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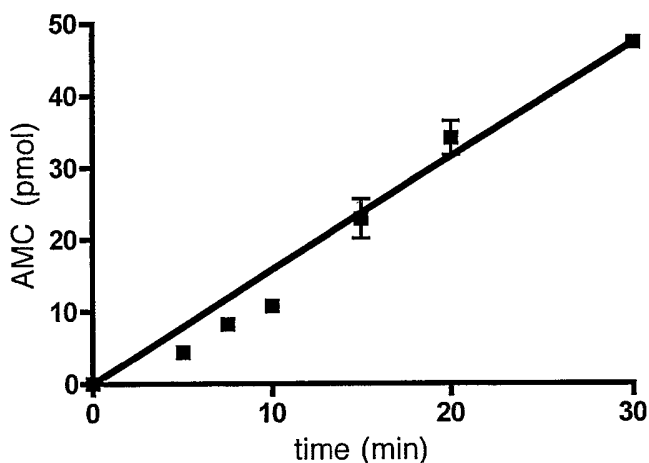
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(54) Title: COMPOUNDS USEFUL AS SUBSTRATES FOR ENZYMES THAT REVERSE NUCLEOTIDYLATED PEPTIDE AND PROTEIN MODIFICATIONS AND METHODS FOR PRODUCTION AND USES THEREOF



(57) Abstract: Compounds which act as substrates for Hint hydrolases and other enzymes that reverse nucleotidylylated peptide and protein modifications and methods for production of these substrates are provided. Also provided are methods for use of these compounds to determine activity of Hint hydrolases and other enzymes that reverse nucleotidylylated peptide and protein modifications and to diagnosis disorders associated with mutated or altered forms of these enzymes.

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Compounds Useful as Substrates for Enzymes that Reverse Nucleotidylylated Peptide and Protein Modifications and Methods for Production and Uses Thereof

5 Introduction

This invention claims the benefit of priority to U.S. Provisional Patent Application Serial No. 60/618,808, filed October 14, 2004, the teachings of which are herein incorporated by reference in their entirety.

This invention was supported in part by funds from the U.S. government
10 (NCI/NIH Grant No. CA75954). The U.S. government may therefore have certain rights in the invention.

Field of the Invention

Hint hydrolases and homologs thereof hydrolyze adenosine monophosphoramide
15 and guanosine monophosphoramide substrates. Activity of these enzymes is believed to be important in neurological function, avian sex determination, and in activation of nucleoside prodrugs. The present invention provides new substrates for Hint hydrolases and other enzymes that reverse nucleotidylylated peptide and protein modifications and methods for production of these substrates. The present invention also provides sensitive
20 quantitative assays for determining activity of Hint hydrolases and other enzymes that reverse nucleotidylylated peptide and protein modifications and for diagnosing disorders associated with these enzymes.

Background of the Invention

25 Hint is a homodimer of approximately 14 kDa subunits that functions as an AMP-lysine hydrolase and positive regulator of Kin28 in yeast, whose most conserved amino acids form the dimer interface and the substrate binding-site (Brenner et al. Nature Struct Biol 1997 4:231-238; Bieganowski et al. J Biol Chem 2002 277:10852-10860; Krakowiak et al. J Biol Chem 2004 279:18711-18716). The Hint active site consists of mostly
30 nonpolar residues that contribute to adenosine binding (Brenner et al. Nature Struct Biol 1997 4:231-238), the histidine that forms a phosphoramide with the substrate α -phosphate (Lima et al. Science 1997 278:286-290), Ser107, which interacts with the leaving group amine (Krakowiak et al. J Biol Chem 2004 279:18711-18716), and Trp123, which

interacts with the alkyl portion of the lysine leaving group across the dimer interface (Krakowiak et al. J Biol Chem 2004 279:18711-18716). Enzymes similar to rabbit Hint 1, including human Hint1, Hint2, Hint3, aprataxin and homologs thereof in other life forms, possess the biochemical activity of hydrolysis of adenosine monophosphoramidate (AMP) substrates and guanosine monophosphoramidate (GMP) substrates such as AMP-lysine and GMP-lysine.

A distinct subset of AMP and/or GMP modified proteins have been identified including, but in no way limited to, yeast Kin28, mammalian Cdk7 and mammalian Xrcc1. Further, ataxia, oculomotor apraxia, the second leading cause of autosomal recessive ataxia is caused by loss of function mutations in a Hint hydrolase (Brenner, C. Biochemistry 2002 41(29):9003-14). Thus, it is believed that protein deadenylation and/or protein deguanlylation are important in neurological function.

The genomics of Hint genes is also indicative of a role of Hint in avian sex determination (Pace, H.C. and Brenner, C. Genome Biol. 2003 4(3):R18.

In addition, there are a variety of nucleoside prodrugs such as AZT that must be converted to nucleoside triphosphates such as AZTTP. However, phosphorylation of the nucleoside to the monophosphate is limiting in the maturation of nucleoside prodrugs. Further, nucleotides such as AZTMP, AZTDP and AZTTP are not transported through the plasma membrane. Thus, researchers in this area have produced monophosphate-amino acid and nucleoside monophosphate-peptide conjugates which are transportable and which make nucleoside monophosphates available inside cells. It is believed that Hint hydrolases are the enzymes responsible for converting these conjugates to the corresponding nucleotides monophosphates.

Thus, assays and components used in assays to measure activity of Hint hydrolases and other enzymes that reverse nucleotidylated peptide and protein modifications are needed to further characterize these enzymes and their roles in biological processes, to identify modulators of these enzymes, and to identify agents, activity of which is modulated by these enzymes.

Summary of the Invention

One aspect of the present invention relates to a compound which acts as a substrate for enzymes that reverse nucleotidylated peptide and protein modifications comprising a nucleoside monophosphate moiety, a peptidyl moiety or other polypeptide sequence

containing a lysine and linked by phosphoramidate linkage to the nucleoside monophosphate moiety, and a reporter group linked by amide or ester linkage to the peptidyl moiety or polypeptide sequence.

Another aspect of the present invention relates to a method for synthesis of a
5 compound of the present invention which comprises linking by phosphoramidate linkage a peptidyl moiety or other polypeptide sequence containing a lysine to a nucleoside monophosphate moiety and linking by amide or ester linkage a reporter group to the peptidyl moiety or other polypeptide sequence.

Another aspect of the present invention relates to an assay to quantify activity of
10 enzymes that reverse nucleotidylated peptide and protein modifications in a sample which comprises incubating a compound of the present invention with the enzyme and measuring any resulting cleavage product of compound following the incubation.

Another aspect of the present invention relates to a method for diagnosing a disorder associated with an enzyme that reverses nucleotidylated peptide and protein
15 modifications which comprises assessing activity of the enzyme in a sample obtained from a subject suspected of suffering from the disorder using a compound of the present invention.

Another aspect of the present invention relates to assay kits for quantifying activity of and/or diagnosing a disorder associated with an enzyme that reverses nucleotidylated
20 peptide and protein modifications and diagnosing disorders associated with these enzymes, said kits comprising a compound of the present invention. Assay kits of the present invention may further comprise a protease that cleaves carboxyl to lysine and which does not recognize nucleotidylated lysine, an enzyme standard or standards, reagents for detection of the reporter group of the compound and/or instructions for use of the assay
25 kit.

Brief Description of the Figures

Figure 1 is a line graph showing time dependence of hydrolysis of an exemplary compound of the present invention, the substrate tBoc-AMPLys-AMC (15 μ M) by 1.95
30 fmol wild-type Hint.

Figure 2 is a line graph showing Hint concentration-dependence of 15-minute incubations with an exemplary compound of the present invention, the substrate tBoc-AMPLys-AMC.

Figure 3 is a bar graph showing the salutary effect of 1 mM EDTA in assays with an exemplary compound of the present invention, the substrate tBoc-AMPLys-AMC (15 μ M), performed with 1.95 fmol wild-type Hint for 15 minutes with the following additives shown from left to right on the X-axis of the bar graph: control
5 with no additive, 1 mM MgCl₂, 1 mM MgCl₂ + EDTA, 1 mM MnCl₂, 1 mM MnCl₂ + 3 mM EDTA, 1 mM EDTA, and 3 mM EDTA.

Detailed Description of the Invention

The present invention relates to compounds which act as substrates for enzymes
10 that reverse nucleotidylated peptide and protein modifications. Examples of such enzymes include, but are in no way limited to rabbit Hint1, human Hint1, human Hint2, human Hint3, aprataxin and homologs thereof in other life forms. In simplest form, a compound of the present invention comprises a nucleoside monophosphate moiety, a peptidyl moiety or polypeptide sequence containing a lysine and a reporter group.
15 Preferred is a compound wherein the nucleoside monophosphate moiety is linked via phosphoramidate linkage to the peptidyl moiety or polypeptide sequence at the lysine ϵ amino group. Also preferred is a compound wherein the reporter group is a fluorogenic or chromogenic reporter group linked by amide linkage to the carbonyl carbon of the C-terminal amino acid of the peptidyl moiety or other polypeptide sequence.

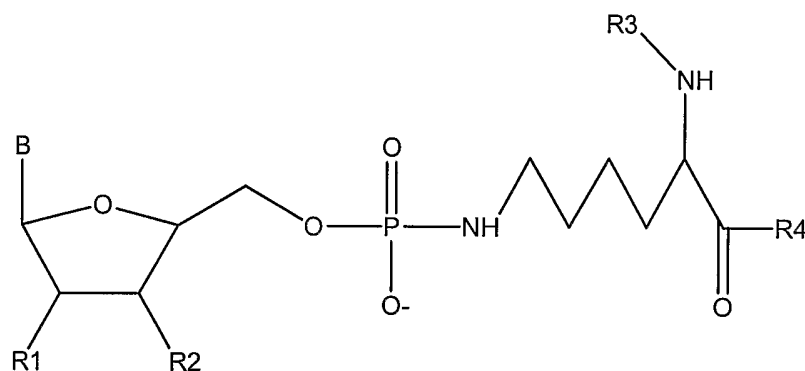
20 Examples of nucleoside monophosphate moieties useful in the present invention include, but are not limited to, adenosine monophosphoramidate (AMP), Tyr-AMP, guanosine monophosphoramidate (GMP), cytidine monophosphoramidate (CMP), thymidine monophosphoramidate (TMP), uridine monophosphoramidate (UMP), deoxyadenosine monophosphate (dAMP), azidothymidine monophosphate (AZTMP), and dideoxyinosine
25 monophosphate (ddIMP).

Example of peptidyl moieties containing a lysine and useful in the present invention include, but are in no way limited to N-Ac-lysine, tert-butoxycarbonyl (tBoc)lysine, fluoromethyloxycarbonyl (fMoc) lysine and peptide-lysine.

Any reporter group with an amine or ester linkage that can be hydrolyzed by a
30 protease and give a specific signal can be used in the present invention. Exemplary reporter groups, which can be used in the compounds of the present invention, include, but are in no way limited to, aminomethylcoumarin (AMC), 7-amino-4-carbamoylmethylcoumarin (ACC; Harris et al. Proc Natl Acad Sci U S A.

2000;97(14):7754-9), and paranitroaniline. Further, as will be understood by those skilled in the art upon reading this disclosure, alternative reporter groups or signaling systems may be incorporated into the compounds of the present invention. For example, in one embodiment, the reporter group or signaling system may be a FRET based system wherein a fluorophore such as fluorescein is placed at one end of the compound and a quencher which absorbs the fluorescent energy when in proximity to the fluorophore is placed at the other end of the compound. Upon cleavage of the compound by a Hint hydrolase or another enzyme that recognizes nucleotidylated peptides the fluorophore will be separated from the quencher thus producing a fluorescent signal.

In one embodiment, compounds of the present invention share a general formula as depicted in Formula I



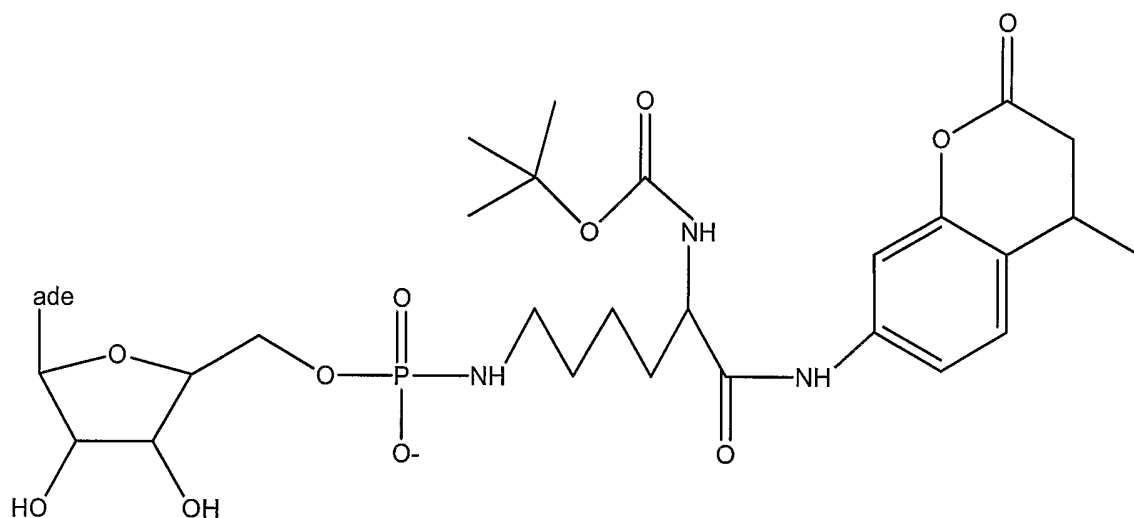
wherein B is a base such as adenine, guanine, cytosine, thymine, uracil or hypoxanthine;

R₁ and R₂ are each selected from the group consisting of H, OH and N₃;

R₃ is selected from the group consisting of H or a peptide and/or a blocking group such as tBoc, fMoc or alpha-n-acetyl; and

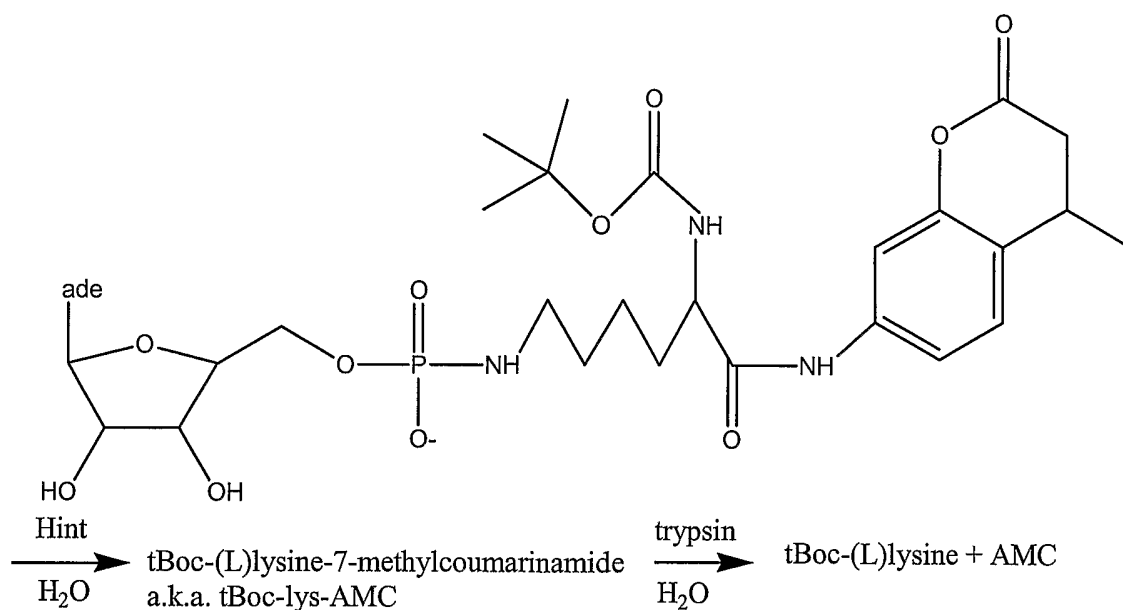
R₄ is a reporter group or a reporter group linked to a peptide or peptide-like group.

An exemplary compound of the present invention is depicted herein in Formula II:



Formula II

and is referred to herein as N-tBoc-(L)lysine- ϵ -AMP-7-methylcoumarinamide or tBoc-
 5 (lys-AMP)-AMC. Hydrolysis of this substrate by a Hint enzyme is depicted in the following Scheme I.



Scheme I

10 Additional exemplary compounds of the present invention include, but are in no way limited to tBoc-Lys-AMP-AMC, fMoc-Lys-AMP-AMC, tBoc, Lys, GMP, AMC, peptide-Lys-AMP-AMC, tBoc-Lys-ddIMP-AMC, tBoc-Lys-AZTMP-AMC, and tBoc-Lys-AZTMP-ACC.

In an alternative embodiment, the reporter group may comprise a signaling system such as FRET and may comprise reporter components at each end of the compound.

The compounds of the present invention provide useful substrates for Hint hydrolases and other enzymes that recognize nucleotidylylated peptides such as AMP-lysine, GMP-lysine and so forth and/or reverse nucleotidylylated peptide and protein modifications. The compounds of the present invention have low fluorescent and/or spectroscopic signals at wavelengths of interest, namely those wavelengths wherein the enzymatically cleaved reporter group is detectable. Further, because of the peptide's nucleotidylylation, these compounds are extremely poor substrates for specific proteases that cleave carboxyl to lysine and which do not recognize nucleotidylylated lysine. Examples of such specific proteases include, but are in no way limited to trypsin, plasmin, thrombin, endopeptidase Lys-C and Kex2. Thus, nonspecific release of the reporter group from the compound should not occur. Specific hydrolyzation of the substrate compound by a Hint enzyme or another enzyme that reverses nucleotidylylated peptide and protein modifications, however, results in a cleavage product of the denucleotidylylated peptide-reporter conjugate which provides an excellent substrate for specific proteases that cleave .

Thus, the present invention also provides useful assays and assay kits and components used in assays and assays kits to measure activity of Hint hydrolases and other enzymes that reverse nucleotidylylated peptide and protein modifications. Such assays and assays kits are useful in further characterization of these enzymes and their roles in biological processes, identification of modulators of these enzymes, and identification of agents, activity of which is modulated by these enzymes.

In one embodiment, a continuous assay of activity of a Hint hydrolase or another enzyme that reverses nucleotidylylated peptide and protein modifications is provided. In this embodiment, a compound of the present invention is incubated in a reaction mixture comprising buffer at a pH of between 5 and 8.5 containing a nonlimiting amount of a specific protease that cleaves carboxyl to lysine and which does not recognize nucleotidylylated lysine and a Hint hydrolase or another enzyme that reverses nucleotidylylated peptide and protein modifications. Concentrations of the compound used will preferably range from about 1 to about 500 μ M. Concentrations of the enzyme reversing nucleotidylylated peptide and protein modifications will preferably range between about 0.1 and about 100 fmol. Incubation times preferably ranges from about 1 minute up to 2 hours. The specific protease is preferably used in a final concentration of

about 10 to about 100 $\mu\text{g}/\mu\text{l}$. An increase in fluorescence is indicative of release of the reporter group concomitant with hydrolysis of the denucleotidylylated peptide-reporter conjugate by the specific protease following cleavage of the compound of the present invention by the Hint hydrolase or other enzyme that reverses nucleotidylylated peptide and protein modifications. Release of the reporter group thus provides an indication of the activity of the Hint hydrolase or other enzyme that reverses nucleotidylylated peptide and protein modifications.

In another embodiment, an endpoint assay of activity of a Hint hydrolase or another enzyme that reverses nucleotidylylated peptide and protein modifications is provided. In this assay, a compound of the present invention is incubated with a Hint hydrolase and other enzyme that reverses nucleotidylylated peptide and protein modifications. Similar concentrations for compound, enzyme and specific protease and similar incubation times to those described above are used. The Hint hydrolase or other enzyme that reverses nucleotidylylated peptide and protein modifications will produce a cleavage product of the denucleotidylylated peptide-reporter conjugate. The enzymatic reaction is terminated after the desired incubation period. Various means for termination can be used including, but not limited to, addition of an alkali, zinc, or an enzyme inhibitor or denaturant or by elevating the temperature of the reaction mixture to deactivate and/or denature the enzyme. The terminated assays are then incubated with a specific protease such as trypsin, plasmin, thrombin, endopeptidase Lys-C or Kex2 which liberates the reporter group from the cleaved denucleotidylylated peptide-reporter conjugate and the amount of Hint hydrolase or other enzyme that reverses nucleotidylylated peptide and protein modifications is quantified based upon the amount of reporter group produced by digestion by the protease. An exemplary endpoint assay and optimal conditions for this exemplary assay are set forth herein in Example 2.

In embodiments wherein the reporter group of the compound comprises a FRET based signaling system further incubations with a specific protease to produce the fluorescent signal is not required.

The assays and kits of the present invention are useful in quantifying amounts of Hint hydrolase in a sample and in assessing the activity of a Hint hydrolase in the presence or absence of an agent suspected of modulating activity of Hint hydrolase. Such assays and kits are also useful in identifying potential prodrugs by examining their ability to compete with the compounds of the present invention for the enzyme.

Compounds of the present invention are also useful in further characterizing Hint hydrolases and other enzymes that reverse nucleotidylated peptide and protein modifications as well as mutants thereof.

For example, the AMP-lysine hydrolase activity of wild-type chicken Hint and the
5 Hint-W123Q mutant was compared using the compound of Formula I, tBoc-LysAMP-AMC.

In these experiments, Hint incubations to liberate tBoc-Lys-AMC were coupled to tryptic digestion and Hint activity was quantitated based upon AMC release. It was found that wild-type chicken Hint hydrolyzed the compound of Formula I (tBoc-LysAMP-AMC)
10 with a k_{cat} of 24 s^{-1} and a K_m of $6.1 \text{ }\mu\text{M}$ (specificity constant = $3,950,000 \text{ M}^{-1} \text{ s}^{-1}$). Consistent with a dominant negative role for the C-terminal Gln of Asw, the Hint-W123Q substitution depressed k_{cat} nine-fold (to 2.6 s^{-1}) and increased K_m two-fold (to $11 \text{ }\mu\text{M}$) for an overall 17-fold decline in k_{cat}/K_m ($229,000 \text{ M}^{-1} \text{ s}^{-1}$). Because an Asw-Hint heterodimer would have only a single Hint active site per dimer, the predicted depression in AMP-
15 lysine hydrolytic activity is > 30 -fold.

In contrast, in experiments with a bulkier adenylylated phosphoramidate substrate, which is followed spectroscopically and is a poor substrate for wild-type rabbit Hint (Krakowiak et al. J Biol Chem 2004 279:18711-18716), wild-type k_{cat} was depressed 470-fold (to 0.051 s^{-1}) and K_m was elevated 15-fold (to $93 \text{ }\mu\text{M}$) for an overall specificity
20 constant of $550 \text{ M}^{-1} \text{ s}^{-1}$ with AMP-pNA. However, Hint-W123Q exhibited 160-fold superior AMP-pNA hydrolase compared to wild-type Hint ($k_{cat} = 1.1 \text{ s}^{-1}$; $K_m = 13 \text{ }\mu\text{M}$; $k_{cat}/K_m = 88,000 \text{ M}^{-1} \text{ s}^{-1}$). The 17-fold depressed AMP-lysine hydrolase activity coupled with 160-fold superior AMP-pNA hydrolase activity represents a 2,700-fold alteration of specificity resulted from a single amino acid substitution. This significant alteration in
25 substrate specificity, demonstrated using compounds and assays of the present invention, is indicative of a mechanistic role for Asw's C-terminal Gln in feminization of developing birds.

Compounds of the present invention were also used to investigate the link between Aprataxin inactivation and ataxia-oculomotor apraxia syndrome 1. Ataxia-oculomotor
30 apraxia syndrome 1 is an early onset cerebellar ataxia that results from loss of function mutations in the *APTX* gene encoding Aprataxin. Aprataxin contains three conserved domains, the forkhead-associated domain which mediates protein-protein interactions with

molecules that respond to DNA damage, a histidine triad domain that is similar to Hint, and a protein NH₂-terminal to a zinc finger.

Using compounds of the present invention it was demonstrated that Aprataxin possesses an active-site-dependent AMP-lysine and GMP-lysine hydrolase activity that depends additionally on the zinc finger for protein stability and on the forkhead associated domain for enzymatic activity. For these experiments, the 342-amino acid *APT*X cDNA was fused to an amino-terminal His-tag, and the resulting protein was purified to homogeneity by metal chelate affinity chromatography. The resulting enzyme was assayed for activity with t-Boc-AMPLys-MCA and t-Boc-GMPLys-MCA, as well as the Fhit substrates GpppBODIPY (Draganescu et al. J. Biol. Chem 2000 275:4555-4560), ApppA, and AppppA. To avoid contamination by the *E. coli* Hint hydrolase, the *APT*X construct was expressed in and purified from *E. coli* strain BB2, which contains a deletion for the Hint-homologous *hinT* gene (Chou et al. J. Biol. Chem 2005280:15356-15361).

Aprataxin exhibited strong k_{cat} discrimination against hydrolysis of the dinucleoside polyphosphates ApppA and AppppA and the dinucleoside polyphosphate analog GpppBODIPY, with 30-, 3-, and 7-fold lower turnover rates with these compounds, respectively, than the turnover rates against substrates with nucleotidylated lysine residues. The enzyme exhibited relatively less K_m discrimination between the nucleotide substrates presented to it and, in fact, displayed a preference for the dinucleoside polyphosphates and their analogs (13 to 39 μ M) versus substrates containing nucleotidylated lysines (47 and 116 μ M). The relatively high K_m values for t-Boc-LysAMP-MCA and t-Boc-LysGMP-MCA did not offset their advantages in the k_{cat} term. As shown in Table III (Example 7), on the basis of the specificity constant, k_{cat}/K_m , Aprataxin is an AMPLysine hydrolase whose activity is limited by the relatively high K_m values of the model peptide substrates presented to it.

Using a compound of the present invention, alleles carrying any of eight recessive mutations associated with ataxia and oculomotor apraxia were found to encode proteins with huge losses in protein stability and enzymatic activity, consistent with a null phenotype. The mild presentation allele, *APT*X-K197Q, associated with ataxia but not oculomotor apraxia was found to encode a protein with a mild defect in stability and activity, while enzyme encoded by the atypical presentation allele, *APT*X-R199H, retained substantial function, consistent with altered and not loss of activity. These data generated using compounds of the present invention suggest that an essential function of Aprataxin

is reversal of nucleotidylylated protein modifications, that all three domains contribute to formation of a stable enzyme, and that the in vitro behavior of cloned APTX alleles can score disease-associated mutations.

Ataxia can be caused by various syndromes. As demonstrated by the above-
5 described experiments, compounds of the present invention provide a useful means for differentially diagnosing ataxia in a subject as caused by ataxia-oculomotor apraxia syndrome 1 based upon a loss in enzymatic activity of apraxia in the subject. Diagnostic methods utilizing the compounds of the present invention for other disorders associated with enzymes that reverse nucleotidylylated peptide and protein modifications that have
10 been altered or mutated can be routinely developed in a similar manner based upon the teachings herein. Such methods will generally comprise obtaining an enzyme sample from a subject suspected of suffering from a disorder associated with mutant or altered enzymes that reverse nucleotidylylated peptide and protein modifications. The enzyme sample is then incubated with a compound of the present invention under conditions which
15 promote cleavage of a denucleotidylylated peptide-reporter conjugate from the compound. The denucleotidylylated peptide-reporter conjugate cleavage product is then incubated with a specific protease which cleaves carboxyl to lysine but does not recognizing nucleotidylylated lysine so that the reporter group is cleaved from the conjugate. The amount of cleaved reporter group in the enzyme sample of the subject is then compared to
20 that measured for normal control enzyme subjected to the same incubation procedure. A difference in measured amounts of cleaved reporter group between the enzyme sample of the subject and normal control enzyme is indicative of the subject having a disorder associated with a mutant or altered enzyme that reverses nucleotidylylated peptide and protein modifications.

25 By "normal control enzyme" as used herein it is meant an non-mutated unaltered enzyme that reverses nucleotidylylated peptide and protein modifications which is obtained from a normal healthy control subject not suffering from a disorder associated with that enzyme.

The present invention also relates to assay kits for quantifying activity of and
30 diagnosing disorders associated with an enzyme that reverses nucleotidylylated peptide and protein modifications. Such assay kits comprise a compound of the present invention. Assay kits of the present invention may further comprise components including, but not limited to, trypsin or another specific protease, which cleaves carboxyl to lysine and which

does not recognize nucleotidylated lysine and thus is capable of cleaving the reporter group from the cleavage product of the compound such as plasmin, thrombin, endopeptidase Lys-C or Kex2, a standard or standards comprising a Hint enzyme or other enzyme that reverses nucleotidylated peptide and protein modifications, reagents for
5 detection of the reporter group of the compound provided with the kit and/or instructions for use of the assay kit.

The following nonlimiting examples are provided to further illustrate the present invention.

10 EXAMPLES

Example 1: tBoc-LysAMP-AMC Synthesis and Characterization

tBoc-LysAMP-AMC was made as a modification of the adenosine 5'phosphoramidate synthesis method of Fu et al. (Chemistry Communications 2003 33:134-135). Under an argon atmosphere at room temperature, 0.25 mmol of tBoc-Lys-
15 AMC (Bachem Biosciences Inc., King of Prussia, PA) and 0.12 mmol ADP (Sigma Chemical Co., St. Louis, MO) were dissolved in 2 ml pyridine plus 0.6 ml trimethylsilyl chloride, added dropwise, and the resulting mixture was stirred for 2 days. The mixture was evaporated and 1 ml of 2M aqueous ammonia was added to hydrolyze the residue. Product was extracted with four 5 ml volumes of diethyl ether, evaporated to dryness, and
20 dissolved in 1.5 ml isopropanol-2M aqueous ammonia-methanol (7:1:2). tBoc-LysAMP-AMC (yield = 20.6%) was purified twice by silica gel column chromatography using a 10 x 1 cm column. Peak material was analyzed by MALDI-MS and NMR. MALDI was performed on an Applied Biosystems Voyager System 6235 in negative ion mode using 3-hydroxypicolinic acid (observed mass = 733.79; calculated mass = 734.26). ³¹P, ¹³C and
25 ¹H NMR data were collected using a 15 mm probe with the compound in DMSO. ¹H NMR (500MHz, DMSO-*d*₆, 50°C) δ = 10.93 (s, 1H), 10.85 (s, 1H), 8.47 (s, 1H), 8.12 (s, 1H), 7.81 (d, *J*=2.0 Hz, 1H), 7.71 (d, *J*=8.5 Hz, 1H), 7.53 (d, *J*=8.8 Hz, 1H), 7.35-7.44 (m, 2H), 7.27 (d, *J*=7.6 Hz, 1H), 7.10-7.16 (m, 1H), 6.25 (d, *J*=1.2 Hz, 1H), 5.89 (d, *J*=5.4 Hz, 1H), 5.57-5.62 (m, 1H), 4.55-4.59 (m, 1H), 4.18-4.21 (m, 1H), 4.07-4.21 (m, 1H), 4.02-4.05
30 (m, 1H), 3.83-3.88 (m, 1H), 3.74-3.80 (m, 1H), 2.72-2.77 (m, 1H), 2.66-2.70 (m, 1H), 2.36 (d, *J*= 4.2 Hz, 3H), 1.64-1.70 (m, 1H), 1.57-1.61 (m, 1H), 1.33 (d, *J*=13.9 Hz, 9H), 1.23-1.25 (m, 1H), 1.00 (d, *J*= 6.1 Hz, 3H); ¹³C NMR (hmqc, DMSO-*d*₆, 50°C, partial) δ =

153.3, 126.5, 116.0, 112.7, 106.2, 87.5, 84.4, 74.5, 71.3, 64.3, 41.5, 40.3, 31.6, 28.8, 28.6, 28.1, 26.0, 18.6; ^{31}P NMR (300MHz, DMSO- d_6) $\delta = 2.66$.

Example 2: tBoc-LysAMP-AMC Assays

5 Time and Hint-concentration dependence of tBoc-LysAMP-AMC hydrolysis were established as follows. All incubations were performed at 20°C in black 96-well plates and quantitated using a Wallac Victor2 multilabel counter. Initially, substrate was hydrolyzed to completion with 1 - 2 μg wild-type Hint for 20 - 40 min in 25 μl 10 mM NaAcetate pH 5.5, 50 mM NaCl. It was determined that addition of 75 μl of T buffer (100
10 mM bis-tris propane pH 9.5, 20 mM CaCl_2 , 200 mM NaCl, and 80 μg / μl trypsin (Worthington Biochemical Corporation, Lakewood, NJ) was sufficient to maximize production of aminomethylcoumarin (AMC) in a 10 minute further incubation. The substrate concentration was corrected by calibration of Hint plus trypsin-dependent fluorescence less the small background of trypsin-dependent fluorescence against a
15 standard curve of AMC in the 100 μl complete reaction without either enzyme. Time-dependence of the reaction required that T buffer be at a sufficiently high pH and/or a sufficiently high concentration of trypsin to arrest Hint activity. Moreover, time-dependence required that any Hint-independent fluorescence arising from potential contamination with tBoc-Lys-AMC or AMC be negligible with respect to Hint-dependent
20 fluorescence. The time-dependence and Hint-dependence of reactions are shown in Figures 1 and 2. Assays to determine cation-dependence of the Hint reaction compared the 10 mM NaAcetate pH 5.5, 50 mM NaCl incubation without cations to those with cations and/or EDTA. As shown in Figure 3, because of the salutary effect of 1 mM EDTA on the reaction, all further reactions included 1 mM EDTA. Kinetic values
25 (depicted below in Table 1) were calculated using reactions that hydrolyzed $\leq 10\%$ of the initial substrate.

Table 1. Kinetic constants for wild-type and W123Q chicken Hint

	tBoc-AMPLys-AMC			AMP-pNA		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
Wild-type	24.1 +/- 1.56	6.14 +/- 1.11	3.95e+6	0.051 +/- 0.011	93.0 +/- 57.6	5.49e+2
W123Q	2.57 +/- 0.473	11.2 +/- 5.11	2.29e+5	1.12 +/- 0.168	12.7 +/- 7.27	8.82e+4

Example 3: tBoc-LysGMP-MCA Synthesis and Characterization

tBoc-LysGMP-AMC was made as a modification of the synthesis of tBoc-LysAMP-AMC in which GDP replaced ADP. Yield was 24.3%.

¹H NMR (500mHz, D₂O, 40°C) δ 7.83 (d, *J*=2.0 Hz, 1H), 7.95 (s, 1H), 7.77 (d, *J*=8.6 Hz, 1H), 7.53 (d, *J*=8.8 Hz, 1H), 7.29 (d, *J*=7.5 Hz, 1H), 6.26 (d, *J*=1.3 Hz, 1H), 5.78 (d, *J*=4.9 Hz, 1H), 4.57-4.61 (m, 1H), 4.34-4.38 (m, 1H), 4.15-4.19 (m, 1H), 3.85-4.01 (m, 4H), 2.61-2.73 (m, 2H), 1.78 (s, 3H), 1.58-1.69 (m, 2H), 1.51-1.55 (m, 2H), 1.35-1.45 (m, 9H), ³¹P NMR (300mHz, DMSO-*d*₆) δ =-0.062

Negative polarity MALDI: peak at 749.01, calculated peak 750.

10

Example 4: Primers used in Plasmid Construction and Mutagenesis**Table II: Primers**

Primer Name	Primer Sequence (Primers are 5'-3')	Mutation
7024	GCGCGGCGTAGCATGGCGCAACGCATGGCTATTTG AAAAAGGAAAATGTCTGTAGGCTGGAGCTGCTTC (SEQ ID NO:1)	
7025	ATGATCAGACGATAGCCATCTTCGGCAATACCTTCT TG CTCAGCAATTTTGAATATCCTCCTTAGTTCC (SEQ ID NO:2)	
7026	GGATGTGGTAAACCTCTTGTC (SEQ ID NO:3)	
7027	CTCGACCATACTCTATACTCG (SEQ ID NO:4)	
7779	CAAGGCTTGAAGATTTTCATATGCAGGACCCCAA (SEQ ID NO:5)	NdeI site
0720	GATTCATATGCAGGACCC (SEQ ID NO:6)	Aptx-168
0721	GGGCTCGAGTCACTGTG (SEQ ID NO:7)	Aptx-168
0730	GATAAATACCCACAGGCCCG (SEQ ID NO:8)	K197Q
0731	CGGGCCTGTGGGTATTTATC (SEQ ID NO:9)	K197Q
0722	CCCAAAGGTCCGTTACC (SEQ ID NO:10)	A198V
0723	GGTAACGGACCTTTGGG (SEQ ID NO:11)	A189V
0732	CAAAGGCCCATACCATTGGC (SEQ ID NO:12)	R199H
0733	GCCAATGGTAATGGGCCTTTG (SEQ ID NO:13)	R199H
7780	CATTGGCTGGTCTTACTGTGGACCTCCATTTC (SEQ	P206L

	ID NO:14)	
7773	AGTATGAGCCATGTAGCTCTTCATGTGATCAGC (SEQ ID NO:15)	H260A
0734	CATCTTCATGGGATCAGCCAG (SEQ ID NO:16)	V263G
0735	CTGGCTGATCCCATGAAGATG (SEQ ID NO:17)	V263G
0728	CAGCCAGGGTTTTGATTC (SEQ ID NO:18)	D267G
0729	GAATCAAAACCCTGGCTG (SEQ ID NO:19)	D267G
7781	AAAAACAAAAACATAGGAATTCTTTCAATACA (SEQ ID NO:20)	W279R
7782	AAAAACAAAAACATTAGAATTCTTTCAATACA (SEQ ID NO:21)	W279X
0724	GCACACTGTTGGGGGAAAAG (SEQ ID NO:22)	689insT
0725	CTTTTCCCCAACAGTGTGC (SEQ ID NO:23)	689insT
0726	CATTGGAATCTTTCAATAC (SEQ ID NO:24)	840delT
0727	GTATTGAAAGATTCCAATG (SEQ ID NO:25)	840delT

Example 5: Expression of Human APTX

Plasmid pB352 and derivatives were used for expression of human *APTX*

- 5 (Clements et al. DNA Repair (Amst) 2004 3:1493-1502). Site-directed mutagenesis of plasmid pB352 was used to create plasmids to express *APTX* alleles K197Q, A198V, R199H, P206L, H260A, V263G, D267G, W279R, W279X, 689insT, and 840delT using primers set forth in Example 4.

10 Example 6: Enzyme Expression and Purification

- E. coli strain BB2-1 was used for protein expression. BB2-1 was produced by disrupting the *E. coli hinT* gene in strain BL21* as described by Datsenko and Warner (Proc. Natl Acad. Sci. USA 97:6640-6645). Primers 7024 and 7025 were used for PCR amplification of the chloramphenicol resistance marker of plasmid pKD3. Stable
- 15 chloramphenicol-resistant transformants of BL21* were tested by PCR with primers 7026 and 7027 to confirm correct recombination of the chloramphenicol resistance marker into the *hinT* locus. *E. coli* transformants carrying *APTX* expression plasmids were aerated at 24 °C. At an A₆₀₀ of 0.5, expression was induced with 100 μM isopropyl μ-D-

thiogalactopyranoside. Cultures were grown 16 hours and harvested by centrifugation. Frozen(-80 °C) and thawed cell pellets (approximately 5 grams wet weight) were lysed using Bugbuster (Novagen) with DNase I and EDTA-free complete protease inhibitor mixture (Roche Applied Science). Clarified lysates were loaded on 1.5 ml Talon columns (Clontech). Aprataxin proteins were eluted with 10 ml of 250 mM imidazole, 10 mM HEPES, pH 7, and concentrated into 100 mM NaCl, 10 mM HEPES, pH 7.2, with 10-kDa cutoff centrifugal concentrators (Amicon). Proteins were transferred to nitrocellulose membranes and detected using an anti-penta-His antibody conjugated to horseradish peroxidase (Qiagen) and SuperSignal West Pico chemiluminescent substrate (Pierce).

Example 7: Enzyme Assays

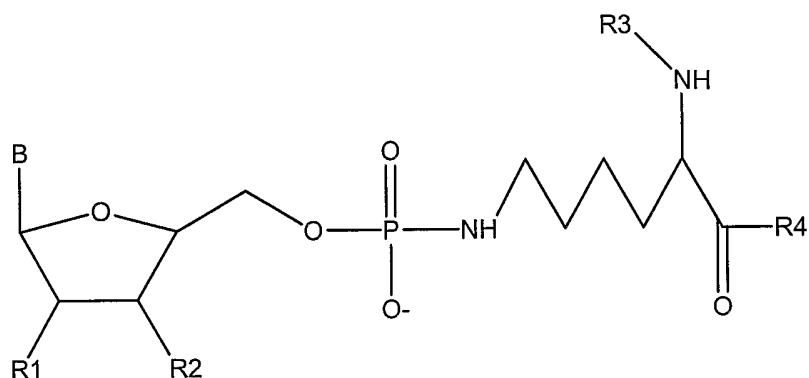
t-Boc-LysAMP-MCA and t-Boc-LysGMP-MCA hydrolytic activities were assayed in 25- μ l volumes containing 2.5–250 μ M substrate, 12 nmol of wild-type enzyme or 12–60 nmol of mutant enzyme, 100 mM NaCl, 10 mM HEPES, pH 7.2, for 30–60 minutes at room temperature. Reactions were initiated by the addition of substrate and stopped by addition of 75 μ l of 80 mg/ml trypsin. After a 10 minute incubation with trypsin, fluorescence (excitation 355 nm, emission 460 nm) was measured with a Wallac 1420 multilabel counter. pH and divalent cation analyses were completed in 10 mM HEPES, with pH from 6.8 to 8.2. GpppBODIPY hydrolysis was assayed as described previously by Draganescu et al. (J. Biol. Chem. 2000 275:4555-4560). Assays were initiated by addition of 12 nmol of enzyme and stopped after 30–60 minutes by the addition of sodium citrate, pH 3. GpppBODIPY concentration ranged between 0 and 25 μ M. Dinucleoside polyphosphate hydrolysis assays were performed using 60 nmol of wild-type enzyme with 5–200 μ M substrate in a 50- μ l volume. Assays were incubated at room temperature for 30 minutes and stopped by addition of 50 μ l of Na₂CO₃, pH 11.6. 50 μ l of the reaction mixture was injected onto a strong anion exchange column (Princeton Chromatography) equilibrated with 10 mM K₂HPO₄, pH 2.58 (Buffer A) on a Varian Prostar HPLC. The elution program was 100% Buffer A for 10 minutes, a 2-minute gradient to 100% 750 mM K₂HPO₄, pH 2.58 (Buffer B), and 100% Buffer B for 6 minutes. Elution times for AMP, ADP, ATP, ApppA, and AppppA were 6.7, 15.3, 16.2, 15.7, and 16.3 minutes, respectively. Product amounts were determined from the peak areas and a standard curve of AMP using Varian Galaxie Software. All assays were performed at least in triplicate. Substrate specificity of aprataxin is shown in the following Table III.

Table III: Substrate specificity of Aprataxin

Substrate	k_{cat} s^{-1}	K_{m} μM	$K_{\text{cat}}/K_{\text{m}}$ $\text{s}^{-1}\text{M}^{-1}$
t-Boc-LysAMP-MCA	0.0028 $\pm$ 0.0002	47 $\pm$ 9	60 $\pm$ 22
t-Boc-LysGMP-MCA	0.003 $\pm$ 0.0004	116 $\pm$ 24	26 $\pm$ 17
GpppBODIPY	0.0004 $\pm$ 0.00005	13.1 $\pm$ 4.7	31 $\pm$ 11
ApppA	0.00008 $\pm$ 0.00002	21 $\pm$ 1.6	3.8 $\pm$ 1.3
AppppA	0.0009 $\pm$ 0.00005	39 $\pm$ 6	23 $\pm$ 8.3

What is claimed:

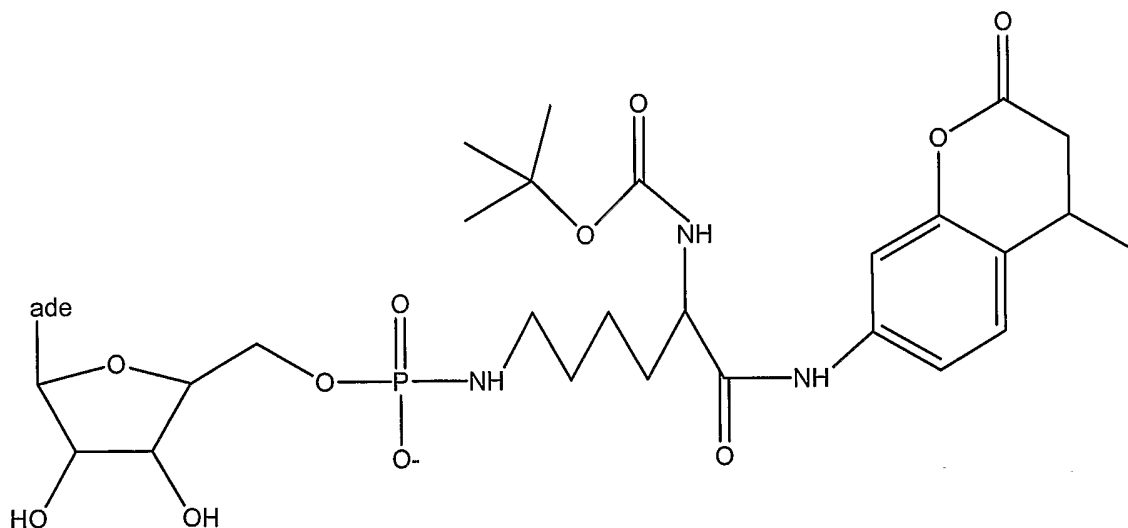
1. A compound which acts as a substrate for enzymes that reverse nucleotidylated peptide and protein modifications, said compound comprising:
 - (a) a nucleoside monophosphate moiety;
 - 5 (b) a peptidyl moiety or other polypeptide sequence terminating in a lysine at its N-terminus and linked by phosphoramidate linkage to the nucleoside monophosphate moiety via the lysine at the N-terminus; and
 - (c) a reporter group linked to the compound.
- 10 2. The compound of claim 1 wherein the reporter group is linked by amide linkage to the C-terminal amino acid of the peptidyl moiety or polypeptide sequence.
3. The compound of claim 1 wherein the reporter group comprises a FRET based signaling system with a quencher attached at one end of the compound and a fluorophore
15 attached to the other end of the compound.
4. The compound of claim 1 wherein the nucleoside monophosphate moiety is selected from the group consisting adenosine monophosphoramidate (AMP), guanosine monophosphoramidate (GMP), cytidine monophosphoramidate (CMP), thymidine
20 monophosphoramidate (TMP), uridine monophosphoramidate (UMP), deoxyadenosine monophosphate (dAMP), azidothymidine monophosphate (AZTMP), and dideoxyinosine monophosphate (ddIMP).
5. The compound of claim 1 wherein the peptidyl moiety comprises N-Ac-lysine,
25 tBoc-lysine, fMoc-lysine, or peptide-lysine.
6. The compound of claim 1 wherein the reporter group is selected from the group consisting of aminomethylcoumarin, 7-amino-4-carbamoylmethylcoumarin and paranitroaniline.
- 30 7. The compound of claim 1 comprising Formula I:



wherein B is a base selected from the group consisting of adenine, guanine, cytosine, thymine, uracil and hypoxanthine;

- 5 R_1 and R_2 are each selected from the group consisting of H, OH and N_3 ;
 R_3 is selected from the group consisting of H or a peptide and/or a blocking group;
 and
 R_4 is a reporter group or a reporter group linked to a peptide or peptide-like group.

- 10 8. The compound of claim 1 comprising Formula II:



9. A method for synthesis a compound of claim 1 comprising:

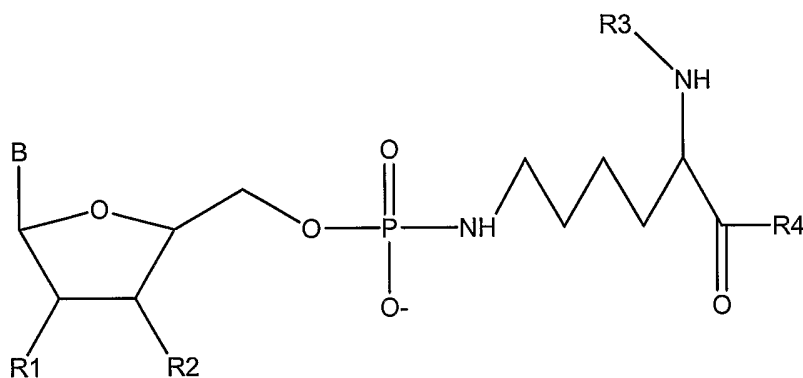
- 15 (a) linking by phosphoramidate linkage a peptidyl moiety or other polypeptide sequence comprising a lysine at its N-terminus to a nucleoside monophosphate moiety via the lysine at the N-terminus of the peptidyl moiety or other polypeptide sequence; and
 (b) linking by amide linkage a reporter group to the C-terminus of the peptidyl moiety or other polypeptide sequence.

10. The method of claim 9 wherein the nucleoside monophosphate moiety of the compound synthesized is selected from the group consisting adenosine monophosphoramidate (AMP), guanosine monophosphoramidate (GMP), cytidine monophosphoramidate (CMP), thymidine monophosphoramidate (TMP), uridine monophosphoramidate (UMP), deoxyadenosine monophosphate (dAMP), azidothymidine monophosphate (AZTMP), and dideoxyinosine monophosphate (ddIMP).

11. The method of claim 9 wherein the peptidyl moiety of the compound synthesized comprises N-Ac-lysine, tBoc-lysine, fMoc-lysine, or peptide-lysine.

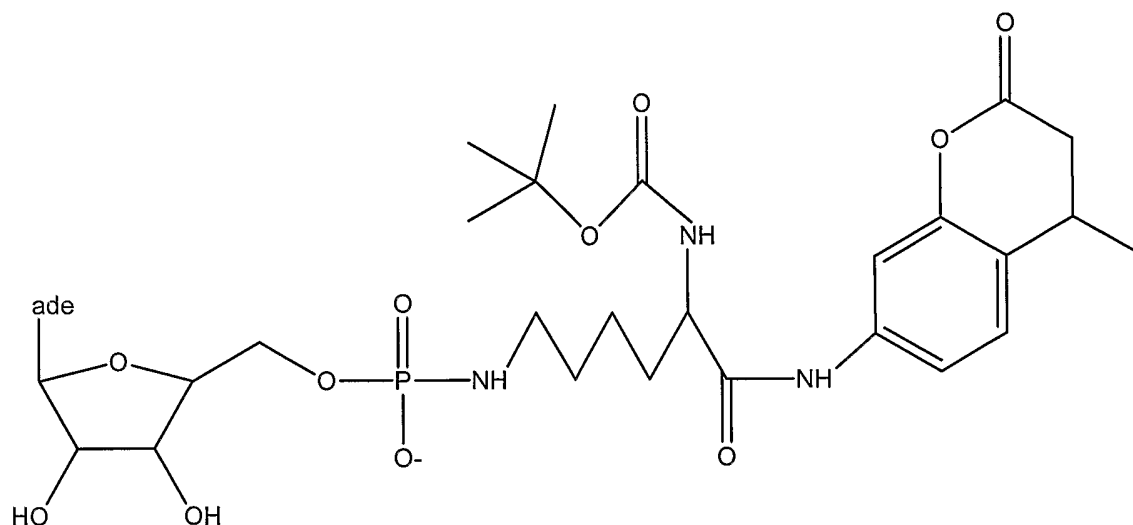
12. The method of claim 9 wherein the reporter group of the compound synthesized is selected from the group consisting of aminomethylcoumarin, 7-amino-4-carbamoylmethylcoumarin and paranitroaniline.

13. The method of claim 9 wherein the compound synthesized comprises Formula I:



wherein B is a base selected from the group consisting of adenine, guanine, cytosine, thymine, uracil and hypoxanthine;
 R₁ and R₂ are each selected from the group consisting of H, OH and N₃;
 R₃ is selected from the group consisting of H or a peptide and/or a blocking group;
 and
 R₄ is a reporter group or a reporter group linked to a peptide or peptide-like group.

14. The method of claim 9 wherein the compound synthesized comprises Formula II:



15. An assay to quantify activity of an enzyme that reverses nucleotidylylated
5 peptide and protein modifications comprising:

(a) incubating the enzyme with a compound of claim 1 under conditions which promote cleavage of a denucleotidylylated peptide-reporter conjugate from the compound; and

(b) incubating the denucleotidylylated peptide-reporter conjugate cleavage product
10 with a specific protease to cleave the reporter group from the conjugate, said specific protease cleaving carboxyl to lysine but not recognizing nucleotidylylated lysine; and
(c) measuring the cleaved reporter group.

16. An assay kit for quantifying activity of an enzyme that reverses
15 nucleotidylylated peptide and protein modifications comprising a compound of claim 1.

17. A method for diagnosing a disorder in a subject associated with an altered or mutated enzyme that reverses nucleotidylylated peptide and protein modifications comprising:

20 (a) incubating an enzyme sample obtained from the subject with a compound of claim 1 under conditions which promote cleavage of a denucleotidylylated peptide-reporter conjugate from the compound;

(b) incubating the denucleotidylylated peptide-reporter conjugate cleavage product with a specific protease to cleave the reporter group from the conjugate, said specific

protease cleaving carboxyl to lysine but not recognizing nucleotidylated lysine;

(c) measuring an amount of cleaved reporter group; and

(d) comparing the measured amount of cleaved reporter group by the enzyme sample obtained from the subject with an amount of cleaved reporter group by normal
5 control enzyme wherein a difference in measured amounts of cleaved reporter group is
indicative of the subject having a disorder associated with an altered or mutated enzyme
that reverses nucleotidylated peptide and protein modifications.

18. The method of claim 17 wherein the disorder is ataxia-oculomotor apraxia
10 syndrome 1.

19. An assay kit for diagnosing a disorder in a subject associated with an enzyme
that reverses nucleotidylated peptide and protein modifications comprising a compound
of claim 1.

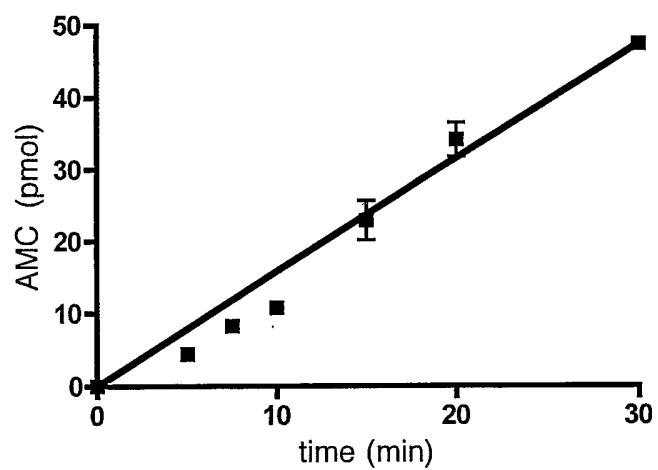


FIGURE 1

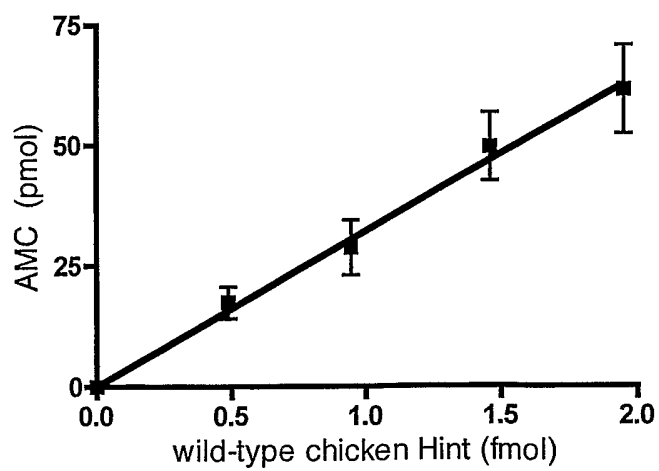


FIGURE 2

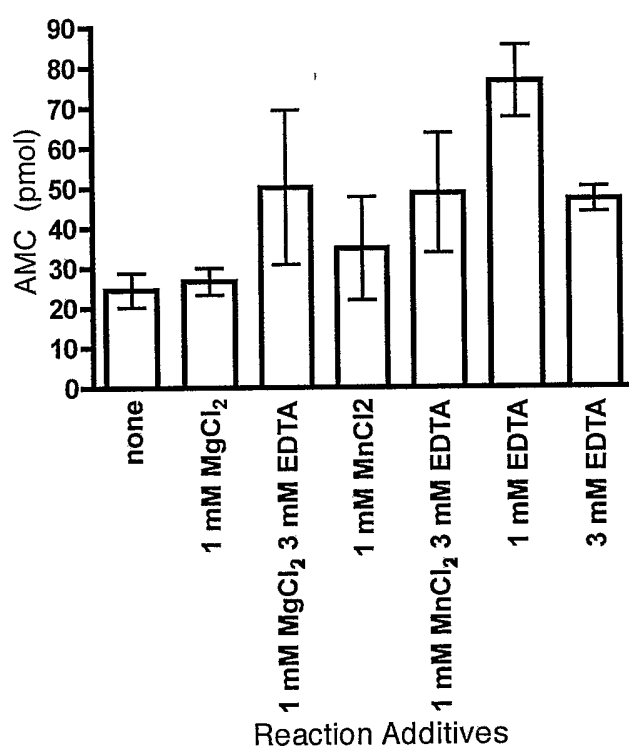


FIGURE 3

SEQUENCE LISTING

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BIOMOL International LP
Brenner, Charles
Howitz, Konrad T
Zipkin, Robert

<120> Compounds Useful as Substrates for Enzymes that Reverse
Nucleotidylylated Peptide and Protein Modifications and Methods for
Production and Uses Thereof

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<151> 2004-10-14

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