% yield).

**Enzymatic Synthesis of $^{36}$S-Methionine (2).** Crude $Na_2{}^{36}S$ (~12 $\mu$mol based on the initial mass of sulfur) was dissolved in 4 mL of 50 mM Tris-HCl (pH 7.4) with *O*-acetylhomoserine **3** (1 mg, 6.2 $\mu$mol) and 500 nM OAHS enzyme. The enzyme reaction was incubated at 60 °C for 16 h in a water bath. The OAHS was then removed by passing the solution through a prewashed 10 kDa molecular weight cut off centrifugal filter device and collecting the flowthrough. The sample was then dried by centrifugation under vacuum. Without further purification, the crude $^{36}$S-labeled HCys **4** was dissolved in 4 mL of 50 mM Tris-HCl (pH 8.8) 50 mM KCl, 10 mM $MgCl_2$, 4 mM betaine, and 5 mM dithiothreitol containing 650 nM BHMT enzyme. The mixture was incubated at 37 °C until all of compound **4** was consumed (as observed by NMR spectroscopy, approximately 16 h). The reaction was then quenched by the addition of $H_2SO_4$ to 10 mM and the resulting $^{36}$S-Met **2** was purified using HPLC method 2. Under these conditions, methionine showed an absorbance maximum at 215 nm and eluted between 4.6 and 6 min. The methionine peaks from multiple injections were combined and lyophilized to give a white solid (0.93 mg, 6 $\mu$mol, 97% yield based on compound **3**). The NMR data matched that for commercial methionine. $^1$H NMR (600 MHz, $D_2O$) $\delta$ 3.78 (dd, $J$ = 7.1, 5.4 Hz, 1H), 2.56 (t, $J$ = 7.6 Hz, 2H), 2.19−1.98 (m, 2H), 2.05 (s, 3H); $^{13}$C{$^1$H} NMR (151 MHz, $D_2O$) $\delta$ 174.2, 53.9, 29.6, 28.8, 13.9; HR-ESI-TOF-MS $m/z$ 154.0530 ([M + H]$^+$), calcd for $[C_5H_{12}NO_2{}^{36}S]^+$ 154.0534.

**Enzymatic Synthesis of Labeled SAM (1).** Isotopically labeled SAM **1** was prepared enzymatically using *E. coli* SAMsyn. Typical 500 $\mu$L reactions contained 1 mM **2** (500 nmol) and 1 mM ATP (500 nmol), in of 20 mM Tris-HCl (pH 8.0), 25 mM $MgSO_4$, 50 mM $K_2SO_4$, 8% 2-mercaptoethanol, and 500 nM SAMsyn. The reaction mixture was incubated at 37 °C for approximately 2 h, until the majority of ATP had been consumed, as observed by analytical HPLC (method 1), and no isomerization of the product **1** had occurred. Compound **1** was then purified using HPLC method 1, where SAM elutes at 3 min. The isolated SAM **1** was dried by centrifugation under vacuum, dissolved in 500 $\mu$L of 50 mM ammonium phosphate (pH 4.0), and further purified using HPLC method 2. These conditions allow for separation of the *S* and *R* sulfonium center isomers of SAM, which elute at 5.5 and 5.7 min, respectively. Isolated products were again dried by centrifugation under vacuum to give **1** as a white solid, and the reaction yield was estimated based on absorbance at 260 nm ($\varepsilon_{260}$ = 15,400 M$^{-1}$ cm$^{-1}$, 198 nmol, 40% yield). $^{36}$S-labeled SAM **1** co-eluted with authentic SAM and was isolated as the (*S*)-isomer at the methyl group in >95% purity, as estimated from HPLC analysis (see Supporting Information). HR-ESI-TOF-MS $m/z$ 403.1382 ([M]$^+$), calcd for $[C_{15}H_{23}N_6O_5{}^{36}S]^+$ 403.1395.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

HPLC and MS data of labeled SAM, MS and NMR spectroscopy data of labeled Met samples, and NMR spectroscopy data for OAc-HCys. This material is available free of charge via the Internet at http://pubs.acs.org/.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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## ■ REFERENCES


(1) Jin, B.; Robertson, K. D. *Adv. Exp. Med. Biol.* **2013**, *754*, 3−29.

(2) Dillon, S. C.; Zhang, X.; Trievel, R. C.; Cheng, X. *Genome Biol.* **2005**, *6*, 227.

(3) Igarashi, K.; Katoh, Y. *Subcell. Biochem.* **2013**, *61*, 105−18.

(4) Ikeguchi, Y.; Bewley, M. C.; Pegg, A. E. *J. Biochem.* **2006**, *139*, 1−9.

(5) Layer, G.; Heinz, D. W.; Jahn, D.; Schubert, W. D. *Curr. Opin. Chem. Biol.* **2004**, *8*, 468−76.




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*J. Org. Chem.* 2015, 80, 5344−5347

(6) Marsh, E. N.; Patwardhan, A.; Huhta, M. S. *Bioorg. Chem.* **2004**, *32*, 326−40.
(7) Cantoni, G. L. *J. Biol. Chem.* **1953**, *204*, 403−415.
(8) Markham, G. D.; Hafner, E. W.; Tabor, C. W.; Tabor, H. *J. Biol. Chem.* **1980**, *255*, 9082−92.
(9) Pajares, M. A.; Markham, G. D. *Adv. Enzymol. Relat. Areas Mol. Biol.* **2011**, *78*, 449−521.
(10) Schramm, V. L. *J. Biol. Chem.* **2007**, *282*, 28297−28300.
(11) Schramm, V. L. *Annu. Rev. Biochem.* **2011**, *80*, 703−732.
(12) Hegazi, M. F.; Borchardt, R. T.; Schowen, R. L. *J. Am. Chem. Soc.* **1979**, *101*, 4359−4365.
(13) Zhang, J.; Klinman, J. P. *J. Am. Chem. Soc.* **2011**, *133*, 17134−7.
(14) Guangqi, E.; Lesage, D.; Ploux, O. *Biochimie* **2010**, *92*, 1454−7.
(15) Cleland, W. W. *FASEB J.* **1990**, *4*, 2899−905.
(16) Cleland, W. W. *Arch. Biochem. Biophys.* **2005**, *433*, 2−12.
(17) So, J.-H.; Boudjouk, P.; Hong, H. H.; Weber, W. P. *Inorg. Synth.* **2007**, 30−32.
(18) Iwama, T.; Hosokawa, H.; Lin, W.; Shimizu, H.; Kawai, K.; Yamagata, S. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1357−61.
(19) Millian, N. S.; Garrow, T. A. *Arch. Biochem. Biophys.* **1998**, *356*, 93−8.
(20) Park, J.; Tai, J.; Roessner, C. A.; Scott, A. I. *Bioorg. Med. Chem.* **1996**, *4*, 2179−2185.
(21) Gross, A.; Geresh, S.; Whitesides, G. *Appl. Biochem. Biotechnol.* **1983**, *8*, 415−422.
(22) Tabor, H.; Tabor, C. W. *Methods Enzymol.* **1971**, *17B*, 393−397.
(23) Nagai, S.; Flavin, M. *Methods Enzymol.* **1971**, *17B*, 423−424.



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