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# An enzyme-coupled colorimetric assay for *S*-adenosylmethionine-dependent methyltransferases

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**Abstract**

We report here an enzyme-coupled colorimetric assay for salicylic acid carboxyl methyltransferase (SAMT), which utilizes *S*-adenosyl-L-methionine (AdoMet or SAM) as the methyl donor. In this assay, *S*-adenosyl-L-homocysteine (AdoHcy or SAH), a common product of AdoMet-dependent transmethylation reactions, is first hydrolyzed by recombinant AdoHcy nucleosidase (EC 3.2.2.9) into adenine and *S*-ribosylhomocysteine. Recombinant LuxS (*S*-ribosylhomocysteinase, EC 3.2.1.148) cleaves the latter compound to form homocysteine. Finally, homocysteine is quantified using Ellman's reagent and the accompanying absorption change at 412 nm through recording using a microplate format. Notably, SAMT and most AdoMet-dependent methyltransferases undergo marked AdoHcy-mediated product inhibition. As such, an additional advantage of this assay which includes AdoHcy nucleosidase is the destruction of AdoHcy, thus alleviating product inhibition. Under our assay conditions, complete substrate conversion is observed and precise kinetic parameters can be determined in a facile and quantitative manner. This assay should be generally applicable to other AdoMet-dependent methyltransferases. Moreover, the procedure is easily amendable to batch assay and high-throughput screening approaches.
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*S*-Adenosyl-L-methionine-dependent methyltransferases are a large family of enzymes with broad biological functions [1–4]. More than 130 members are listed by the Enzyme Commission (EC 2.1.1.1–147), and others have been described but have not yet been numbered. Such enzymes act on small molecules, lipids, proteins, polysaccharides, or polynucleotides. These modifications result in a myriad of crucial biological functions. For instance, protein methylation together with phosphorylation and acetylation are integral components of the "histone code," joining the genetic code as a critical determinant of chromosomal inheritance [5]. Because of their biological importance, there is a growing need to precisely determine the kinetic parameters of methyltransferases and to identify inhibitors for these enzymes. However, a general, convenient colorimetric assay is not presently available for the majority of AdoMet[1]-dependent methyltransferases. This is because the common substrate of all these methyltransferases, AdoMet, and the AdoHcy product derived from it display little spectroscopic difference. Only when the methyl-accepting substrates and the methylated products display distinguishable spectra can the reaction course be spectroscopically monitored. A limited number of such examples include catechol *O*-methyltransferase (COMT, EC 2.1.1.6), thiopurine *S*-methyltransferase (TPMT, EC 2.1.1.167), and salicylic acid

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[1] *Abbreviations used:* AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatograph; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; SAMT, salicylic acid carboxyl methyltransferase; TNB, 2-nitro-5-thiobenzoic acid; Tris, tris(hydroxymethyl)aminomethane.

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carboxyl methyltransferase (SAMT) as we report here [6–9].

Currently, most methyltransferase activity assays are based on radioactive labeling, using $^{14}C$- or tritium-labeled AdoMet as the substrate. The radioactive assays require subsequent chromatographic separation or selective extraction procedures of substrates and products, which is often laborious, slow, and only semiquantitative. A more serious limitation of the current assays is the marked product inhibition exhibited by most AdoMet-dependent methyltransferases due to AdoHcy accumulation. AdoHcy, a common product of the methyl transfer reactions, is a potent feedback inhibitor for all or most methyltransferases [10]. As a result, it is tedious and technically challenging to accurately measure the kinetic parameters of these methyltransferases.

We report here an enzyme-coupled colorimetric assay for AdoMet-dependent methyltransferases and a strategy to alleviate product inhibition from AdoHcy. Salicylic acid carboxyl methyltransferase was used as a model system [11]. As shown in Scheme 1, SAMT catalyzes the methylation of the carboxylate group in salicylic acid using AdoMet as the methyl donor to form methyl salicylate and AdoHcy. This enzyme was first discovered in the flowers of the California annual *Clarkia breweri*, where it is responsible for the biosynthesis of the scent volatile methyl salicylate [12]. SAMT belongs to a novel family of plant methyltransferases, the SABATH family [11,13]. Structurally related enzymes that catalyze the formation of other plant volatiles that are involved in plant–insect interactions and hormonal regulation, such as methylbenzoate and methyl jasmonate, have been described by several groups (reviewed in [11,13]).

In our coupling assay, AdoHcy, a common product of the AdoMet-dependent transmethylation reactions, is first hydrolyzed by recombinant AdoHcy nucleosidase (EC 3.2.2.9) into adenine and *S*-ribosylhomocysteine [14,15]. The latter compound is further cleaved by recombinant LuxS (*S*-ribosylhomocysteinase, EC 3.2.1.148, formerly EC 3.3.1.3) to form homocysteine [16–19]. Finally, homocysteine is quantified using Ellman's reagent and the absorption change at 412 nm as measured using a microplate format. The AdoHcy nucleosidase present in our assay mixtures effectively cleaves AdoHcy, thus alleviating product inhibition from AdoHcy. Under our assay conditions, complete substrate conversion is observed and precise kinetic parameters can be determined easily and quantitatively.

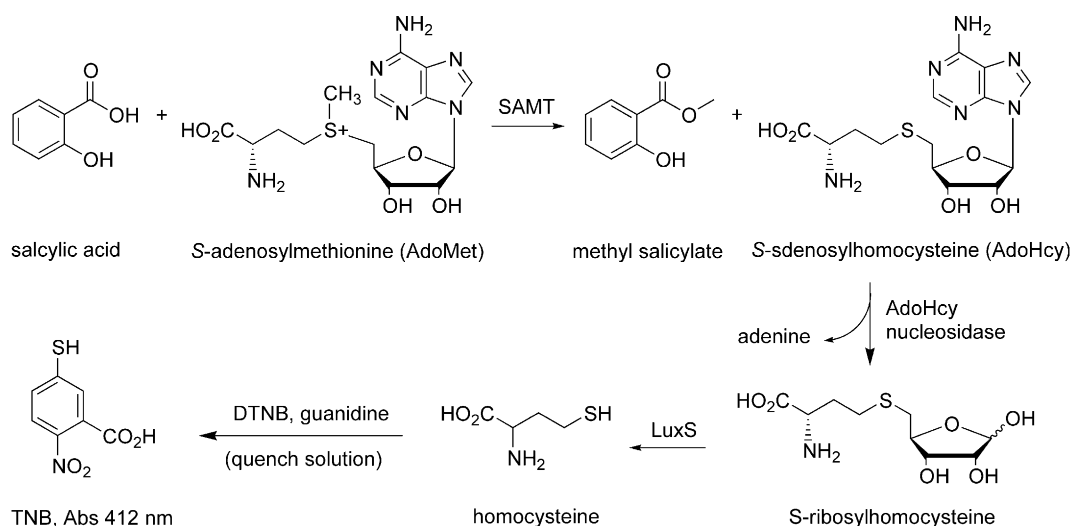
## Materials and methods

### *Materials*

AdoMet *p*-toluenesulfonate salt and AdoHcy were purchased from Sigma. 5,5-Dithiobis-2-nitrobenzoic acid (DTNB; Ellman's reagent) and methyl salicylate were purchased from Aldrich. All other chemicals were of the highest purity available. Recombinant AdoHcy nucleosidase and LuxS, both containing histidine tags, were expressed and purified as previously described [15,17].

### *Preparation of reagents*

A quenching solution for the reaction was prepared by dissolving guanidine-hydrochloride, ethylenedinitrilotetraacetic acid (EDTA), and *N*-(2-hydroxyethyl)-




Scheme 1. Coupling assay of SAMT activity. The TNB generated from the coupled assay is quantified by absorption at 412 nm using a microplate reader.

piperazine-$N'$-2-ethanesulfonic acid (Hepes) in Milli-Q water to final concentrations of 8 M, 0.5 mM, and 100 mM, respectively, with gentle heat, followed by pH adjustment to 8.0 with concentrated NaOH solution. A stock solution of 10 mM salicylic acid was prepared in 100 mM potassium phosphate, pH 7.0. An AdoMet solution was prepared in 1 mM HCl to a final concentration of 3.54 mM and stored at −20 °C. The AdoMet and AdoHcy concentrations were determined by measuring absorbance at 260 nm ($\varepsilon = 15,400\ M^{-1}\ cm^{-1}$) [20]. Prior to the assay, the AdoMet solution was thawed and kept on ice before mixing with other assay components.

*Procedure for stoichiometric analysis of AdoHcy conversion to TNB*

Nitrogen gas was bubbled through a 100 mM Hepes, pH 8.0, solution for 20 min prior to initiation of the assay. This treatment was to remove oxygen from the solution, preventing oxidation of the thiol group in homocysteine. DTNB (10 mM in 100 mM Hepes, pH 8.0, solution) was added to the 8 M guanidine stock solution to a final concentration of 133 μM. The assay solutions contained various amounts of AdoHcy, 8.6 μM LuxS, 0.1 M Hepes at pH 8.0 in a total volume of 270 μL. After equilibrium at 37 °C for 10 min, the reactions were initiated by adding AdoHcy nucleosidase (0.1 μM, final concentration). Aliquots of 60 μL reaction mixtures were quenched with 180 μL DTNB-guanidine solution in a 96-well microtiter plate at 0, 5, 10, and 20 min. The resulting mixtures were incubated at room temperature for an additional 5 min before measuring the absorption at 412 nm on a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA). The path length of the mixture was determined to be 0.78 cm. An extinction coefficient of 13,700 $M^{-1}\ cm^{-1}$ at 412 nm was used to quantify TNB formation [21].

*Procedure for the enzyme-coupled colorimetric assay*

Nitrogen gas was bubbled through the assay buffer solution (50 mM Tris–HCl, 100 mM KCl at pH 7.5) for 20 min prior to the start of the assay, minimizing oxidation of the thiol group in homocysteine and the active site cysteine in LuxS [17,18,22]. A typical assay mixture contained 222 μM salicylic acid, 430 μM AdoMet, 0.27 μM SAMT, 0.1 μM AdoHcy nucleosidase, 20 μM LuxS, 50 mM Tris–HCl, 100 mM KCl at pH 7.5 in a total volume of 1.4 mL. After equilibrium at 37 °C, the reactions were initiated by adding SAMT. Absorption at 312 nm was continuously recorded on a Cary 100 UV-Visible spectrophotometer (Varian, Palo Alto, CA) under kinetic mode. In addition, 60-μL aliquots of the reaction mixtures were quenched with 180-μL DTNB-guanidine solutions in a 96-well microtiter plate at various times. Quantification of TNB formation was performed as described in the previous section.

*High-performance liquid chromatograph (HPLC) analysis of reaction products*

HPLC analysis of the methyl salicylate product was performed on an Apollo C-18 reverse-phase column (4.6 mm × 25 cm, Alltech, Deerfield, Il). The column was isocratically eluted with a mixture of acetonitrile (80%) and 50 mM ammonium bicarbonate at pH 7.8 (20%) with a 1.5 mL/min flow rate while being monitored at 312 nm.

## Results and discussion

The stoichiometry of the conversion of AdoHcy to TNB under our assay conditions was investigated first. A linear correlation between the amount of AdoHcy added and the amount of TNB formed was observed (Fig. 1). The two coupling enzymes in our assay, AdoHcy nucleosidase and LuxS, have been shown to stoichiometrically convert their substrates to the corresponding products [14–16,18]. Considering that the assay mixture (60 μL) was diluted fourfold with the DTNB-guanidine quenching solution (180 μL), a conversion factor of 25% was expected from AdoHcy to TNB. An actual conversion factor of 26.2 ± 0.7% was determined from Fig. 1. This result indicates that AdoHcy was converted into TNB in a stoichiometric fashion under our assay conditions.

To yield valid kinetic parameters from our coupled assay, it is essential that the coupling enzymes used are not rate limiting, so that the measured rate is determined solely by the SAMT activity. In other words, the methylation product (methyl salicylate) and the analyte generated from the coupled assay (TNB) should be

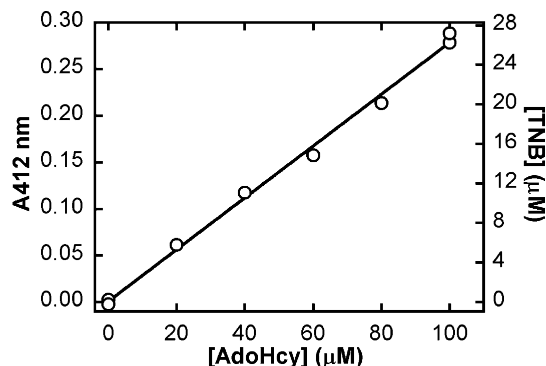



Fig. 1. Determining stoichiometry for enzyme-coupled conversion of *S*-adenosylhomocysteine to TNB. The assay mixture was diluted fourfold with the quench solution. A conversion factor of 25% was expected for the conversion from AdoHcy to TNB, and 26.2 ± 0.7% was determined from the plot.

formed at equal rates. To ensure that our assay meets this requirement, we chose the concentrations of SAMT and the coupling enzymes based on their specific activities. The $k_{cat}$ values for SAMT, AdoHcy nucleosidase, and LuxS are about 0.28, 50, and 0.07 $s^{-1}$, respectively [11,15,18]. Under our typical assay conditions (0.27 μM SAMT, 0.1 μM AdoHcy nucleosidase, 20 μM LuxS), the calculated maximum activities of the coupling enzymes were at least 50-fold higher than those of the SAMT enzyme.

We also found that methyl salicylate formation could be continuously monitored on an ultraviolet-visible spectrophotometer. Using authentic samples, we determined that salicylic acid and methyl salicylate absorbed maximally at 296 and 303 nm, respectively, under our assay conditions. When the acid is converted into the ester, the largest absorbance increase occurs at 312 nm with an extinction coefficient of 1970 $M^{-1}$ $cm^{-1}$. This value was used to quantify methyl salicylate formation in all of our studies, and the results obtained were in close agreement with the data collected from HPLC separation and subsequent quantification (see Fig. 3).

A total time course of the SAMT-catalyzed methylation of salicylic acid (222 μM) was performed (Fig. 2), with salicylate methyl ester formation quantified by absorption changes at 312 nm (solid line) and TNB concentration (after fourfold dilution) determined from the absorption at 412 nm recorded on a microplate reader (filled circles). The data indicate that methyl salicylate and TNB were formed at the same rates. In addition, complete conversion from salicylic acid to methyl salicylate was observed after 170 min. The formation of methyl salicylate in the coupled-enzyme assay was confirmed by HPLC analysis through comparison with an authentic sample (Fig. 3). The reaction product was also verified by coinjection with an authentic sample of methyl salicylate. Furthermore, methyl salicylate

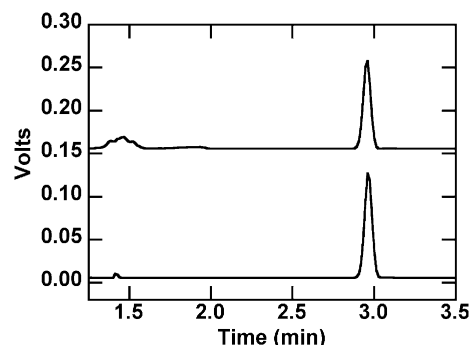



Fig. 3. HPLC traces of authentic methyl salicylate (222 μM, bottom trace) and an aliquot of the reaction mixture after the reaction was completed as judged by absorption changes at 312 nm (222 μM was expected for complete substrate conversion, top trace).

concentrations determined from quantitative HPLC analysis (data not shown) agreed with those shown in Fig. 2, the latter of which were obtained from absorption changes at 312 nm and the TNB assay.

Using our assay, the specific activity of SAMT was determined (Fig. 4). Reactions were carried out in the presence and absence of SAMT and monitored by the continuous spectroscopic assay and the enzyme-coupled assay. Again, methyl salicylate and TNB were formed at comparable rates. When no SAMT was added to the reaction mixture, essentially no methyl ester of salicylate or TNB was formed. With increased SAMT concentrations, the initial rates of the reaction also increased (data not shown). Under our assay conditions, the specific activity was calculated as $0.28 \pm 0.01$ $s^{-1}$ at 37 °C. In comparison, a $k_{cat}$ value of $0.092 \pm 0.0077$ was reported at 25 °C [11]. Together, the results shown in Figs. 2 and 4 demonstrate that the SAMT activity was rate limiting in the enzyme-coupled assay.

As mentioned in the introduction, a serious limitation of the current assays for AdoMet-dependent methyl-

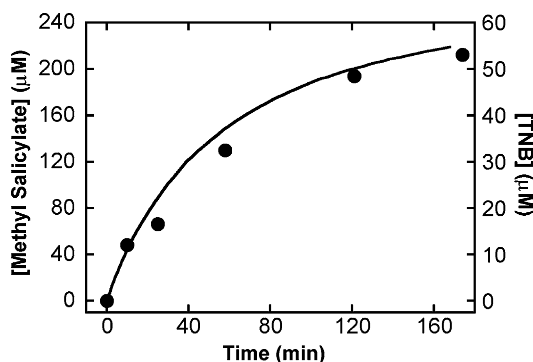



Fig. 2. Total time course of the SAMT-catalyzed methylation of salicylic acid (222 μM). The salicylate methyl ester formation was monitored continuously on a UV-visible spectrophotometer and quantified by the absorption changes at 312 nm (solid line). TNB formation in the discontinuous coupling assay was quantified by absorption at 412 nm on a microplate reader (filled circles).

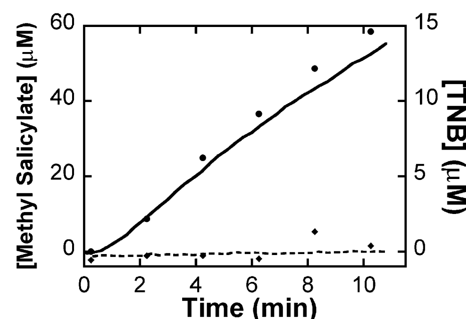



Fig. 4. Initial time course of the SAMT-catalyzed methylation of salicylic acid (222 μM). The salicylate methyl ester formation was monitored continuously on a UV-visible spectrophotometer and quantified by the absorption changes at 312 nm (solid and dashed lines). TNB formation in the discontinuous coupling assay was quantified by absorption at 412 nm on a microplate reader (closed circles and diamonds). Top line was in the presence of SAMT, and bottom line was in the absence of SAMT.

transferase is the ever present AdoHcy-dependent product inhibition. AdoHcy, a common product of the methyl transfer reactions, is a potent feedback inhibitor for nearly all methyltransferases [10]. For the bovine brain protein L-isoaspartate *O*-methyltransferase (EC 2.1.1.77), the $K_m$ value for AdoMet is 2 μM and the $K_i$ value for AdoHcy is 0.08 μM. To a first approximation, the AdoHcy product binds 25-fold tighter than the AdoMet substrate [23]. We decided to investigate whether the SAMT enzyme was subject to AdoHcy inhibition also. Methylation reactions of salicylic acid were monitored in the presence and absence of added AdoHcy nucleosidase, and both proceeded at similar initial rates (Fig. 5). However, as the AdoHcy concentration increased to about 50 μM (at 25% of the complete conversion), the reaction lacking AdoHcy nucleosidase stalled. In contrast, the reaction including AdoHcy nucleosidase proceeded smoothly to completion (see Figs. 5 and 2). This experiment clearly demonstrates that AdoHcy severely inhibits SAMT and that addition of AdoHcy nucleosidase can relieve this significant product inhibition through irreversible cleavage of AdoHcy. A similar result was observed for thiopurine *S*-methyltransferase (TPMT, EC 2.1.1.167) in our laboratory [7]. These experiments suggest that addition of AdoHcy nucleosidase is a general strategy to remove product inhibition due to AdoHcy in AdoMet-dependent methyltransferase assays.

To overcome AdoHcy inhibition, most of the reported assays are carried out with a large excess of AdoMet, often in the radioactive form. As a result, it is tedious and technically challenging to accurately measure the kinetic parameters of these methyltransferases. Alternatively, a strategy that is similar to the one we report here has been employed to alleviate AdoHcy inhibition of AdoMet-dependent methyltransferases. In that system, AdoHcy deaminase (EC 3.5.4.4) was added to convert AdoHcy into *S*-inosylhomocysteine, which shows little inhibitory activity toward several AdoMet-dependent methyltransferases [24–26]. In addition, conversion of AdoHcy to *S*-inosylhomocysteine is associated with an absorbance decrease at 265 nm, allowing methyl transfer reactions to be followed spectroscopically [25,27,28]. However, because AdoHcy deaminase is not readily available in pure form and its gene has yet to be cloned [29], this method is seldom used. In comparison, the recombinant AdoHcy nucleosidase and LuxS enzymes are expressed as fusion proteins with polyhistidine tags, allowing facile purification using metal affinity chromatography [15,17]. We routinely obtain about 20–50 mg of purified protein from 1 L of Luria–Bertani broth, and the purified proteins are stable and robust for at least several months when stored at −80 °C. As such, AdoHcy nucleosidase and LuxS can be used as common reagents for the coupled assay that we report here. Furthermore, the stability and robustness of the coupling enzymes confer a high degree of reproducibility. For example, $k_{cat}$ values of 0.273, 0.291, and 0.277 $s^{-1}$, respectively, were obtained from experiments carried out on 3 separate days and with various batches of enzyme.

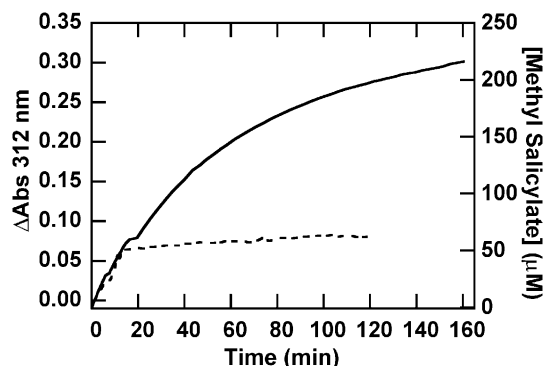



Fig. 5. Total time course of the SAMT-catalyzed methylation of salicylic acid (222 μM) in the presence (solid line) and in the absence (dotted line) of AdoHcy nucleosidase.

In this paper, we report an enzyme-coupled colorimetric assay for SAMT, an AdoMet-dependent methyltransferase. Our method has two significant advantages over the current methyltransferase activity assays. First, the addition of AdoHcy nucleosidase to the reaction mixture greatly reduces product inhibition from AdoHcy. Consequently, our method will allow kinetic characterization of methyltransferases with greater precision. Second, TNB generated from the coupling assay can be quantified at 412 nm on readily available spectrophotometers and in a microplate format. Compared to the radioactive assay commonly used, our assay does not require tedious separation of substrate and product. As shown in Figs. 2 and 4, conversion from AdoHcy to TNB is linear from 5 μM to at least 200 μM product formed. The higher limit of the assay is restricted only by the linear range of individual spectrophotometers at 412 nm and can be extended simply by further dilution of the TNB solutions. The lower limit of our assay is determined by the sensitivity and precision of the spectrophotometer, e.g., 4 μM of AdoHcy formation (1 μM TNB after a fourfold dilution) causes an absorbance change of about 0.01 at 412 nm, which is near the lower detection limit for most instruments. In summary, this assay should be generally applicable to other AdoMet-dependent methyltransferases and is amendable to batch assays and high-throughput screening methods.

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