Biocatalysis

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Facile Chemoenzymatic Strategies for the Synthesis and Utilization of *S*-Adenosyl-L-Methionine Analogues**

Shanteri Singh, Jianjun Zhang, Tyler D. Huber, Manjula Sunkara, Katherine Hurley, Randal D. Goff, Guojun Wang, Wen Zhang, Chunming Liu, Jürgen Rohr, Steven G. Van Lanen, Andrew J. Morris, and Jon S. Thorson**

Abstract: *A chemoenzymatic platform for the synthesis of S-adenosyl-L-methionine (SAM) analogues compatible with downstream SAM-utilizing enzymes is reported. Forty-four non-native S/Se-alkylated Met analogues were synthesized and applied to probing the substrate specificity of five diverse methionine adenosyltransferases (MATs). Human MAT II was among the most permissive of the MATs analyzed and enabled the chemoenzymatic synthesis of 29 non-native SAM analogues. As a proof of concept for the feasibility of natural product "alkylrandomization", a small set of differentially-alkylated indolocarbazole analogues was generated by using a coupled hMAT2–RebM system (RebM is the sugar C4′-O-methyltransferase that is involved in rebeccamycin biosynthesis). The ability to couple SAM synthesis and utilization in a single vessel circumvents issues associated with the rapid decomposition of SAM analogues and thereby opens the door for the further interrogation of a wide range of SAM utilizing enzymes.*

Enzyme-catalyzed late-stage group-transfer-based tailoring reactions contribute to the structural and functional diversity of many complex natural products (NPs).[1] Representative examples include amination, acylation, alkylation, glycosylation, halogenation, phosphorylation, and sulfation. In some cases, the enzymes responsible for such transformations display notable flexibility with respect to their substrate scope.[2] Such promiscuity is an enabling feature of chemoenzymatic NP diversification platforms as exemplified by NP glycorandomization (a platform for the differential glycosylation of natural products/drugs).[3] Enzyme-catalyzed alkylation is a highly prevalent modification that leads to the N, O, S, and/or C alkylation of NPs (Figure 1).[4] A platform to co-opt natural product methyltransferases (MTs) for broad natural product differential alkylation (alkylrandomization) would thus be anticipated to dramatically expand the potential scope of NP chemical diversity.

The typical alkyl donor for MT-catalyzed alkylation is *S*-adenosyl-L-methionine (SAM) and importantly, we and others have demonstrated that NP,[5] protein,[6] and nucleic acid[7] MTs transfer alternative alkyl groups in the presence of suitably modified SAM analogues. However, reminiscent of the restriction imposed upon glycorandomization by the availability of sugar nucleotides, access to arrays of stable SAM analogues similarly restricts the development of NP alkylrandomization. Current state-of-the-art for the chemical synthesis of SAM analogues relies upon multistep syntheses of diastereomeric mixtures of SAM analogues and requires HPLC purification to remove the starting material, *S*-adenosyl-homocysteine (SAH, a strong inhibitor of MTs).[8] The purified SAM analogues from this process are also markedly unstable, therby limiting their practical development as stand-alone synthetic reagents and/or biological probes. Within this context, a general platform to enable the generation and direct utilization of SAM analogues, starting from stable precursors, would be advantageous.

To address this need, we herein describe the broad substrate specificity assessment of a representative set of five distinct methionine adenosyltransferases (MATs) from various sources. MAT (EC 2.5.1.6; also sometimes referred to as AdoMet synthetase or SAM synthetase) is the primary catalyst for the biosynthesis of SAM from adenine triphosphate (ATP) and L-methionine (Met; Scheme 1 a) and there is preliminary evidence to suggest that certain MATs are capable of utilizing Met analogues.[6e,9] Consistent with this notion, the studies highlighted herein reveal that human MAT II (hMAT2) enables the cumulative synthesis of a broad

[*] Dr. S. Singh, Dr. J. Zhang, T. D. Huber, Prof. J. S. Thorson
Center for Pharmaceutical Research and Innovation
College of Pharmacy, University of Kentucky
Lexington, KY 40536 (USA)
E-mail: ssingh555@uky.edu
jsthorson@uky.edu

Dr. S. Singh, Dr. J. Zhang, T. D. Huber, Dr. G. Wang, Prof. J. Rohr, Prof. S. G. Van Lanen, Prof. J. S. Thorson
Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky
789 South Limestone Street, Lexington, KY 40536 (USA)

W. Zhang, Prof. C. Liu
Molecular and Cellular Biochemistry, University of Kentucky
College of Medicine, University of Kentucky
Lexington, KY 40536 (USA)

M. Sunkara, Prof. A. J. Morris
Division of Cardiovascular Medicine, Gill Heart Institute
University of Kentucky, Lexington, KY 40536 (USA)

K. Hurley
Department of Biochemistry, University of Wisconsin-Madison
Madison, WI 53705 (USA)

Dr. R. D. Goff
Western Wyoming Community College
2500 College Dr., Rock Springs, WY 82902-0428 (USA)

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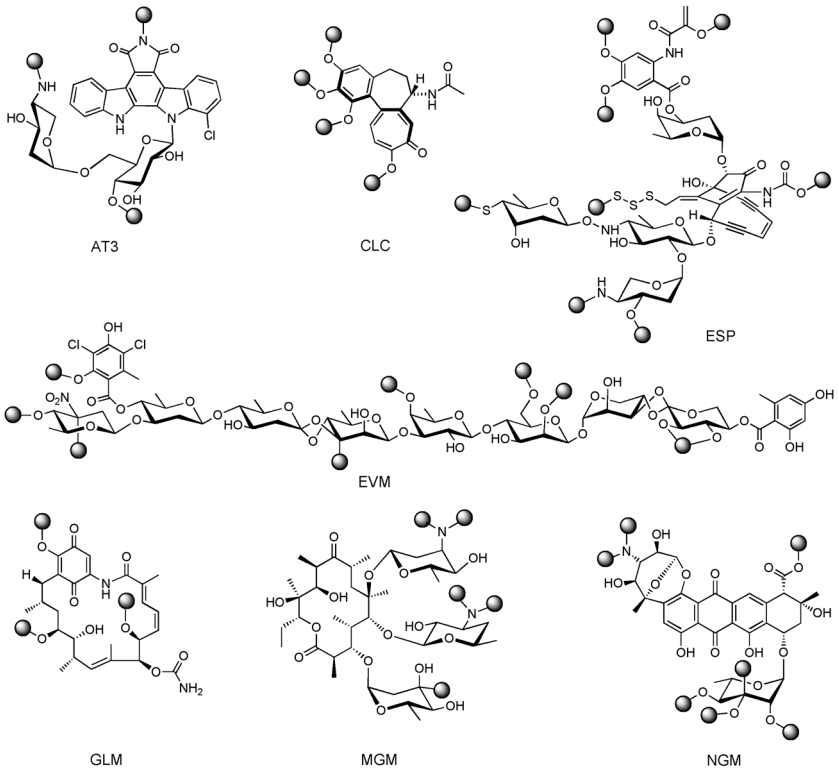
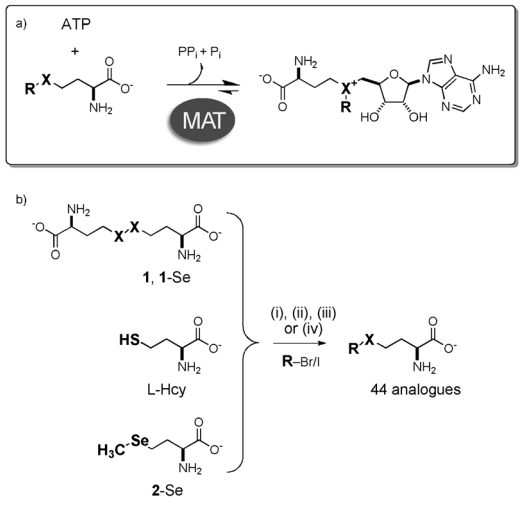


Figure 1. Representative methylated natural products in which methyl groups deriving from SAM through MT-catalyzed methylation are highlighted as spheres. AT3 = AT2433; CLC = colchicine; ESP = esperamicin; EVM = evernimicin; GLM = geldanamycin; MGM = megalomicin; NGM = nogalamycin.

panel of unnatural SAM analogues (29 analogues detected) starting from synthetic S/Se-alkylated Met analogues (44 analogues) or commercial sources (3 analogues). To demonstrate the feasibility of NP alkylrandomization, this study also highlights the subsequent generation of a small set of indolocarbazole analogues by using a coupled hMAT2–RebM system, where RebM is the sugar C4′-*O*-MT that is involved in rebeccamycin biosynthesis.[5a,10] This study both offers a strategic advance in terms of NP diversification and reveals that hMAT2 displays surprising substrate permissivity.



Scheme 1. a) The methionine adenosyltransferase (MAT)-catalyzed reaction, in which L-Met (R = CH_3, X = S) is the native substrate and the native product is S-adenosylmethionine (R = CH_3, X = S). The variability of both R and X are assessed within the current study. b) General methodology for the synthesis of *S*- and *Se*-L-Met analogues (X = S and Se, respectively): i) Na, NH_3, −78 °C with **1** or **1**-Se; ii) K_2CO_3, acetone with L-Hcy; iii) $NaBH_4$/NaOH, H_2O, THF, 5 h with **1**-Se; iv) HCl, Reflux with **2**-Se (see Table S1 in the Supporting Information).

Given the conflicting reports regarding the relative stability of S- versus Se-SAM[6c,d,11] and the fact that Se-SAM is considered a better alkyl donor than S-SAM in MT-catalyzed reactions owing to the inherently longer and weaker Se–C bond,[11b] both S- and Se-Met analogues were synthesized for this study. The general strategy for the synthesis of the S- and Se-Met analogue panel employed direct or reductive alkylation of L-homocystine, the oxidized dimer of L-homocysteine (L-Hcy) [**1** or **1**-Se, Scheme 1 b, (i) and (iii), respectively], L-Hcy [Scheme 1 b, (ii)], or **2**-Se [Scheme 1 b, (iv)] following simple modifications of previously reported strategies.[12] The desired S- and Se-Met analogues were purified from the crude reaction mixtures by reverse-phase high-pressure liquid chromatography (RP-HPLC), C_{18} RP flash chromatography, or Dowex 50W8X-200 resin to afford yields of isolated product ranging from 20–90 % (Table S1 in the Supporting Information).

A representative set of MATs from various sources, including bacterial, archaeal, and mammalian orthologues, were selected for the initial assessment. The exploratory set included the human MAT II catalytic alpha and regulatory beta subunits (hMAT2),[13] the human MAT II catalytic alpha subunit alone (hMAT2A),[13] the human MAT I catalytic alpha subunit (hMAT1A),[13] *Escherichia coli* MAT (eMAT),[14] and the thermophilic *Methanocaldococcus jannaschii* MAT (mMAT).[15] The basis for the inclusion of the human homologues was an interest in interrogating the substrate specificities of disease-relevant hMATs,[16] while the additional bacterial and archaeal homologues were selected to compare and contrast the current study with prior work relating to their substrate specificities.[9a,17]

Standard uniform assay conditions [Met analogue (2 mM), ATP (1 mM), MAT (5 μM), tris(hydroxymethyl)aminomethane (Tris; 25 mM), $MgCl_2$ (5 mM), KCl (50 mM), pH 8, 4 h] were adopted to facilitate this broad assessment. For the thermophilic counterpart (mMAT), the reactions were conducted at 65 °C, while all other reactions were incubated at 37 °C. The production of the SAM analogues was determined by a simple RP-HPLC end-point assay to afford complete analysis of the desired SAM analogue and/or any major corresponding SAM analogue degradation products. The formation of the putative SAM analogues and SAM-derived degradation products was subsequently confirmed by HRMS

analysis for all of the positive reactions (Table S2 in the Supporting Information).

A cumulative comparison of the substrate specificities of all five MAT model systems based on RP-HPLC is illustrated in Figure 2, in which 5′-methyl-thio(seleno)-5′-deoxyadenosine (MTA) production (as measured by RP-HPLC) was interpreted as product production based on the well-established SAM decay pathways that indicate that MTA is directly derived from SAM (and not ATP).[6d,11a,18] This cumulative analysis revealed hMAT2 to be the most permissive of the MATs tested at 37 °C, while the thermophilic mMAT also displayed notable permissivity at 65 °C. Of the 47 putative substrates tested with hMAT2, 10 led to appreciable (> 50 %) production of the corresponding SAM analogue, an additional 8 led to moderate (25–50 %) conversion, while 11 offered detectable product (< 25 %) under the conditions described. In general, smaller alkyl substitutions were better tolerated, thus suggesting that steric infringement perhaps prohibits larger substitutions. Interestingly, in the case in which direct comparisons could be made, the degree of unsaturation correlated with a reduction in turnover (e.g., propyl > allyl > propargyl). Also, in most cases, Se analogues were preferred over their S counterparts, most notably for hMAT2. Consistent with previous studies,[19] the regulatory beta subunit of MAT2A improved the overall proficiency of hMAT2 and, in some cases, this increase in proficiency translated to a slight increase in permissivity. Finally, the addition of inorganic pyrophosphatase in an attempt to drive the reaction forward through degradation of the pyrophosphate coproduct did not appreciably increase turnover.

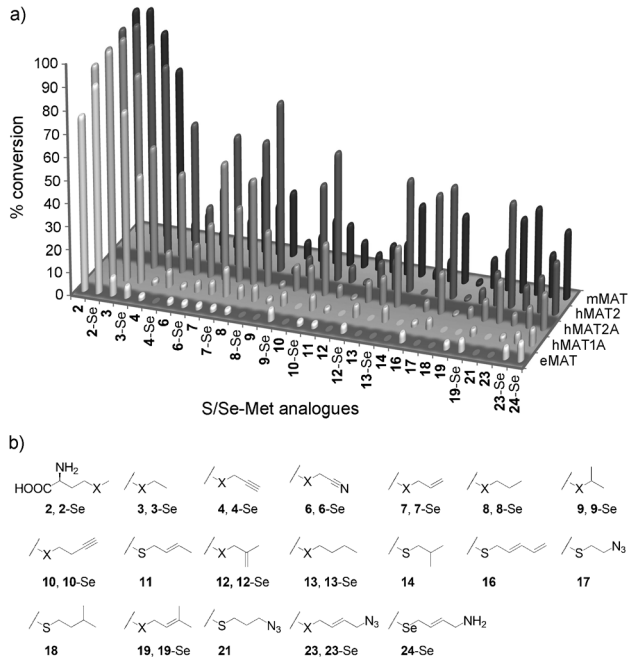


Figure 2. a) Turnover of S/Se-Met analogues to the corresponding S(Se)AM analogues catalyzed by selected MATs (as measured by RP-HPLC, average percent error ≤ 5 %; see the Supporting Information). No product formation was observed in the absence of MAT, S/Se-Met analogues or ATP. b) Structures of the S/Se-Met analogues listed in Figure 2a (X = S, Se).

Based on these results, the corresponding MATs can be generally classified as having broad (hMAT2/hMAT2A), medium (mMAT), or stringent (eMAT and hMAT1A) substrate specificities. Superimposition of the available X-ray structures (hMAT1A, hMAT2A, and eMAT) reveals a root mean square deviation (rmsd) of 0.268 Å between hMAT2A and hMAT1A (sequence identity ≈ 85 %) and approximately 0.56 Å between eMAT and hMAT1A or hMAT2A (sequence identity ≈ 58 %; Figure S3 in the Supporting Information). Structural comparison of the permissive (hMAT2A) and stringent (hMAT1A and eMAT) MATs highlights key distinctions at certain residues (Figure 3). Interestingly, while hMAT2A active-site mutagenesis has led to a slight improvement in turnover with an unnatural analogue,[6e] the current structural comparison suggests the broad substrate specificity of hMAT2A to be predominately mediated by residues within the secondary shell and/or on the solvent-exposed surface distal to active-site loops that likely contribute to interdomain movement/dynamics. Whether or not these residues contribute to the hMAT2 alpha/beta interface is currently unknown.

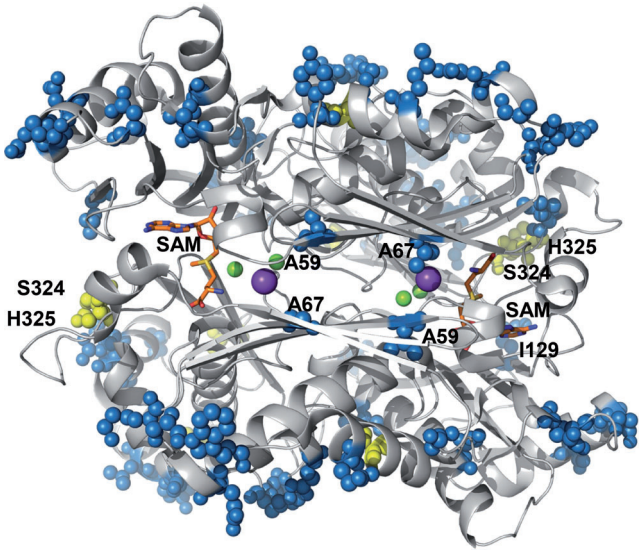
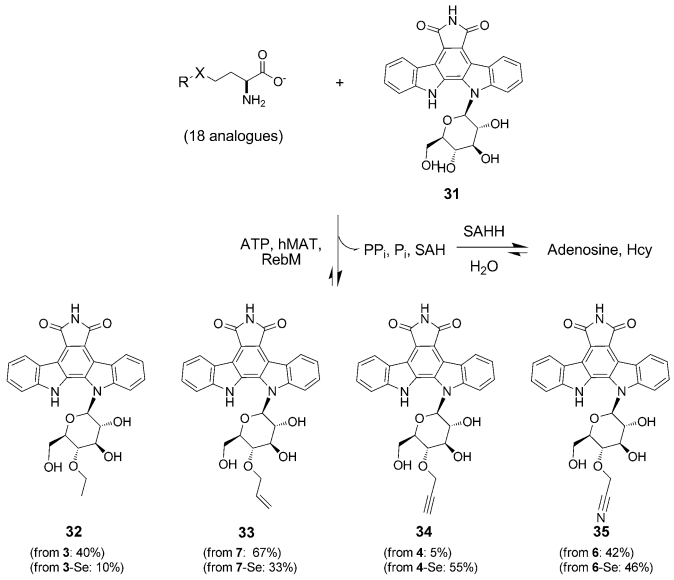


Figure 3. Mapping of the residue positions that differ between eMAT (PDB ID:1RG9), hMAT2A (PDB ID:2PO2) and hMAT1A (PDB ID:2OBV) on the hMAT2A homodimeric structure. Residues conserved across all three MATs are indicated by grey ribbons; residues that differ between the three MATs are highlighted as blue ball and stick models; and residues that are conserved in the "stringent" MATs (hMAT1A and eMAT) but differ in the more permissive hMAT2A are highlighted as yellow ball and stick models. The purple and green spheres within the active site represent K^+ and Mg^{+2}, respectively.

To assess the feasibility of "alkylrandomization" through a single-vessel coupled MAT/MT reaction, the rebeccamycin MT RebM was selected. RebM is the sugar C4′-*O*-MT that is involved in rebeccamycin biosynthesis and has previously been demonstrated to tolerate both alternative acceptors and alternative alkyl donors.[5a,10] The coupled system also

employed standard assay conditions [Met analogue (2 mM), ATP (1 mM), rebeccamycin congener **31** (50 μM), hMAT2 (5 μM), RebM (10 μM), SAH-hydrolase (5 μM), Tris (25 mM), $MgCl_2$ (5 mM), KCl (50 mM), pH 8, 24 h at 37 °C] and included only those S/Se-Met analogues that afforded ≥ 20 % turnover with hMAT2 under standard conditions (Figure 2). From this pilot study, 8 out of 18 of the selected S/Se-Met series led to the production of the corresponding differentially alkylated indolocarbazoles in appreciable yields (≥ 40 %, Scheme 2 and



Scheme 2. Products of the hMAT2A–RebM coupled reaction in the presence of **3**/**3**-Se, **7**/**7**-Se, **4**/**4**-Se, and **6**/**6**-Se (see Figure 2). The percentage product formation for each S/Se-Met analogue utilized is noted in parentheses (average percent error ≤ 5 %, see the Supporting Information).

Figure S4b). Importantly, inclusion of SAH-hydrolase (SAHH) in the coupled reaction to prevent the product inhibition associated with most if not all MTs[20] improved the overall product yields by 15–40 %. Notably, this is the first report of MT-catalyzed acetonitrilylation (**35**; derived from L-Met analogue **6** or **6**-Se).

In conclusion, the broad substrate specificity analysis presented reveals that hMAT2/2A and mMAT display notably broad substrate tolerance. Of these, hMAT2A is overexpressed in a number of tumor types,[21] in which inhibition of hMAT2A by small-molecule inhibitors or siRNA affords dramatic tumor reduction.[22] The discovered substrate malleability of hMAT2A, in conjunction with the known aberrant cancer-specific hMAT2A overexpression, may offer the potential to employ suitable S/Se-Met analogues as cell-based metabolic probes for investigating the role of methylation in cancer. In addition, the work highlighted herein enables one of the first facile syntheses of SAM analogues directly compatible with downstream SAM-utilizing enzymes, including MTs, in a method with significant advantages over other recently reported chemoenzymatic strategies that depend on synthetic non-native alkylated amino acid donors and non-native nucleoside acceptors.[6f] As such, this platform circumvents a major liability of prior SAM analogue/MT strategies, namely the decomposition of the SAM analogues, and thereby opens the door to further interrogation of a host of MTs that operate on macromolecular (protein/nucleic acid) and small-molecule (natural products) substrates. Finally, the strategy presented unveils a single vessel proof of concept for natural product "alkylrandomization", which, while currently somewhat limited by enzyme specificity, is expected to be further advanced through MT/MAT directed evolution and/or structure-based engineering in a manner reminiscent to that used for advancing glycorandomization.[3,23]

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