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(54) Title: CHEMOENZYMATIC SYNTHESIS OF S-NUCLEOSYL AMINO ACIDS (SNA), ANALOGS OF S-ADENO-
SYL-L-METHIONINE AND S-ADENOSYL-L-HOMOCYSTEINE AND USES THEREOF

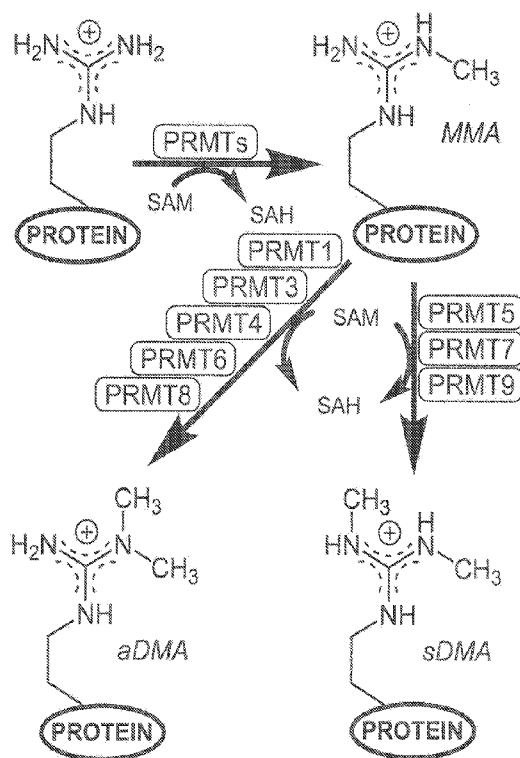


FIG. 1

(57) Abstract: Disclosed are methods for
chemoenzymatic synthesis of S-Nucleosyl
Amino acid probes (SNA), analogs of S-aden-
osyl-L-methionine and S-adenosyl-L-homo-
cysteine, analogs synthesized by the methods,
and uses thereof.



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CHEMOENZYMATIC SYNTHESIS OF *S*-NUCLEOSYL AMINO ACIDS (SNA),
ANALOGS OF *S*-ADENOSYL-L-METHIONINE AND *S*-ADENOSYL-L-
HOMOCYSTEINE AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/290,502, filed on February 3, 2016, the contents of which are hereby incorporated by reference.

STATEMENT OF GOVERNMENT INTEREST

[0002] This invention was made with government support under grant number GM108646 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Protein methylation is a significant regulator of biological function and its misregulation is increasingly implicated in oncogenesis and tumor progression. Protein arginine methyltransferases (PRMTs), a family of nine enzymes in humans, methylate arginines in many proteins, including histones, spliceosomal factors, and ribosomal proteins (Figure 1). Since PRMTs are critical components of a range of biological processes and are frequently misregulated in cancer, these enzymes are emerging targets for chemotherapy. In particular, PRMT5 is overexpressed in tumors and its elevated activity is highly correlated with poor clinical prognosis.

[0004] *S*-adenosyl-L-methionine (SAM) is the universal methyl donor substrate for all methyltransferases and thus a scaffold for drug design. The present invention addresses the need for analogs of SAM and *S*-adenosyl-L-homocysteine (SAH) for therapeutic treatments.

SUMMARY OF THE INVENTION

[0005] The present invention provides various strategies to prepare *S*-Nucleosyl Amino acid probes (SNA). A ‘tool-box’ composed of recombinant promiscuous enzymes (*e.g.* kinases, methionine adenosyltransferases, SAM-dependent chlorinase, and/or *S*-adenosyl-L-homocysteine hydrolase) is introduced for the preparation of SAM-cofactor and SAH analogs with broad chemical diversity. Using an immunoassay and/or a radioactive assay, the SNA library is screened against various methyltransferases to pin-point valuable chemical scaffolds and to further assist in the synthesis of selective methyltransferases’ inhibitors.

[0006] The present invention provides methods of synthesizing an analog of *S*-adenosyl-L-methionine (SAM) comprising: i) reacting a nucleoside or nucleoside analog with a nucleoside triphosphate in the presence of one or more of adenosine kinase, deoxynucleoside kinase and deoxycytidine kinase to form a monophosphate nucleoside analog; ii) forming a triphosphate nucleoside analog by a) reacting the monophosphate nucleoside analog with a nucleoside triphosphate in the presence of one or both of myokinase and cytidylate kinase to form a diphosphate nucleoside analog; and reacting the diphosphate nucleoside analog with a nucleoside triphosphate in the presence of one or both of pyruvate kinase and nucleoside-diphosphate kinase to form a triphosphate nucleoside analog; and/or b) reacting the monophosphate nucleoside analog with phosphoenolpyruvic acid and pyrophosphate in the presence of pyrophosphate phosphate dikinase to form a triphosphate nucleoside analog; and iii) reacting the triphosphate nucleoside analog with methionine or a methionine analog in the presence of methionine adenosyltransferase to form an analog of SAM.

[0007] Also provided are methods of synthesizing an analog of *S*-adenosyl-L-methionine (SAM) comprising reacting a 5'-chlorinated nucleoside analog with methionine or a methionine analog in the presence of a SAM-dependent chlorinase to form an analog of SAM.

[0008] Also provided are methods of synthesizing an analog of *S*-adenosyl-L-homocysteine (SAH) through reaction between a nucleoside or nucleoside analog with homocysteine or an analog of homocysteine (*e.g.* cysteine) in the presence of *S*-adenosyl-L-homocysteine hydrolase to form an analog of SAH.

[0009] Also provided are disclosed analogs and their use in treating cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Fig. 1. All protein arginine methyltransferases (PRMTs) catalyze methylation of arginine residues to generate monomethyl arginine (MMA). While the PRMT1, PRMT3, PRMT4, PRMT6 and PRMT8 catalyze the formation of asymmetric dimethyl arginine (aDMA), the symmetric dimethyl arginine mark (sDMA) is deposited by the PRMT isozymes 5, 7 and 9.

[0011] Fig. 2. SAM substrate and SAH analog interaction with the human methyltransferases Dot1L, CARM1 (*i.e.* PRMT4) and PRMT1. Overview of Dot1L structure (ribbon and surface representation) with close-ups on the cofactor binding pocket (SAM-bound in first frame, PDB: 3QOW, and *N*₆-methyl SAH inhibitor-bound in second frame,

PDB: 3SR4; black sticks). Table of inhibition constants (K_i ; μM) for several molecules (1, 2 and 3, depicted on far-right) against human Dot1L, CARM1 and PRMT1.

[0012] Fig. 3A-3C. An overview of the SAM binding pocket from three members of the PRMT family. Ribbon and surface shown, far left. Close-ups of the cofactor binding pocket depict the ‘adenosine’ and the ‘methionine’ binding mode (left and right, respectively). Black stick representation show SAM or its bound analogs. **A.** human PRMT5 (PDB: 4GQB; *HsPRMT5*). **B.** rat PRMT1 (PDB: 1ORH; *RnPRMT1*). **C.** human PRMT4 (PDB: 4IKP; *HsCARM1*). Dashed lines = hydrogen bonds; structural water molecules are depicted with spheres.

[0013] Fig. 4A-4C. Chemoenzymatic approach to the synthesis of a SAM-cofactor library is more efficient than chemical synthesis. **A.** Comparison between the tedious lengthy chemical approach (shaded) and the two chemoenzymatic roads leading to the correct SAM stereoisomer. Strategies first employ a nucleoside activation (left arrow) followed by a nucleophilic displacement (right arrow). The activation steps through phosphorylation are catalyzed by enzymes (oval shapes): adenosine kinase (AK), myokinase (MK) and pyruvate kinase (PK). Then, nucleophilic displacement with methionine is catalyzed either by SAM-synthetases (MAT) on the nucleoside triphosphates (top approach) or by SAM-dependent chlorinase (SalL) on the 5'-chloro-5'-deoxy adenosine (bottom approach). **B.** Coomassie-stained SDS-PAGE showing, from left to right, recombinant enzymes from *Salinispora tropica* (StSalL), *Methanococcus jannaschii* (MjMAT), *Sulfolobus solfataricus* (SMAT), *Neisseria meningitidis* (NmMAT) and *Campylobacter jejuni* (CjMAT). **C.** Sampling of the chemical space around the SAM methyl donor includes alterations onto the nucleobase (“BASE”), the sugar moiety (“SUGAR”) and the amino-acid tail (“METHIONINE”). Modifications are represented by atoms (where X, Y and Z are either a nitrogen, a carbon, a sulfur, or an oxygen atom) and functional groups where R_{1-10} are either a hydrogen-, an oxygen-, a fluorine-, a chlorine-, or a sulfur-atom, a hydroxyl-, an ether-, an ester-, a carboxylic acid-, a cyano-, an azido-, a primary- secondary- or tertiary-amino-, or a hydrocarbon-group (e.g. methyl, ethyl).

[0014] Fig. 5A-5C. Approaches to the preparation of SAM and SAH analogs. **A.** Enzyme ‘tool box’ for the synthesis of nucleoside triphosphates. Enzymes (oval shapes) used include: deoxynucleoside kinase (*d*NK), deoxycytidine kinase (*d*CK), cytidylate kinases (*d*CMPK, CMPK), nucleoside-diphosphate kinase (NDK) and pyrophosphate phosphate dikinase (PPDK). **B.** *Lupinus luteus* adenosylhomocysteinase (*L*/SAHH) assisted synthesis of

SAH analogs. *S*-adenosyl-L-cysteine is synthesized through the *L*/SAHH-catalyzed reaction using adenosine (ADO) and cysteine (n=1). C. Chemical synthesis of 5'-triphosphate adenosines (ADO).

[0015] Fig. 6. An overview of the methionine analog specificity for recombinant SAM-synthetases (MAT) and SAM-dependent chlorinase from *Salinispora tropica* (*StSalL*). The relative enzymatic efficiencies of four molecules (M1-4) were measured. These values are compared for the enzymes from *M. jannaschii* (*MjMAT*; black), *N. meningitidis* (*NmMAT*; stripped pattern), *C. jejuni* (*CjMAT*; grey) and *S. tropica* (*StSalL*; squared pattern).

DETAILED DESCRIPTION OF THE INVENTION

[0016] The invention provides various strategies to prepare *S*-Nucleosyl Amino acid probes (SNA). A 'tool-box' composed of recombinant promiscuous enzymes (*e.g.* kinases, methionine adenosyltransferases, SAM-dependent chlorinase, and/or *S*-adenosyl-L-homocysteine hydrolase) is introduced for the preparation of SAM-cofactor and SAH analogs with broad chemical diversity. Using an immunoassay and/or a radioactive assay, the SNA library is screened against various methyltransferases to pin-point valuable chemical scaffolds and to further assist in the synthesis of selective methyltransferases' inhibitors.

[0017] The invention provides a method of synthesizing an analog of *S*-adenosyl-L-methionine (SAM) comprising

i) reacting a nucleoside or nucleoside analog with a nucleoside triphosphate in the presence of one or more of adenosine kinase, deoxynucleoside kinase and deoxycytidine kinase to form a monophosphate nucleoside analog;

ii) forming a triphosphate nucleoside analog by

a) reacting the monophosphate nucleoside analog with a nucleoside triphosphate in the presence of one or both of myokinase and cytidylate kinase to form a diphosphate nucleoside analog; and reacting the diphosphate nucleoside analog with a nucleoside triphosphate in the presence of one or both of pyruvate kinase and nucleoside-diphosphate kinase to form a triphosphate nucleoside analog; and/or

b) reacting the monophosphate nucleoside analog with phosphoenolpyruvic acid and pyrophosphate in the presence of pyrophosphate phosphate dikinase to form a triphosphate nucleoside analog; and

iii) reacting the triphosphate nucleoside analog with methionine or a methionine analog in the presence of methionine adenosyltransferase to form an analog of SAM.

[0018] Any of the nucleoside triphosphates can be, for example, adenosine triphosphate (ATP) or cytidine triphosphate (CTP).

[0019] The adenosine kinase can be, for example, *Anopheles gambiae* adenosine kinase. The deoxynucleoside kinase can be, for example, *Bacillus anthracis* deoxynucleoside kinase. The deoxycytidine kinase can be, for example, *Homo sapiens* deoxycytidine kinase. The cytidylate kinase can be, for example, *Coxiella burnetii* cytidylate kinase. The nucleoside-diphosphate kinase can be, for example, *Coxiella burnetii* nucleoside-diphosphate kinase. The pyrophosphate phosphate dikinase can be, for example, from *Zea mays* or *Clostridium symbiosum*. The methionine adenosyltransferase can be, for example, from *Methanococcus jannaschii*, *Sulfolobus solfataricus*, *Neisseria meningitidis* or *Campylobacter jejuni*.

[0020] Also provided is a method of synthesizing an analog of *S*-adenosyl-L-methionine (SAM) comprising reacting a 5'-chlorinated nucleoside analog with methionine or a methionine analog in the presence of a SAM-dependent chlorinase to form an analog of SAM. The SAM-dependent chlorinase can be, for example, *Salinispora tropica* enzyme.

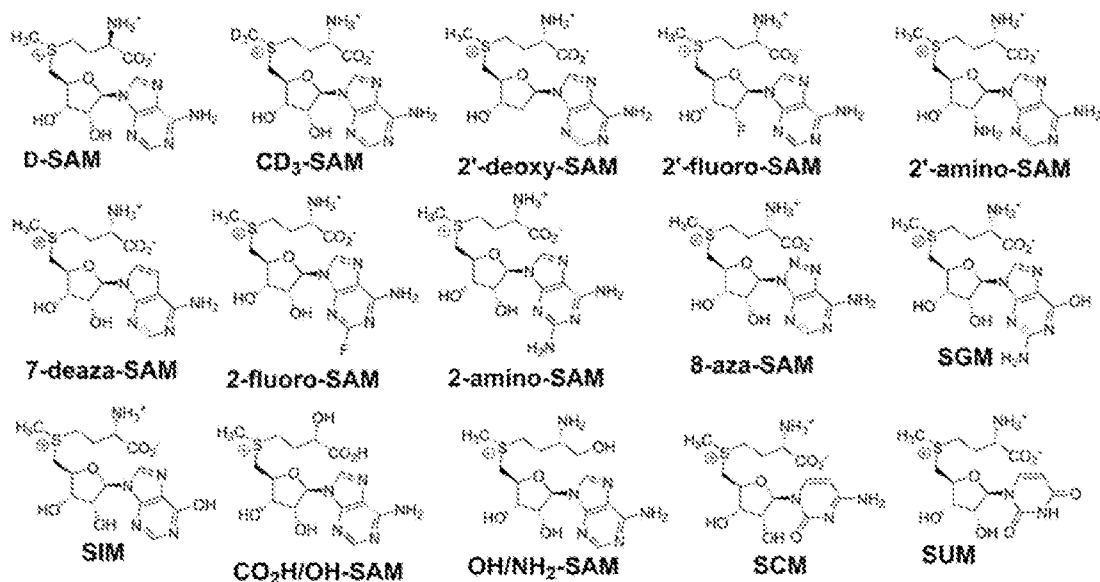
[0021] Also provided are methods of synthesizing an analog of *S*-adenosyl-L-methionine (SAH) comprising reacting a nucleoside or nucleoside analog with homocysteine or an analog of homocysteine (e.g. cysteine) in the presence of *S*-adenosyl-L-homocysteine hydrolase to form an analog of SAH. The *S*-adenosyl-L-homocysteine hydrolase can be, for example, *Lupinus luteus* enzyme.

[0022] The nucleoside or nucleoside analog can be a ribonucleoside or ribonucleoside analog or a 2'-deoxyribonucleoside or 2'-deoxyribonucleoside analog or a 3'-deoxyribonucleoside or 3'-deoxyribonucleoside analog. The nucleoside or nucleoside analog can be, for example, adenosine, 2'-deoxyadenosine, 3'-deoxyadenosine, an analog of adenosine, an analog of 2'-deoxyadenosine, or an analog of 3'-deoxyadenosine. The nucleoside analog can be substituted, for example, with one or more of methyl, ethyl, benzyl, -NH₂, -OH, -SH, -NHNH₂, -NHOH, -NO₂, -N₃, -F, -Cl, =O and -CD₃. Examples of nucleoside analog that can be used include, but are not limited to, tubercidin, vidarabine, 2-amino adenosine, 2-fluoro adenosine, 2'-fluoro-2'-deoxy adenosine, 2'-amino-2'-deoxy adenosine and *N*₆-methyl adenosine.

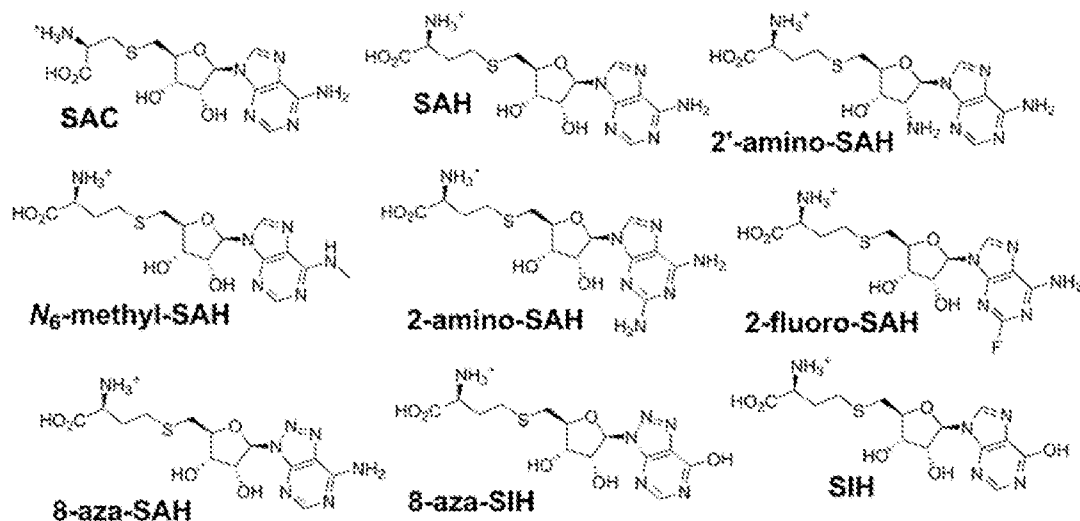
[0023] One or more of adenosine kinase, deoxynucleoside kinase, deoxycytidine kinase, myokinase, cytidylate kinase, pyruvate kinase, nucleoside-diphosphate kinase, pyrophosphate phosphate dikinase, methionine adenosyltransferase, SAM-dependent chlorinase and *S*-

adenosyl-L-homocysteine hydrolase used in the methods disclosed herein can be a recombinant enzyme expressed, for example, in *E. coli*.

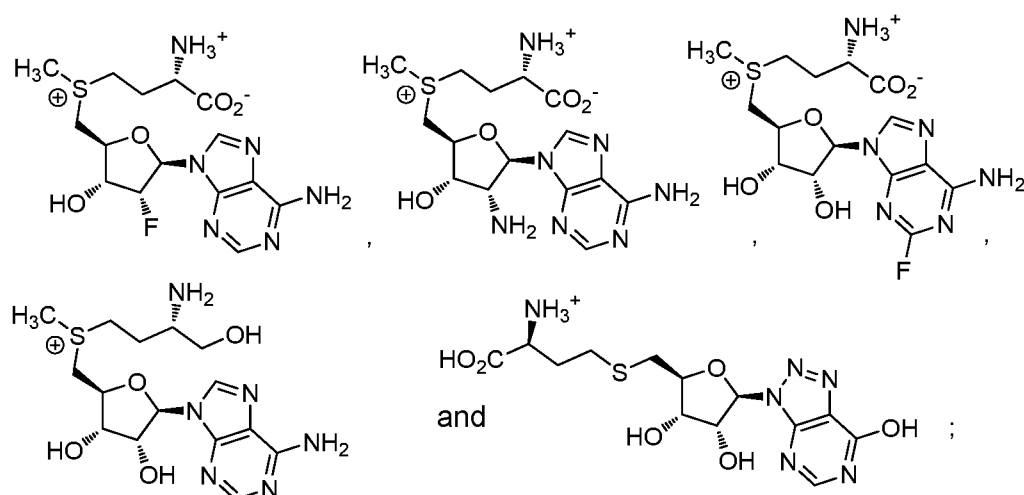
[0024] SAM analogs that can be used, include for example, the following:



[0025] SAH analogs that can be used, include for example, the following:



[0026] The invention also provides a compound selected from the group consisting of



or a pharmaceutically acceptable salt thereof.

[0027] Pharmaceutically acceptable salts that can be used with compounds of the present invention include, e.g., non-toxic salts derived, for example, from inorganic or organic acids including, but not limited to, salts derived from hydrochloric, sulfuric, phosphoric, acetic, lactic, fumaric, succinic, tartaric, gluconic, citric, methanesulphonic and p-toluenesulphonic acids.

[0028] Also provided is a pharmaceutical composition comprising one or more of the compounds disclosed herein and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers and diluents that can be used herewith encompasses any of the standard pharmaceutical carriers or diluents, such as, for example, a sterile isotonic saline, phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsions. The pharmaceutical compositions can be formulated to be advantageous for the selected route of administration to a subject.

[0029] Also provided is a method of treating a cancer in a subject comprising administering to the subject one or more of the compounds disclosed herein in an amount effective to treat a cancer in a subject. The compound can be administered in an amount effective to inhibit a methyltransferase involved in cancer development or progression in a subject.

[0030] As used herein, “treating” or “treat” a cancer means to alleviate or ameliorate or eliminate a sign or symptom of the cancer that is being treated. For example, treatment with the compound can reduce or eliminate the cancer in the subject, or retard the growth, development, progression or spread of the cancer in the subject.

[0031] The compounds and compositions of the present invention can be administered to subjects using routes of administration known in the art. The administration can be systemic

or localized to a specific site. Routes of administration include, but are not limited to, intravenous, intramuscular, intrathecal or subcutaneous injection, oral or rectal administration, and injection into a specific site.

[0032] All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0033] This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

Introduction

[0034] All methyltransferases use the universal *S*-adenosyl-L-methionine (SAM) methyl-donor. Recent computational studies have demonstrated the druggability of the SAM binding pocket for methyltransferases, including PRMTs. Disclosed herein is a chemoenzymatic approach for production of SAM/SAH analogs with broad chemical diversity. These molecules are further referred to as *S*-Nucleosyl Amino acids or SNA. As a proof of concept, starting from commercial nucleoside, methionine and homocysteine analogs, fourteen SNA were synthesized through simple use of recombinant enzymes expressed in *E. coli*. *In vivo*, methionine adenosyltransferase (MAT) catalyzes the formation of SAM from L-methionine and adenosine triphosphate (ATP). The present approach uses an array of recombinant enzymes, including MAT proteins with broad substrate specificity. This unique combination of enzymes is the key to a fast and stereoselective ‘one-pot’ preparation of SAM/SAH analogs. This method will promote a completely new approach to the development of small molecules to target methyltransferases, including PRMT-specific inhibitors.

[0035] Several attempts have been made to identify inhibitors selective over different human PRMTs, yet many were not truly isozyme-specific (for example, selectivity for PRMT5/6 over CARM1 below 30). Although PRMTs have a broad range of methyl acceptors, the arginine substrate is often isozyme-specific so novel bi-substrate scaffolds occupying the arginine and SAM binding sites were synthesized. Yet these inhibitors display low potency and poor selectivity. Instead, the properties described are reminiscent of the naturally occurring inhibitor *S*-adenosyl-L-homocysteine by-product of all methyl-transferase reactions (SAH; Figure 2, molecule 1).

[0036] To advance the design and utility of inhibition of arginine methyltransferases for cancer chemotherapy, scaffolds of SAM or SAH analogs are expected to be effective and selective inhibitors of PRMTs. Recent computational analyses based on structural biology studies predict that the SAM cofactor binding site is chemically tractable. Phylogenetic sequence alignment of methyltransferase active sites predicts high similarity between: 1) PRMTs, 2) small molecule methyltransferases (SMMTs) and 3) the disrupter of telomere silencing 1-like enzyme (Dot1L). SAH is a potent, yet non-specific, inhibitor of methyltransferases (Figure 2; molecule 1, first row from table) and the genetic similarities mentioned above support this observation. SAM and ATP share similarities (*i.e.* adenosyl group) and so do their binding pockets. Nucleoside analogs have highly proven effectiveness in the clinic as there are various examples of selective and specific kinases inhibitors, supporting the approach for SAM analogs.

[0037] In support of this hypothesis, SAM or SAH analogs harboring subtle chemical modifications display potent affinity toward distinct methyltransferases. *S*-(5'-adenosyl)-3-thiopropylamine (decarboxylated analog of SAH) is 10-fold more selective for PRMT1 over PRMT6. The *N*₆-methyl SAH molecule (Figure 2, molecule 2) perfectly illustrates how selectivity can emerge from a slight alteration of the SAM/SAH original scaffold as it is 350- and 80-fold selective for Dot1L over CARM1 and PRMT1, respectively (Figure 2; second row in table). Several cofactor PRMT binding pocket differences emerge upon structural analysis, further supporting the hypothesis (Figure 3). The occupancy for the nucleoside binding site in PRMT5 (Figure 3A; first close-up) diverges from the highly exposed 'adenosine' moiety in PRMT1 (Figure 3B; first close-up) and the moderate disposition for CARM1 to interact with solvent (Figure 3C; first close-up). The L-homocysteine binding site is often filled with structural water molecules (Figure 3; far-right close-ups). Thus, chemical alterations to enhance the hydrogen bond network in this portion of the active site may lead to the discovery of unnatural cofactor substrates with unprecedented isozyme specificity.

Experimental Procedures

1.1 Production of Nucleoside Triphosphate Building Blocks

[0038] 1.1.a 'One-pot' chemoenzymatic synthesis of nucleoside triphosphates to access SAM analogs. Synthetic chemical approaches yield a 1:1 mixture of the two SAM stereoisomers (Figure 4A, shaded middle frame). The enzymes that catalyze the formation of SAM from ATP and L-methionine *in vivo* are used to produce SAM analogs (MAT; Figure 4A, top enzymatic approach). Adenosine phosphorylation is performed by kinases (*e.g.*

adenosine kinase 'AK', myokinase 'MK' and pyruvate kinase 'PK'; Figure 4, top enzymatic approach). MAT proteins are well characterized and several mechanistic and structural biology reports make these enzymes the preferred biocatalyst for rapid access to SAM analogs. *Methanococcus jannaschii* MAT has been purified (MjMAT; Figure 4B). The promiscuity of this SAM-synthetase and its ability to utilize an array of nucleoside triphosphates other than ATP (*e.g.* dATP, GTP, ITP, CTP, UTP) was confirmed using high-performance liquid chromatography (HPLC).

[0039] *Library starting material.* Customized libraries of adenosine analogs can be acquired from commercial sources (*i.e.* Carbosynth and Granlen). These molecules will cover chemical alterations on the 'sugar' (Figure 4C; R₁, R₂, R₃ and X) and the 'base' (Figure 4C; R₄, R₅, R₆, R₇, Y and Z). Each single point variation will either increase hindrance (*e.g.* *N*- or *O*-methyl/ethyl substitutions) or modulate hydrogen bonding capacity (*e.g.* -NH₂, -OH, -SH, -NHNH₂, -NHOH, -NO₂, -N₃, -F, -Cl, -CH₃) of the final compound.

[0040] *Synthesis of nucleoside triphosphate analogs.* To produce nucleoside triphosphate analogs, AK, MK and PK enzymes are used to catalyze the formation of nucleoside mono-, di- and triphosphate analogs, respectively (Figure 4A; top enzymatic approach). The MK and PK enzymes are commercially available (Sigma), and AK from *Anopheles gambiae* (AgAK) was expressed and purified in *E. coli*. Importantly, AgAK displayed good catalytic efficiency ($k_{\text{cat}}/K_{\text{m}} \sim 1.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) for several adenosine analogs tested (*e.g.* tubercidin, vidarabine, 2-amino adenosine, 2-fluoro adenosine, 2'-fluoro-2'-deoxy adenosine, 2'-amino-2'-deoxy adenosine, *N*₆-methyl adenosine) showing its utility for the screen in milligram quantities with >95% yields within 3 hours. Upon removal of the enzymes by ultrafiltration, the nucleoside triphosphate (NTP) can be purified by reverse-phase HPLC. Following optimization, preparations can be scaled-up (25 mg nucleoside; 1 mL reaction) and purification carried by semi-preparative reverse-phase HPLC. A last desalting step (*i.e.* HPLC) can be performed to provide salt-free NTPs. After concentration by freeze-drying, NTPs can be stored in -80°C at a 100 mM stock concentration. These NTPs are enzymatically combined with methionine analogs to produce SNMs.

[0041] *1.1.b Overcoming poor enzymatic conversions.* Although the AK/MK/PK enzymatic system is promiscuous, a few analogs are resilient to this approach. Molecules bearing an arabinofuranosyl configuration (Figure 5A; *e.g.* 9-[2'-deoxy-2'-fluoro-β-D-arabinofuranosyl]adenine) displayed low reactivity so complementary enzymes were prepared. The preparation of these enzymes was straightforward since two of them were

already purified by the New York Structural Biology Center at Einstein (NYSBC): deoxynucleoside kinase (*d*NK; from *Bacillus anthracis*, DNASU clone ID FLH249881.01F), deoxycytidine kinase (*d*CK; from *Homo sapiens*, DNASU clone ID FLH271744.01L), cytidylate kinases (*d*CMPK, CMPK; from *Coxiella burnetii*, NYSBC clone ID CBU_0527), nucleoside-diphosphate kinase (NDK; from *Coxiella burnetii*, NYSBC clone ID CBU_1258). Another two enzymes, the pyrophosphate phosphate dikinases (PPDK) from *Zea mays* and *Clostridium symbiosum*, are also useful (Figure 5B). These PPDK proteins catalyze the conversion from mono- to nucleoside triphosphate using PEP and pyrophosphate (PPi). Finally, a chemical approach may be used to synthesize nucleoside triphosphates (Figure 5C).

1.2 SMN assembly from NTPs and methionine analogs

[0042] An enzyme array to incorporate a broad spectrum of methionine analogs. To expand the SNA library, the reactivity of several methionine analogs (Figure 4C, amino-acid tail labelled 'METHIONINE') was screened toward *Mj*MAT in the presence of ATP. Since only L- and D-methionine (M0 and M1, respectively; Figure 6) were efficiently processed by *Mj*MAT, other species' MAT enzymes originally described for synthesis of sulfonium-alkyl SAM-analogs and ester derivatives were screened. MAT enzymes from *Neisseria meningitidis* (*Nm*) and *Campylobacter jejuni* (*Cj*) (Figure 4B) were expressed and purified. To demonstrate the utility of these enzymes, α -methyl-methionine (M2, Figure 6) was a substrate of *Nm*- and *Cj*MAT (Figure 6) and (*S*)-(-)-methioniol (M3; Figure 6) was an excellent substrate for *Cj*MAT.

[0043] Since reactivity was not observed toward analog M4 for any MAT enzyme, a complementary approach to the chemoenzymatic synthesis of SNA probes was used (Figure 4A, bottom enzymatic road). The unique SAM-dependent chlorinase from the marine organism *Salinispora tropica* (*St*SalL) is a key enzyme for salinosporamide A biosynthesis. Although the occurrence of high chloride concentration (*i.e.* 546 mM) in sea water shifts the chemical equilibrium away from SAM synthesis, this reaction is reversible *in vitro*. *St*SalL (Figure 4B) was expressed and purified, and the methionine analog specificity for this enzyme was determined in the presence of 5'-chloro-5'-deoxy adenosine (5'-CIDA; Figure 6). Reactivity was observed toward 2-hydroxy-4-(methylthio) butanoic acid (M4; Figure 6).

1.3 Combine the Above Tools to Produce a SAM Analog Library

[0044] Nucleophilic displacements by methionines to yield *S*-Nucleosyl Amino acid probes (SNA). Small scale reactions for each catalyst were performed using the NTP, methionine analogs and HPLC method. The best enzyme for the synthesis of each SNM is

determined including the determination of corresponding kinetic parameters (Figure 4A, production of PPi from MAT reaction) or by HPLC. Finally, the SNM analogs are produced on a larger scale, with purification by HPLC for subsequent screening. Molecules are characterized through nuclear magnetic resonance (NMR). Chemical identity is also confirmed by mass spectrometry.

[0045] *Alternative chemoenzymatic routes.* A few SNA probes are difficult to prepare. To broaden the substrate diversity, the reversible reaction catalyzed by *S*-adenosyl-L-homocysteine hydrolase from *Lupinus luteus* (LISAHH) that is displaced toward SAH production *in vitro* was used (Figure 5B). The enzyme had a poor nucleoside specificity and could utilize both L-homocysteine and L-cysteine (n=2 and n=1, respectively; Figure 5B). This powerful catalyst was a useful tool for production of *S*-nucleosyl-L-homocysteine and *S*-nucleosyl-L-cysteine analogs (SNH and SNC, respectively). Unlike SAM analogs, SNH and SNC probes are not substrates for PRMTs, so they are assayed as competitive inhibitors.

2.1 Preparation of arginine substrate acceptors for PRMT screens

[0046] SAM analogs will be assayed toward each methyltransferases and PRMT isoforms using specific arginine substrates. Nucleoplasmin has been used to test profiling of HsPRMT5-MEP50. Histone H4 is also used as a substrate for the methyltransferase catalyzed by PRMT1 and PRMT5 (H4R3me1).

2.2 Antibody detection of methylated product as a screen readout

[0047] Previously described primary antibody is used to detect the MMA mark. This antibody displays affinity toward H4R3me1, H2AR3me1 and NpmR187me1. In support of the experimental setup, the MMA antibody was used to detect the PRMT5 product NpmR187me1. The experiments are performed under typical nitrocellulose blocking/wash/incubation protocols with an ultrasensitive HRP substrate (TMA-6, Lumigen). Light output was directly quantified with a LAS4000 16-bit digital imager (GE) and had a linear response over a wide range of product concentrations, making this approach a good tool for the screen.

2.3 Competitive assay and scintillation as a screen readout

[0048] Previously described filter binding assays (*e.g.* P81 phosphocellulose) are used to separate radioactive SAM cofactor ($[^3\text{H-methyl}]\text{SAM}$ or $[^{14}\text{C-methyl}]\text{SAM}$) from the radioactive methylated product (*e.g.* small peptide or full length protein methylated at lysine or arginine residue). At pH=8 (bicarbonate buffer), the methylated radioactive product of the

methyltransferase reactions is isolated onto filter binding surface and the radioactivity is further quantified using a scintillation detector.

2.4 Profiling of PRMTs using antibody detection

[0049] *Protocol for PRMT profiling with the SAM analog library.* Briefly, buffered 50 μL reactions contain an optimized concentration of PRMT5-MEP50 (or other methyltransferase), sub-saturating levels of acceptor (*i.e.* concentration equal to K_m for the protein substrate) and 25 μM of SAM or SNA probes. Reaction samples are deposited onto nitrocellulose membrane (*e.g.* 10 μL) and these DotBlots are further analyzed for detection of the MMA mark. In addition to this first reactivity screen, a second experiment is performed using similar conditions where SAM concentration is kept constant (25 μM) and SNA probes are added to compete with the cofactor (final 125 μM concentration). Analysis with the DotBlot approach permits identification of SAM competitive inhibitors. To complete the profiling, K_m and k_{cat} are measured for substrate analogs and K_i for inhibitors (SNH or SNC probes). These kinetic parameters are determined under discontinuous conditions through detection of products (K_m , k_{cat}) or SAH (K_i) by reverse-phase UPLC.

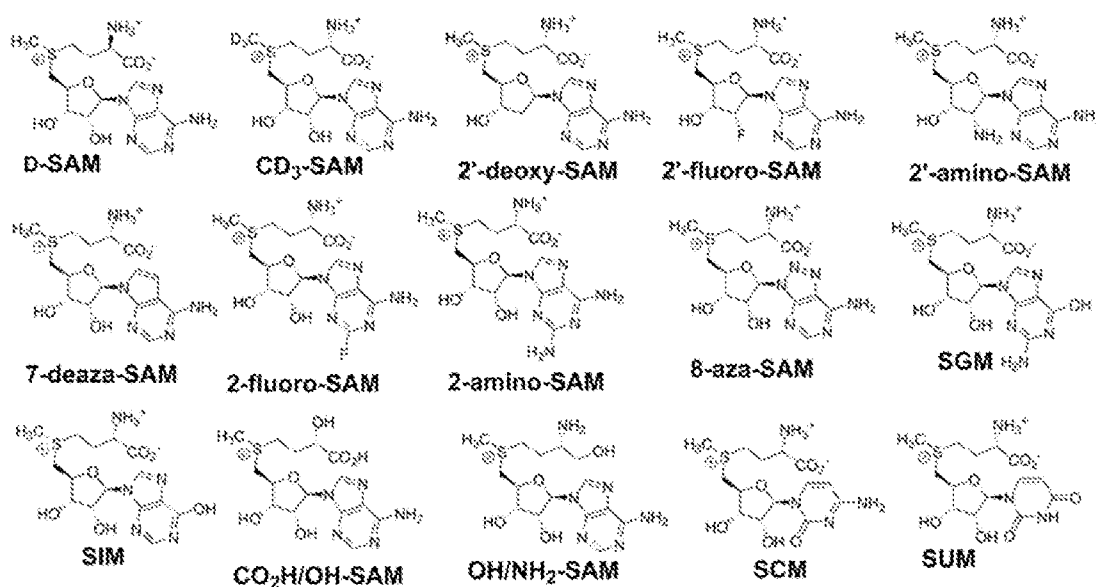
2.5 Profiling of PRMTs using filter assay and scintillation counter

[0050] *Protocol for PRMT profiling with the SAM analog library.* Briefly, buffered 50 μL reactions contain an optimized concentration of PRMT5-MEP50 (or other methyltransferase), sub-saturating levels of acceptor (*i.e.* concentration equal to K_m for the protein substrate), 25 μM of radioactive SAM ($[^3\text{H}\text{-methyl}]$ or $[^{14}\text{C}\text{-methyl}]$) and increasing concentrations of each SNA probes. Reaction samples are deposited onto filter membranes (*e.g.* P81 phosphocellulose). Membranes are further washed with bicarbonate buffer (pH=8) to remove excess radioactive SAM substrate, leaving the radioactive methylated peptide/protein product onto the filter surface. Dried-out filters are further incubated with scintillation liquid and radioactive signal is determine through scintillation counting. To complete the profiling, K_m and k_{cat} are measured for substrate analogs and K_i for inhibitors. These kinetic parameters are determined under discontinuous conditions.

Results

[0051] SAM analogs and SAH analogs have been synthesized using the enzymatic approach. These *S*-Nucleosyl Amino acid probes (SNA) are prepared with excellent yields (>70%) and purified by HPLC. Compounds are characterized using Mass Spectrometry to confirm exact mass.

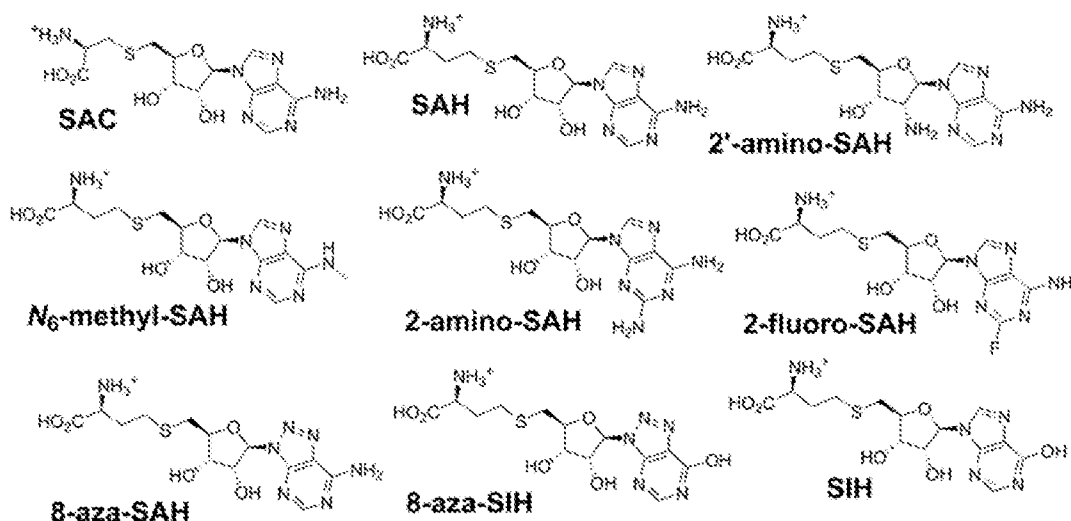
[0052] The SAM analogs are shown below:



	m/z		HPLC (C18 4.6 X 250 mm)	
	<i>predicted</i>	<i>experiment</i>	RT (min)	Abs _{max} (nm)
D-SAM	399.1445	399.1444	4.78	260
2'-deoxy-SAM	383.1496	383.1495	9.85	260
2'-fluoro-SAM	401.1402	401.1401	9.80	260
2'-amino-SAM	398.1605	398.1602	3.93	260
7-deaza-SAM	398.1493	398.1496	6.56	272
2-fluoro-SAM	417.1351	417.1343	5.85	262
2-amino-SAM	414.1554	414.1547	4.37	257/280
SGM	415.1394	415.1390	3.58	253
8-aza-SAM	400.1398	400.1398	4.67	279
SIM	400.1285	400.1289	3.16	248
CO ₂ H/OH-SAM	400.1285	400.1284	9.71	260
OH/NH ₂ -SAM	385.1653	385.1652	4.03	260
SCM	375.1333	375.1332	2.77	269
SUM	376.1173	376.1172	2.85	260

HPLC Method (Luna2 C18, 4.6X250 mm, 5 μm): Buffer A (Acetic acid and Triethylamine 100 mM, pH 6.0) and Buffer B (same as Buffer A with 30% Acetonitrile). 0-4 min, 1 mL/min 99% A; gradient to 10 min, 1 mL/min 10% A; ramp to 2 mL/min to 10.25 min; constant till 16.25 min with 10% A; maintained at 2 mL/min and return to 99% A to 16.75 min; constant till 22.75 min; ramp down to 0 mL/min at 23 min.

[0053] The SAH analogs are shown below:



	m/z		HPLC (C18 4.6 X 250 mm)	
	<i>predicted</i>	<i>experiment</i>	RT (min)	Abs _{max} (nm)
SAC	371.1132	371.1132	10.08	260
2'-amino-SAH	384.1448	384.1447	10.19	260
N ₆ -methyl-SAH	399.1445	399.1445	10.83	260
2-amino-SAH	400.1398	400.1397	10.16	257/280
2-fluoro-SAH	403.1194	403.1195	10.63	262
8-aza-SAH	386.1241	386.1242	10.24	278
8-aza-SIH	387.1081	387.1081	11.60	254
SIH	-	-	-	-

HPLC Method (Luna2 C18, 4.6X250 mm, 5 μm): Buffer A (Acetic acid and Triethylamine 100 mM, pH 6.0) and Buffer B (same as Buffer A with 30% Acetonitrile). 0-4 min, 1 mL/min 99% A; gradient to 10 min, 1 mL/min 10% A; ramp to 2 mL/min to 10.25 min; constant till 16.25 min with 10% A; maintained at 2 mL/min and return to 99% A to 16.75 min; constant till 22.75 min; ramp down to 0 mL/min at 23 min.

Discussion

[0054] Disclosed herein is an approach to rapidly produce a combinatorial collection of *S*-Nucleosyl Amino acid probes (SNA) that can serve as preferred methyl transferase substrates or inhibitors toward methyltransferases. Specifically, a fast enzymatic synthesis of a compound library of SNA probes can be produced from commercial building blocks using unique biocatalysts. The chemical probes from the initial library display a single point variation compared to the natural SAM/SAH and convolutions with 3 or more point variation can lead to a potential 10⁶ molecule library. This approach is useful to obtain inhibitors with improved isozyme specificity. Since many of these enzymes are oncogenic or otherwise

involved in human disease, such targeted compounds will enhance personalized medicine and reduce potential side effects due to off-target inhibition.

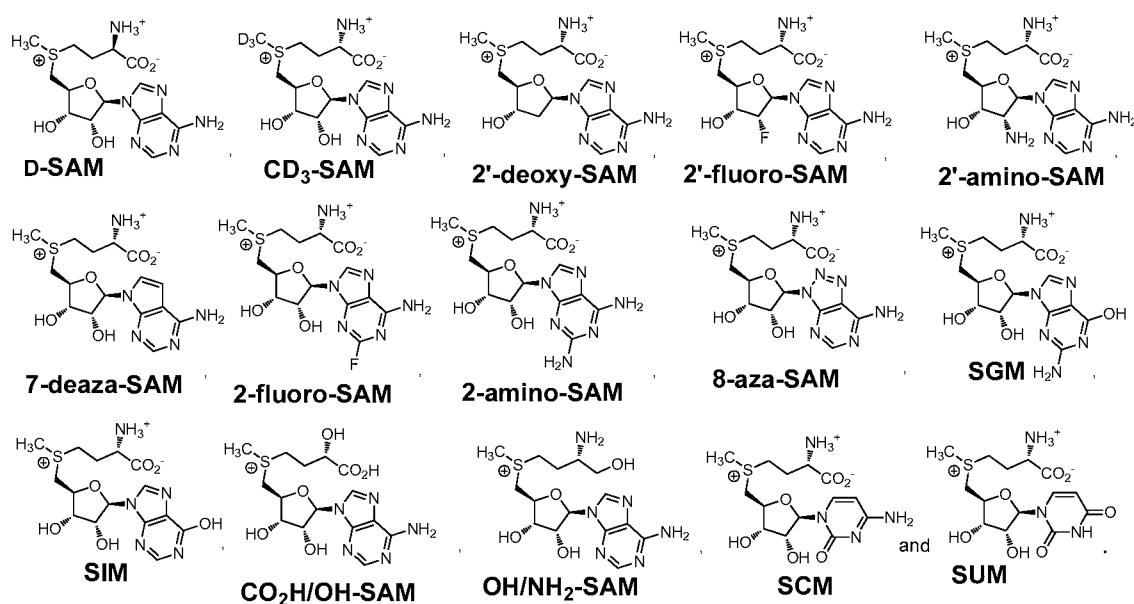
[0055] The present approach is highly innovative because the platform permits facile screening of a large chemical library to determine SAM cofactor binding specificity for methyl transferases. Small molecules targeting the cofactor binding pocket of these enzymes are likely to yield isozyme-specific inhibitors. Tedious multi-step chemical synthesis of a small number of SAM/SAH analogs was performed in the 1970s and assayed toward four SMMTs. However, these had poor yields (10-30%) with no control over their stereochemistry, showing that a synthetic chemistry approach will be difficult to accomplish. In contrast, the present use of a unique set of natural and powerful catalysts is the key to the stereoselective, cleaner and more efficient synthesis of these analogs.

What is claimed is:

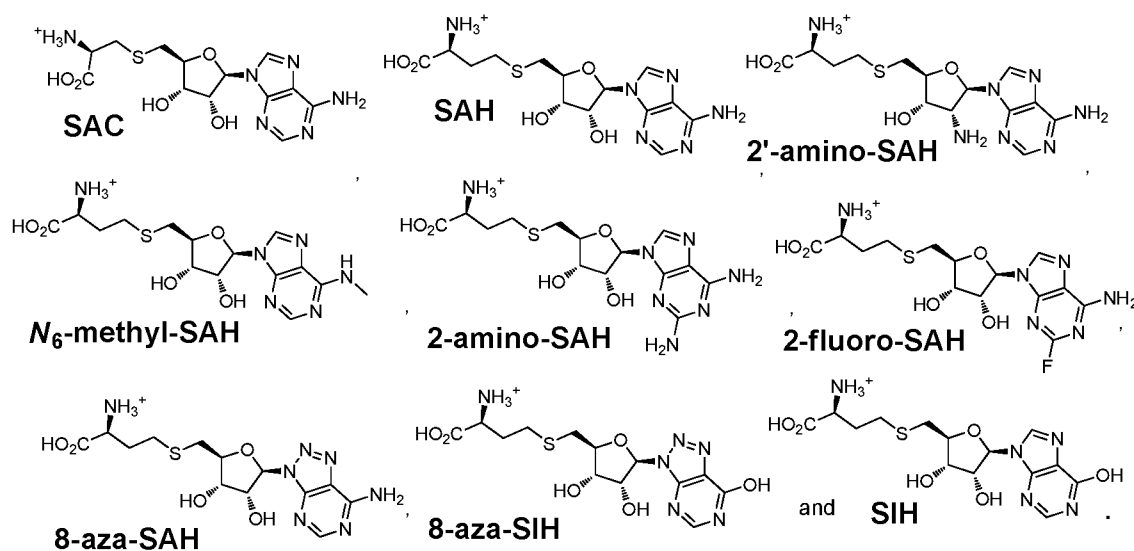
1. A method of synthesizing an analog of *S*-adenosyl-L-methionine (SAM) comprising
 - i) reacting a nucleoside or nucleoside analog with a nucleoside triphosphate in the presence of one or more of adenosine kinase, deoxynucleoside kinase and deoxycytidine kinase to form a monophosphate nucleoside analog;
 - ii) forming a triphosphate nucleoside analog by
 - a) reacting the monophosphate nucleoside analog with a nucleoside triphosphate in the presence of one or both of myokinase and cytidylate kinase to form a diphosphate nucleoside analog; and reacting the diphosphate nucleoside analog with a nucleoside triphosphate in the presence of one or both of pyruvate kinase and nucleoside-diphosphate kinase to form a triphosphate nucleoside analog; and/or
 - b) reacting the monophosphate nucleoside analog with phosphoenolpyruvic acid and pyrophosphate in the presence of pyrophosphate phosphate dikinase to form a triphosphate nucleoside analog; and
 - iii) reacting the triphosphate nucleoside analog with methionine or a methionine analog in the presence of methionine adenosyltransferase to form an analog of SAM.
2. The method of claim 1, wherein any nucleoside triphosphate is adenosine triphosphate (ATP) or cytidine triphosphate (CTP).
3. The method of claim 1 or 2, wherein the adenosine kinase is *Anopheles gambiae* adenosine kinase.
4. The method of any of claims 1-3, wherein the deoxynucleoside kinase is *Bacillus anthracis* deoxynucleoside kinase.
5. The method of any of claims 1-4, wherein the deoxycytidine kinase is *Homo sapiens* deoxycytidine kinase.
6. The method of any of claims 1-5, wherein the cytidylate kinase is *Coxiella burnetii* cytidylate kinase.

7. The method of any of claims 1-6, wherein the nucleoside-diphosphate kinase is *Coxiella burnetii* nucleoside-diphosphate kinase.
8. The method of any of claims 1-7, wherein the pyrophosphate phosphate dikinase is from *Zea mays* or *Clostridium symbiosum*.
9. The method of any of claims 1-8, wherein the methionine adenosyltransferase is from *Methanococcus jannaschii*, *Sulfolobus solfataricus*, *Neisseria meningitidis* or *Campylobacter jejuni*.
10. A method of synthesizing an analog of *S*-adenosyl-L-methionine (SAM) comprising reacting a 5'-chlorinated nucleoside analog with methionine or a methionine analog in the presence of a SAM-dependent chlorinase to form an analog of SAM.
11. The method of claim 10, wherein the SAM-dependent chlorinase is *Salinispora tropica* enzyme.
12. A method of synthesizing an analog of *S*-adenosyl-L-homocysteine (SAH) comprising reacting a nucleoside or nucleoside analog with homocysteine or an analog of homocysteine in the presence of *S*-adenosyl-L-homocysteine hydrolase.
13. The method of claim 12, wherein the analog of homocysteine is cysteine.
14. The method of claim 12 or 13, wherein the *S*-adenosyl-L-homocysteine hydrolase is from *Lupinus luteus*.
15. The method of any of claims 1-14, wherein the nucleoside or nucleoside analog is a ribonucleoside or ribonucleoside analog.
16. The method of any of claims 1-14, wherein the nucleoside or nucleoside analog is a 2'-deoxyribonucleoside or 2'-deoxyribonucleoside analog.
17. The method of any of claims 1-11, wherein the nucleoside or nucleoside analog is a 3'-deoxyribonucleoside or 3'-deoxyribonucleoside analog.

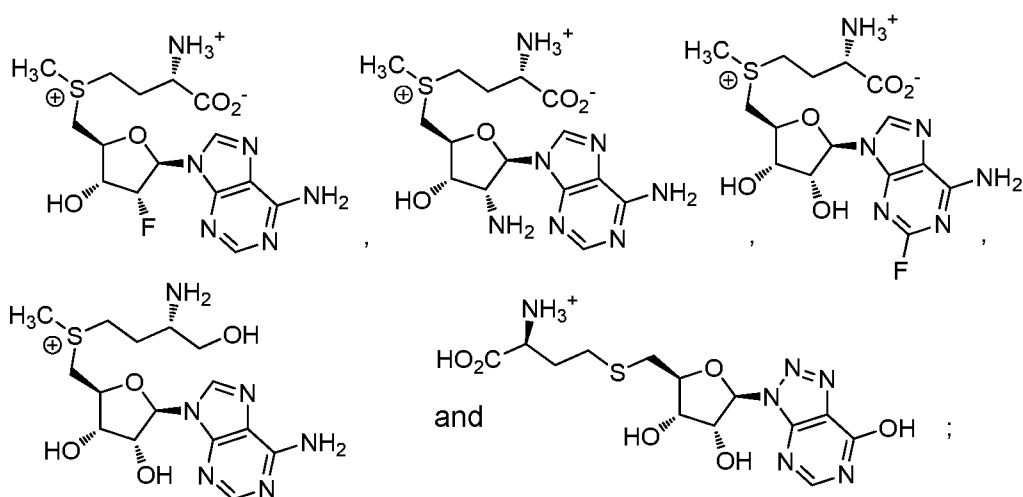
18. The method of any of claims 1-11, wherein the nucleoside or nucleoside analog is adenosine, 2'-deoxyadenosine, 3'-deoxyadenosine, an analog of adenosine, an analog of 2'-deoxyadenosine, or an analog of 3'-deoxyadenosine.
19. The method of any of claims 12-14, wherein the nucleoside or nucleoside analog is adenosine, 2'-deoxyadenosine, an analog of adenosine, or an analog of 2'-deoxyadenosine.
20. The method of any of claims 1-19, wherein the nucleoside analog is substituted with one or more of methyl, ethyl, benzyl, -NH₂, -OH, -SH, -NHNH₂, -NHOH, -NO₂, -N₃, -F, -Cl, =O and -CD₃.
21. The method of any of claims 1-14, wherein the nucleoside analog is tubercidin, vidarabine, 2-amino adenosine, 2-fluoro adenosine, 2'-fluoro-2'-deoxy adenosine, 2'-amino-2'-deoxy adenosine or *N*₆-methyl adenosine.
22. The method of any of claims 1-21, wherein one or more of adenosine kinase, deoxynucleoside kinase, deoxycytidine kinase, myokinase, cytidylate kinase, pyruvate kinase, nucleoside-diphosphate kinase, pyrophosphate phosphate dikinase, methionine adenosyltransferase, SAM-dependent chlorinase and *S*-adenosyl-L-homocysteine hydrolase is a recombinant enzyme expressed in *E. coli*.
23. The method of any of claims 1-11 or 15-22, wherein the SAM analog is selected from the group consisting of



24. The method of any of claims 12-22, wherein the SAH analog is selected from the group consisting of



25. A compound selected from the group consisting of



or a pharmaceutically acceptable salt thereof.

26. A pharmaceutical composition comprising one or more of the compounds of claim 25, and a pharmaceutically acceptable carrier.

27. A method of treating a cancer in a subject comprising administering to the subject one or more of the compounds of claim 25 in an amount effective to treat a cancer in a subject.

28. The method of claim 27, wherein the compound is administered in an amount effective to inhibit a methyltransferase involved in cancer development or progression in a subject.

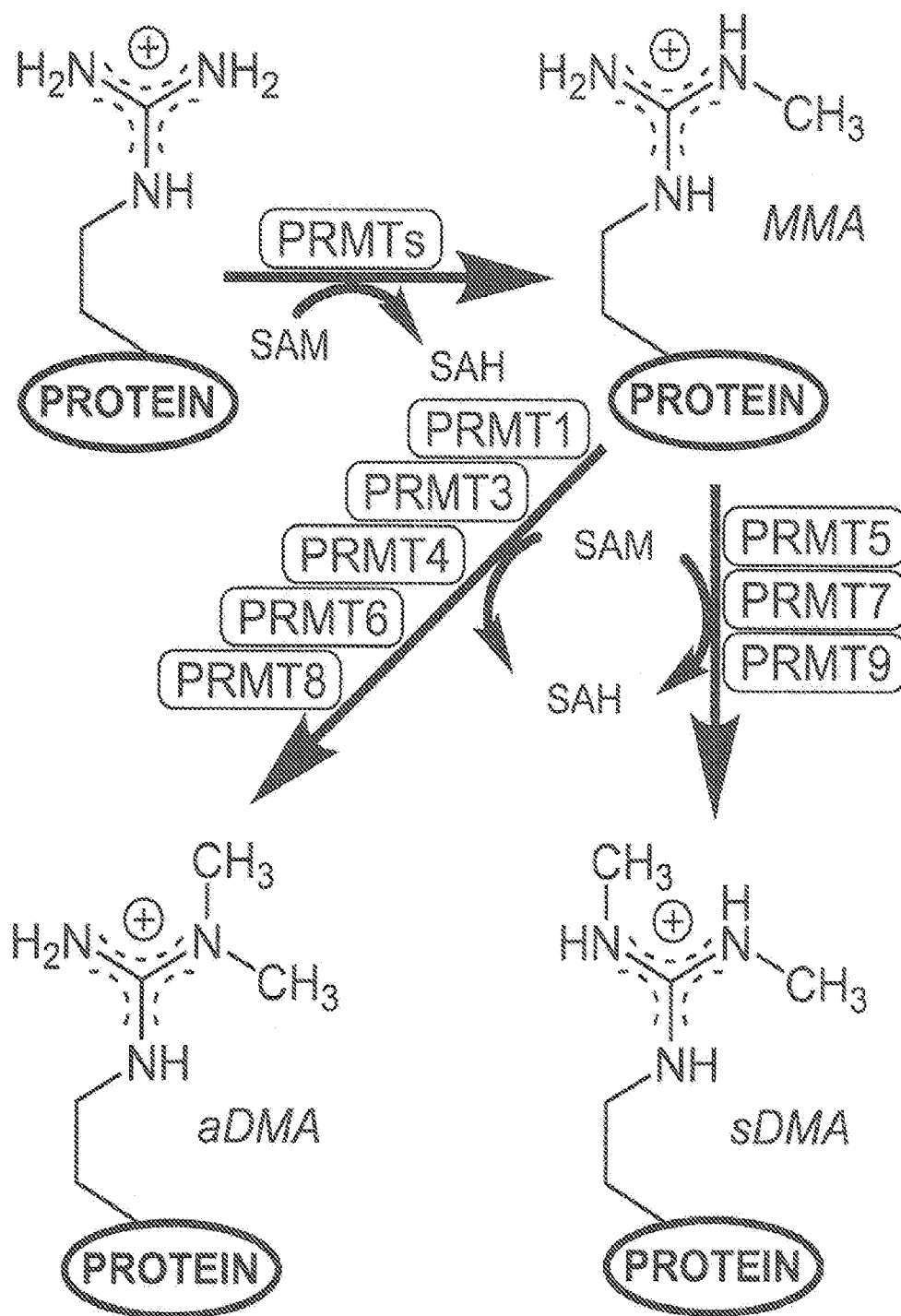
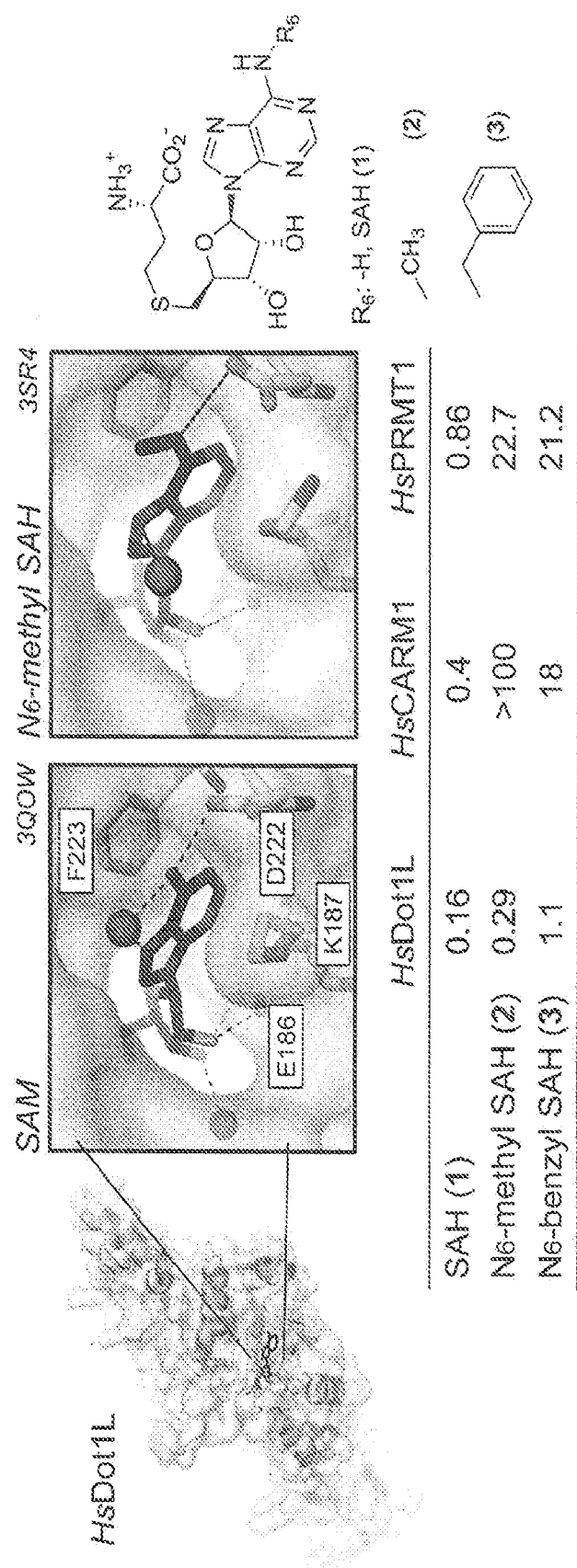


FIG. 1



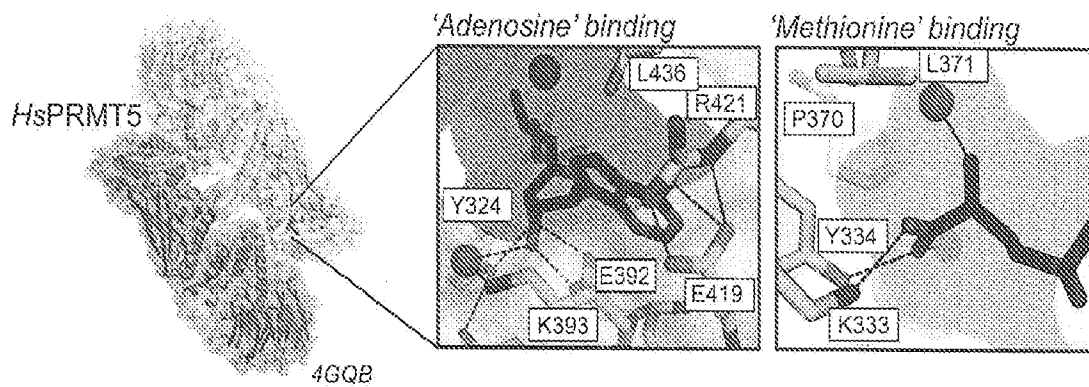


FIG. 3A

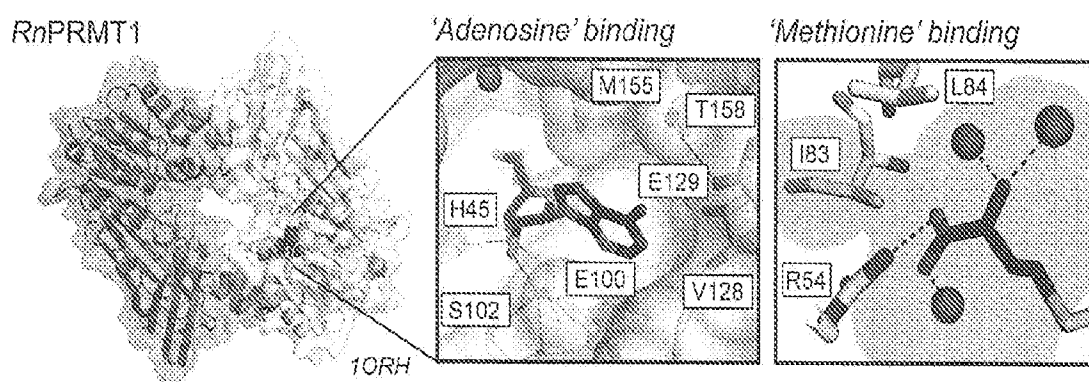


FIG. 3B

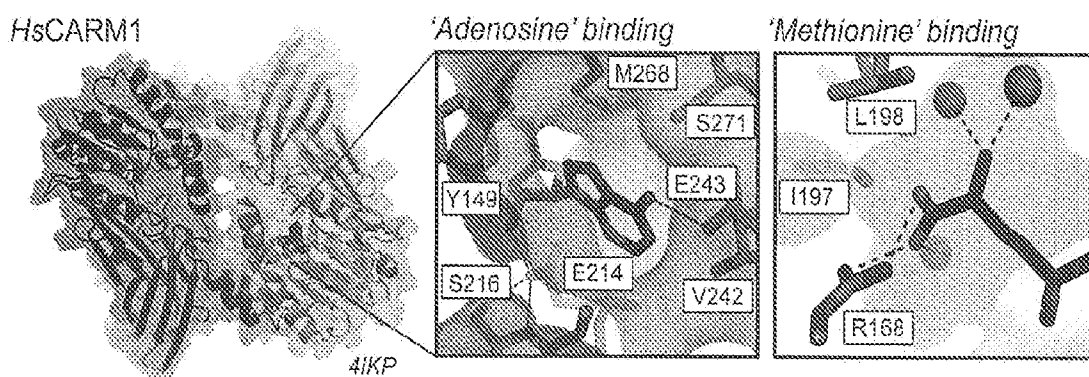


FIG. 3C

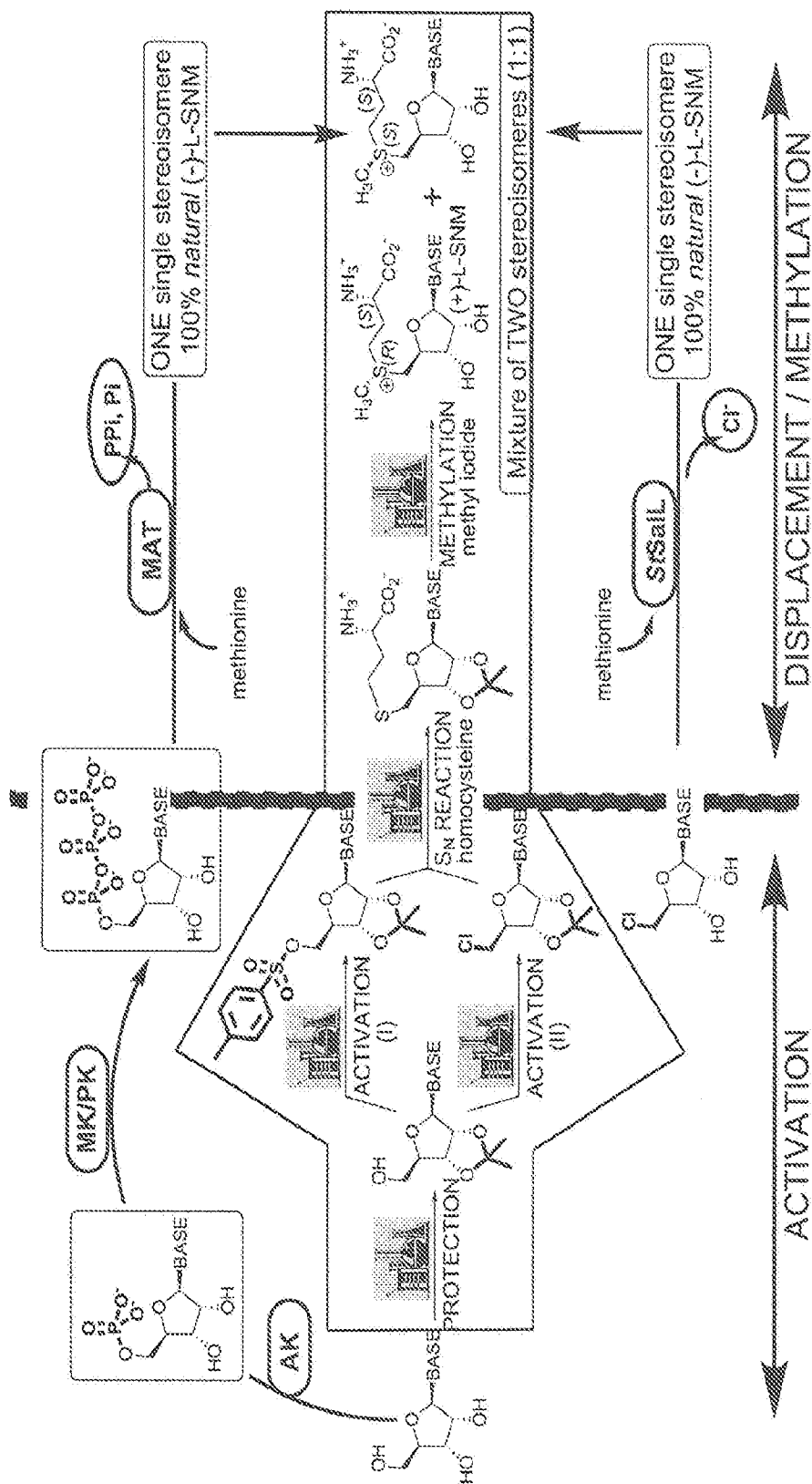


FIG. 4A

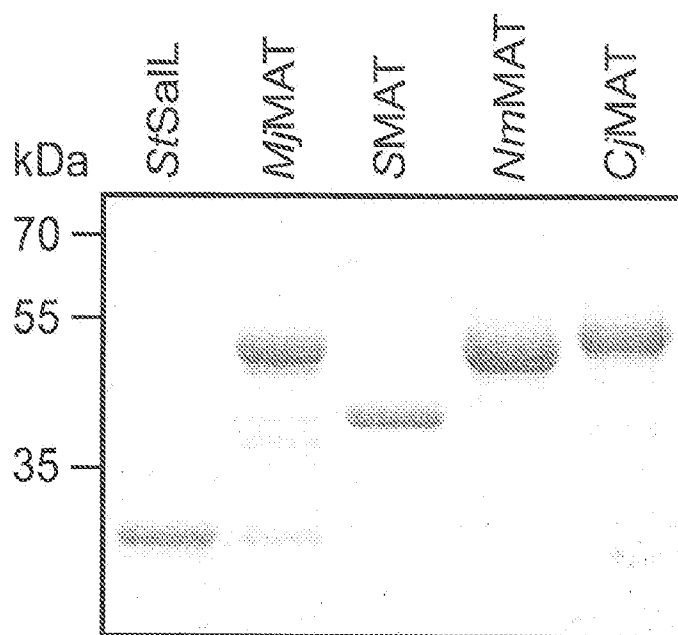


FIG. 4B

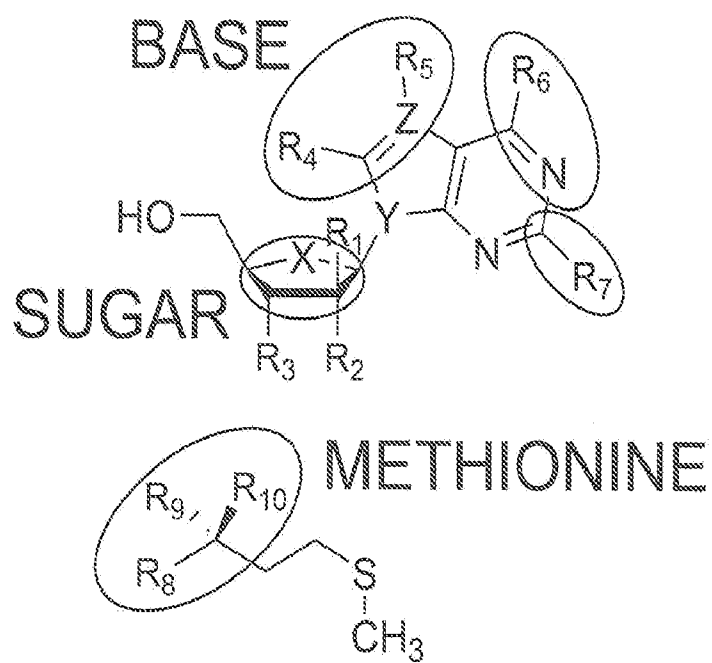
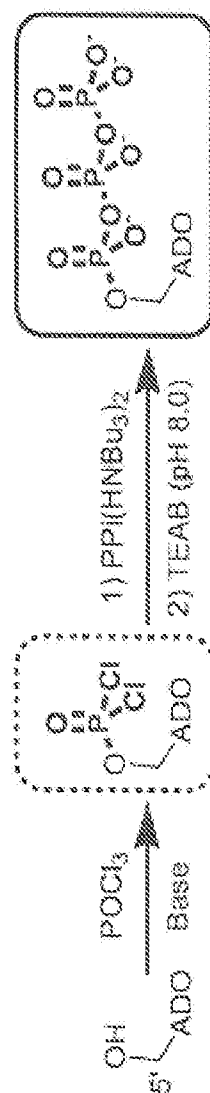
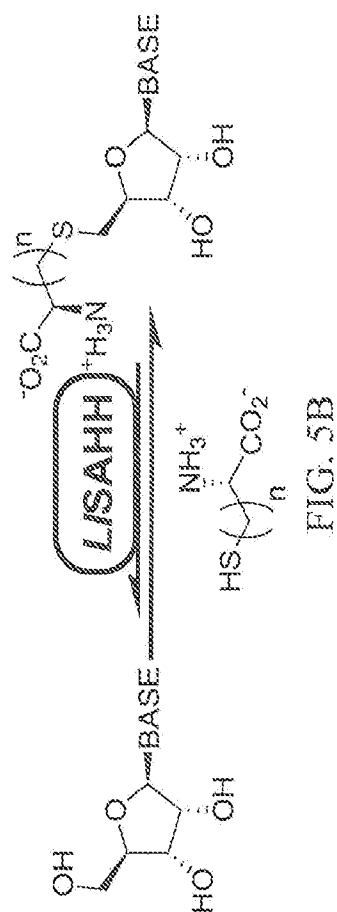
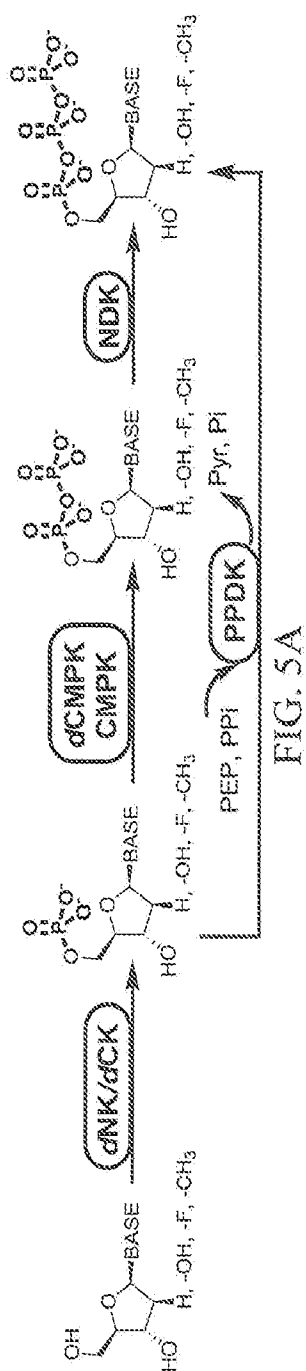


FIG. 4C



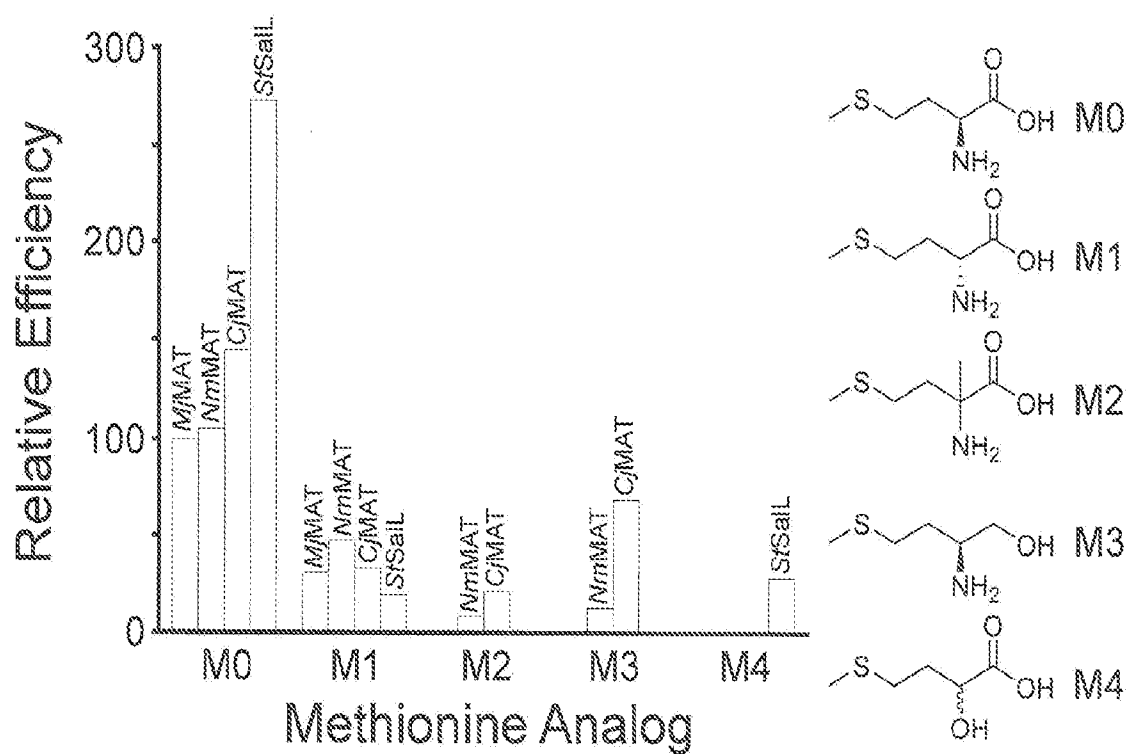


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/014804

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/519; A61K 31/525; A61K 31/4415; C12N 9/14; C12Q 1/34; C12Q 1/48 (2017.01)

CPC - A61K 31/519; A61K 31/525; A61K 31/4415; C12N 9/14; C12Q 1/34; C12Q 1/48 (2017.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,605,625 A (YAMADA et al) 12 August 1986 (12.08.1986) entire document	12, 13
A	US 2009/0197310 A1 (LAM et al) 06 August 2009 (06.08.2009) entire document	1-3, 10-14
A	WO 2015/200680 A2 (EPIZYME INC) 30 December 2015 (30.12.2015) entire document	1-3, 10-14
A	US 4,609,626 A (YAMADA et al) 02 September 1986 (02.09.1986) entire document	1-3, 10-14

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 May 2017

Date of mailing of the international search report

06 JUN 2017

Name and mailing address of the ISA/US

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PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/014804

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-9, 15-24
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Extra Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 10-14

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/014804

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I: claims 1-3 and 10-14 are drawn to methods of synthesizing an analog of S-adenosyl-L-methionine (SAM).

Group II: claims 25-28 are drawn to compounds of the instant invention, pharmaceutical compositions thereof, and methods of treating cancer in a subject.

The inventions listed in Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I, methods of synthesizing an analog of S-adenosyl-L-methionine (SAM), are not present in Group II; and the special technical features of Group II, compounds of the instant invention, pharmaceutical compositions thereof, and methods of treating cancer in a subject, are not present in Group I.

The Groups I and II share the technical features of an analog of S-adenosyl-L-methionine (SAM). However, these shared technical features do not represent a contribution over the prior art.

Specifically, WO 2015/200680 A2 to Epizyme Inc. teaches an analog of S-adenosyl-L-methionine (SAM) (Para. [0524],... S-adenosylhomocystein (SAH)...).

The inventions listed in Groups I and II therefore lack unity under Rule 13 because they do not share a same or corresponding special technical feature.