Published in final edited form as:

Chembiochem. 2013 May 27; 14(8): 950–953. doi:10.1002/cbic.201300221.

A Tandem Chemoenzymatic Methylation via S-Adenosyl-L-methionine

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Keywords

chemoenzymatic synthesis; methyltransferase; DNA methylation; natural products; alkylation

The utility of chemoenzymatic synthesis lies in the ability of enzymes to perform regio- and stereoselective transformations on complex molecules. Access to these methods has recently offered facile junctions for small molecule synthesis as illustrated elegantly in the total syntheses of bacillithiol,^[1] hyperiones,^[2] gangloside LLG-3,^[3] (+)-clividine,^[4] (+)-amabine,^[5] (+)-(R)-aureothin,^[6] and enterocin.^[7] Like its synthetic counterpart, advances in chemoenzymatic methods, particularly those that can be conducted in a single vessel or device, offer avenues to new synthetic designs. When practical, these methods expand our access to biological probes, materials and therapeutics.

Most enzymatic transformations require the use of cofactors, many of which are expensive and unstable, and methods have been developed to prepare the active forms of cofactors *in situ*. These include adenosine triphosphate (ATP),^[8] nicotinamide adenine dinucleotide (NADH),^[9] and 3'-phosphoadenosine-5'-phosphosulfate (PAPS).^[10] However, no such method currently exists for S-adenosyl-L-methionine (AdoMet), the second most common cofactor in metabolism.^[11]

AdoMet, also commonly referred to as SAM, participates in a wide range of enzymatic processes. Its primary roles are methylation, polyamine biosynthesis, and radical chemistry.^[12] AdoMet-dependent methyltransferases are a large class of enzymes responsible for the methylation of nitrogen, oxygen, sulfur, and carbon in DNA, RNA, proteins, and metabolites.^[13] These enzymes offer particular utility for *in vitro* applications, as methylation often confers important activity and regulatory control to biomolecules. However, AdoMet is both expensive and unstable, degrading rapidly under neutral or alkaline conditions.^[14] Therefore, a practical method for *in situ* AdoMet generation remains

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significant. Here we demonstrate a chemoenzymatic method for producing AdoMet *in situ* through the reversible halogenase SalL (Scheme 1).

In 2007, the discovery of the salinosporamide A biosynthetic pathway from the marine bacterium *Salinispora tropica* unveiled the enzyme SalL.^[15] This enzyme uses AdoMet and chloride ions for the production of 5'-chloro-5'-deoxyadenosine (CIDA), a key intermediate in the biosynthesis of chloroethylmalonyl-CoA.^[16] In nature, both the abundance of chloride in seawater and the reactivity of AdoMet drive the reaction to completion. However, the reverse reaction, AdoMet production, can be promoted at low chloride concentrations. This reaction can be further driven toward AdoMet with the addition of an excess of L-methionine (L-Met) (Scheme 1). CIDA is commercially available and can be inexpensively produced by the reaction of adenosine with SOCl₂.^[17] We therefore set out to explore the use of recombinant SalL, with these stable and inexpensive reagents, for *in situ* generation of AdoMet for enzymatic synthesis.

We began by demonstrating that recombinant SalL (see Supporting Information)^[15] was able to produce AdoMet when incubated with CIDA and L-Met at 37 °C. LC-MS analysis confirmed the production of a peak that matched AdoMet and 5'-methylthioadenosine (MTA), a breakdown product of AdoMet, with characteristic ionization at *m/z* values of 399, 320, and 298 corresponding to [AdoMet]⁺, [MTA+Na]⁺, and [MTA+H]⁺, respectively (see Supporting Information).^[18] We then used HPLC analysis to screen for optimal activity and determined that the reaction could be conducted in a variety of buffers including phosphate, Tris and HEPES when maintained at an optimal pH 7–8 (see Supporting Information). One key limitation was that this procedure requires low salt concentration, as high concentrations of chloride ion converted AdoMet back to CIDA.^[15]

To illustrate the utility of this method, we coupled the production of AdoMet with the methylation of DNA by HhaI methyltransferase.^[20] Activity was analyzed by the use of the isoschizomeric endonuclease HinP1I (Scheme 2),^[21] which cleaves only unmethylated DNA. The coupled reaction was monitored by gel electrophoresis over a gradient in the concentration of SalL and HhaI. Lambda DNA was completely protected from digestion at high concentrations of SalL (Figure 1A) and HhaI (Figure 1B). Control reactions in which SalL, L-Met, and CIDA were substituted with a pure sample of AdoMet in an equivalent concentration also showed full protection. Both control reactions in which either SalL or the HhaI methyltransferase was omitted showed significant digestion. This result confirmed the suitability of this procedure for the *in situ* production of AdoMet with a concomitant transfer of the methyl group to a target molecule. Significantly, it demonstrated that the conditions for the SalL reaction do not meaningfully hinder the methylation activity of a common methyltransferase.

Next, we probed the utility of this method for small molecule applications. We chose to explore the methyltransferase MtfA from the chloroeremomycin biosynthetic pathway in *Amycolatopsis orientalis* that incorporates an *N*-methyl group in vancomycin-type glycopeptides.^[22] It has been shown that MtfA can be used to methylate other lipoglycopeptide antibiotics, including teicoplanin and its aglycone (Scheme 3).^[22] The activity of recombinant MtfA towards teicoplanin was confirmed using commercial AdoMet with a 98% yield of *N*-methylteicoplanin (based on peak area obtained during HPLC analysis) after incubation at 37 °C for 24 h. The major [M+H]⁺ ion for *N*-methylteicoplanin was found to be 1894 by MS analyses.

MtfA was then analyzed for compatibility with *in situ* AdoMet generation by incubation of the SalL reaction mixture with teicoplanin and MtfA under similar conditions (Figure 2). The resulting mixture was analyzed by LC-ESI-MS and the conversion of teicoplanin to *N*-

methylteicoplanin was determined to proceed with a 66% yield (yields described herein are based on peak area obtained during HPLC analysis). To optimize the yield, a period of pre-incubation for AdoMet generation by SalL was analyzed over 3h. Pre-incubation for 2h resulted in maximal yield improvement to 83%. This enhancement is believed to derive from a larger initial pool of AdoMet, thus maintaining an increased levels of AdoMet relative to *S*-adenosyl-*L*-homocysteine (AdoHcy) throughout the reaction. AdoHcy, a product of AdoMet-dependent methylation, has been shown to inhibit methyltransferases, including MtfA.^[23] It is also possible that AdoHcy inhibits SalL, as suggested by evidence from homologous fluorinases FDAS.^[24]

The effect of product inhibition on turnover was evaluated using samples of AdoMet and AdoHcy. We found that 0.8 mM AdoHcy causes a 30% decrease in overall yield, indicating that pre-incubation may be required to drive initial velocity of the methyltransferase reaction (data not shown). Isolation of the chemoenzymatically produced *N*-methylteicoplanin was accomplished using Affigel resin terminated with a D-Ala-D-Ala dipeptide (see Supporting Information).^[25] NMR and MS confirmation of *N*-methylteicoplanin validated this method as an effective technique for chemoenzymatic synthesis with methyltransferases (see Supporting Information).

As a means of demonstration, we developed an application that used ¹³C NMR spectroscopy to monitor the reaction process. As shown in Figure 3, we were able to sequentially monitor the transfer of a ¹³C label from *L*-Met-(methyl-¹³C) to AdoMet and then to teicoplanin, resulting in the production of *N*-methylteicoplanin. This process was advanced by use of the high ¹³C sensitivity available on an Agilent X-Sens probe. As shown in Figure 3, we observed the production of methyl-¹³C-AdoMet at 24.6 ppm within 1 h followed by conversion of teicoplanin to ¹³C-*N*-methylteicoplanin within 9 h. A broad ¹³C signal at 33.7 ppm was observed for *N*-methylteicoplanin, which was comparable to the reported *N*-methyl group in vancomycin (33.2 ppm in DMSO-*d*₆ and 33.5 ppm in 200 mM phosphate pH 6).^[26]

While it was not possible to determine the yield of this reaction using ¹³C NMR (due to fact that teicoplanin could not be observed), HPLC analyses showed a 74% conversion to *N*-methylteicoplanin under these conditions. The product was clearly identified as mono-¹³C-labeled *N*-methylteicoplanin by the observation of [M+H]⁺ with *m/z* at 1893.5809 as compared to the expected [M+H]⁺ with *m/z* of 1893.5829 for C₈₈¹³C₁H₁₀₀Cl₂N₉O₃₃ (formula from methylation of the major isomer, teicoplanin A2), the mass was increased by one due to the addition of one ¹³C-enriched carbon.

AdoMet-dependent methyltransferases are an important class of tailoring enzymes that act upon a variety of large and small biomolecules. These modifications have been shown to be critical in signaling, gene regulation, and bioactivity.^[27] The utility of these methyltransferases for chemoenzymatic synthesis has historically been precluded by the severe instability and expense of AdoMet. We have shown that the AdoMet-dependent chlorinase SalL can effectively be harnessed to generate AdoMet *in situ* from *L*-Met and CIDA. The resulting AdoMet preparation can be used without purification as a methyl donor for methyltransferase-catalyzed reactions and as a facile means of isotope incorporation. The preparative methylation, using this methodology, demonstrates the synthetic utility of this new cofactor generation, thereby unlocking a toolbox of AdoMet-dependent enzymes for use in chemoenzymatic synthesis. This finding now offers an alternate route for the preparation of AdoMet and enables access to modified materials such as ¹³C-labeled AdoMet.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding was provided by NIH R01GM095970, NIH R01CA127622, Drug Research Academy Program, and the Novo Nordisk STAR program. We would like to thank Prof. G. D. Wright (McMaster University) for the MtfA gene construct. We also thank Dr. J. Beld (UC San Diego) and Dr. Y. Su (UC San Diego) for mass spectral analyses, and Dr. A. Mrse (UC San Diego) and Dr. X. Huang (UC San Diego) for assistance with acquiring NMR spectral data. J.M.L (UC San Diego) and M.T. (UC San Diego, a visiting graduate student from R. P. Clausen's laboratory) share first authorship.

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Figure 1.

Inspection of the methylation of lambda DNA by HhaI methyltransferase. A) Reduction in production of AdoMet by decreasing the concentration of SalL results in reduced DNA methylation by the methyltransferase HhaI and increased DNA cleavage. A 50 ng/μL sample of N6-methyladenine free lambda DNA was incubated with SalL, 4 U/μL HhaI, 15 mM L-Met and 200 μM CIDA in 50 mM Tris•HCl, 10 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.5 at 37 °C. After 1h, the reactions were stopped by heat denaturation at 80 °C for 5 min followed by treatment with 0.1 U/μL of the isoschizomeric endonuclease HinP1I. Control (-) indicates N6-methyladenine free lambda DNA. B) DNA methylation by *in situ* produced AdoMet offers a reliable blockage in HhaI cleavage. Treatment of 50 ng/μL N6-methyladenine free lambda DNA with HhaI, 930 nM SalL, 15 mM L-Met and 200 μM CIDA were incubated in 50 mM Tris•HCl, 10 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.5 at 37 °C. After 1h, the reactions were stopped by heat denaturation at 80 °C for 5 min followed by treatment with HinP1I. DNA bands are identified as cleaved (C) and uncleaved (U) forms.

Figure 2.

HPLC analysis. A) An HPLC trace depicting the sample of teicoplanin. B) A HPLC trace after incubating 0.83 mM teicoplanin, 0.23 μ M SalL, 8.3 mM L-Met, 3.3 mM CIDA, 0.83 mg/mL BSA, and 45 μ M MtfA in 5.3 mM sodium phosphate, 41 mM HEPES pH 7.3 after 24 h at 37 $^{\circ}$ C. Analysis was conducted on C-18 column using an isocratic flow of 25% aq. acetonitrile with 0.1% formic acid.

Figure 3.

Monitoring the two-step alkylation of teicoplanin via ^{13}C -NMR analysis. A) ^{13}C -NMR spectra depicting the time course analysis of 1.4 mM teicoplanin, 28.4 mM L-Met-(methyl- ^{13}C), 2.8 mM CIDA, 280 nM SalL and 280 nM MtfA in 10 mM phosphate buffer pH 7.5 containing 10% DMSO- d_6 . B) ^{13}C -NMR spectra depicting the time course analysis of the control reaction without MtfA with 1.4 mM teicoplanin, 28.4 mM L-Met-(methyl- ^{13}C), 2.8 mM CIDA, 280 nM SalL in 10 mM phosphate buffer pH 7.5 containing 10% DMSO- d_6 . ^{13}C -NMR spectra were collected for 64 scans with delay time (d1) of 5 sec on an Agilent X-Sens probe. Spectra were collected from the same sample monitored over

time. A window depicting the region between 20–38 ppm was shown; full spectra are provided in the Supporting Information.

Scheme 1.

Coupled enzymatic production of AdoMet by SalL and subsequent methylation by a methyltransferase. Blue sphere represents DNA, RNA or a metabolite.

Scheme 2.

Coupling of *in situ* AdoMet synthesis by SalI and DNA methylation. A) Close up of depicting an unmodified DNA segment bound to the HhaI (not shown).^[19] Arrow denotes the position of methylation. B) Schematic depiction of the coupled reaction of *in situ* AdoMet synthesis by the action of SalI on L-Met and ClDA and methylation of lambda DNA by HhaI methyltransferase. This methylation prevents cleavage by HindP1I.

Scheme 3.

Schematic depiction of the methylation of teicoplanin by the methyltransferase, MtfA using *in situ* produced AdoMet.