**Title:** PROTON NMR SPECTROSCOPY FOR BIOCHEMICAL SCREENING

**Abstract:** High-Throughput Screening (HTS) of large compound libraries is the method of choice for discovery of lead compounds for drug development. The present invention provides novel, sensitive and broadly applicable NMR screening methods in which the H8 signal of a purine-containing substrate or product, or the H6 signal of a pyrimidine-containing substrate or product, is used to monitor enzymic reactions that utilize such substrates. Experiments can be performed in real time or in an endpoint assay format using protein and substrate concentrations comparable to the ones used by other HTS techniques. Application of the NMR method to the assay of the enzymes creatine kinase and phosphodiesterase 2A is presented. The methods are applicable to any of a multitude of enzymes that utilize purine nucleoside substrates (e.g. ATP, GTP) or pyrimidine nucleoside substrates (e.g. CTP, UDP-GlcNac), including protein kinases, GTPases, and lipid kinases.

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The ability to identify small molecules that interact with a biological target is a fundamental step in drug discovery. Currently, high throughput screening (HTS) through random screening of corporate compound collections is the technique of choice in the pharmaceutical industry to identify potent protein inhibitors {Dove, 1999 #45; Smith, 2002 #46}. HTS has undoubtedly produced high quality lead molecules for many drug discovery programs. However, initial hits are commonly susceptible to a high rate of false positives, resulting from interference of compounds associated with the fluorimetric or colorimetric detection or/and from compound aggregation, especially in high concentration screens of low molecular weight fragments in fragment-based drug discovery {Rishton, 1997 #47; Feng, 2005 #48}. Ways to devise more reliable biochemical assays for the identification of new lead molecules are continually being sought, as it is now well accepted that quality is more important than quantity in such a screen {Smith, 2002 #46}.

Nuclear magnetic resonance (NMR) spectroscopy has been well recognized for its unique ability to directly monitor protein-ligand interactions with high resolution. It has also recently been developed as a screening tool, and complements HTS for the identification of small molecules that bind and modulate biological targets implicated in disease pathways {Pellecchia, 2008 #49; Hajduk, 1999 #50}. Over the past decade or so, NMR-based screening has become an important component in an integrated arsenal of biophysical and biochemical methods designed to discover and optimize drug hits and leads in pharmaceutical research {Hajduk, P. J. et al. Quarterly Reviews of Biophysics 32, 211 240 (1999); Stockman, B. J. et al. Prog. NMR Spectr. 41, 187 231 (2002); Meyer, B. et al. Chem. Int. Ed. 42, 864 (2003)}.

One major advantage of NMR biophysical screening methods, such as saturation transfer difference (STD), water-LOGSY, line broadening and protein
chemical shifts perturbation experiments, in comparison to other screening technologies, is that they can detect weak interactions, and are thus particularly suitable for fragment-based drug discovery (Shuker, 1996 Ref #51; Mayer, 1999 #52; Dalvit, 2000 #53; Hajduk, 1997 #54; Dalvit, 2002 #55). However, those approaches have suffered a number of drawbacks, mainly due to the requirements for a high magnetic field spectrometer (typically ≥ 600 MHz), high protein concentration (usually ∼1µM - 1mM) (Albert, 2007 #56; Lepre, 2004 #57; McCoy, 2005 #34), and low to medium throughput. Separately, NMR biochemical screens with a lower consumption of proteins and higher throughput have been developed, through observing the formation of products, for the identification of enzyme inhibitors. While tactically interesting, several screens reported have practical limitations in their applications because they often require the modification of a natural substrate to incorporate an unnatural 19F tag (Dalvit, 2004 #36; Stockman, 2008 #37; US patent 7,255,885) and/or have lower throughput due to the use of less sensitive NMR spectroscopy methods, using for example 13C, 19F or 31P ((Chiyoda, 1998 #58; Milner-White, 1983 #59; Mendoza, R et al. (2001) Bioorganic & Med. Chem. Letrs, 21:5248-5250); Mbefo, M.K. et al. (2010) J. Biol Chem. 285(4): 2807-2822; Marshall, C.B. et al. (2009), www.SCIENCESIGNALING.org, 2(55) ra3, pages 1-10). A review of the scientific literature indicates that NMR assays involving the direct use of the simplest and most sensitive nucleus, 1H, have rarely been reported (e.g. see {Stockman, 2007 #38}; Yang, T. et al. (2009) J. Biol Chem. 284(32): 21526-21535), and that its potential as a biochemical screening technique appears to have been largely unexplored.

[4] None of the current NMR enzymic assay methods have characteristics that readily permit broad applicability to multiple enzyme targets. In contrast, the novel proton NMR assay methods described herein can be utilized for the assay of a wide variety of enzyme targets that are potentially important in drug discovery, e.g. any protein kinase.

**SUMMARY OF THE INVENTION**

[5] Herein is disclosed a versatile 1H NMR-based activity assay method that detects resolved purine or pyrimidine proton resonances, and can be used for high
throughput screening of compound libraries for identifying inhibitors of most enzymes that catalyze the modification of purine-ring-containing or pyrimidine-ring-containing substrates, including kinases, ATPases, GTPases, phosphodiesterases and sulfotransferases. It permits generic, rapid and reliable biochemical screening of enzyme systems that catalyze the modification of purine-ring-containing or pyrimidine-ring-containing substrates, including those catalyzing nucleotide conversions, e.g. ATP→ADP, ATP→AMP, ATP→cAMP, ADP→AMP, GTP→GDP, GTP→GMP, GTP→cGMP, GDP→GMP, UDP→UDPGlCNac, or CTP→CDP conversions. This method, developed using a standard 400 MHz NMR spectrometer, provides a user-friendly platform, that is amenable for adoption by both small and large scale drug discovery laboratories, and has many practical benefits over other NMR-based biophysical and biochemical screens in terms of throughput, resource requirement, and applications. It may be used as a secondary screen complementary to HTS, or as a primary screen where HTS capability or biochemical assay is not readily available. In two of its most broadly applicable embodiments, this method directly monitors either the purine H8 resonance or the pyrimidine H6 resonance of the enzyme substrate and/or product free in solution, does not require extensive assay development, and can also be performed in real-time, if required. The application of this method to enzyme kinetics and inhibitor screening is demonstrated using the enzyme creatine kinase, where the ATP and ADP H8 resonances are monitored, and the enzyme phosphodiesterase 2A, where the cGMP and GMP H8 resonances are monitored.

[6] In one aspect the present invention provides a method for identifying an inhibitor of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as an inhibitor of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test
compound has inhibited the enzymic conversion of the substrate to the product resulting therefrom.

[7] In another aspect the present invention provides a method for identifying an agonist of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as an agonist of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of the substrate to the product resulting therefrom.

[8] This invention further provides a method for identifying an inhibitor of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the intensity of a pyrimidine H6 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyrimidine H6 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as an inhibitor of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of the substrate to the product resulting therefrom.

[9] This invention also provides a method for identifying an agonist of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the intensity of a pyrimidine H6 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a
pyrimidine H6 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as an agonist of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of the substrate to the product resulting therefrom.

[10] For purine-ring-containing substrates, the H8 proton on the heterocyclic purine ring is used as the probe signal. The H8 signal provides the best resolved peak between cofactor pairs (i.e. substrate and product), and is very easily quantifiable due to its strong singlet NMR multiplicity, and location in a lower field region (~8.5 ppm) that is separated from buffer and solvent resonances. For similar reasons, the H6 proton (doublet) provides the best probe signal for pyrimidine-ring-containing substrates. In some cases other protons can also be used such as H1' in AMP/adenosine, H1' in AMP/cAMP, H2 in adenosine/adenine, and H1" of UDP-GlcNac in UDP/UDP-GlcNac. All proton resonances that can be used for detection are listed in Figures 10-18. Proton signals not shown are not suitable for the assay due to resonance overlap between substrate and product, between different protons in the same compound, or interference from buffer and solvents.

[11] The present invention also provides NMR methods to identify or characterize an unknown enzymic function of a protein by its interaction with a purine-ring-containing or pyrimidine-ring-containing substrate, e.g. a purine or pyrimidine nucleoside. By this method, for example, novel substrates for protein or lipid kinases of unknown specificity may be determined.

[12] The present invention further provides NMR methods to identify novel interactions between proteins, and to identify modulators of protein-protein interactions.

**BRIEF DESCRIPTION OF THE FIGURES**

[13] **Figure 1. The H NMR spectra of the H8 proton of ATP and ADP resonances in the RMCK NMR activity assay.** A, the reaction with 0.5 mM MgATP and without RMCK, quenched at 20 min with EDTA. B, the reaction with
0.5 mM MgATP and 10 nM RMCK, quenched at 20 min with EDTA. C, the reaction with 0.5 mM MgADP and 10 nM RMCK, with EDTA added before MgADP. In addition to MgATP / MgADP and RMCK, the solutions also contained 40 mM creatine, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris-dn in D$_2$O, pH 9.0. A total of 64 scans were recorded for each spectrum (6 min acquisition time).

[14] Figure 2. The $^1$H chemical shifts difference of the H8 protons of ATP and ADP resonances as a function of pH. The solution contained 0.4 mM ATP or ADP, 0.1 mM DSS in 50 mM Tris-dn (●), or 0.4 mM ATP or ADP, saturating concentration of magnesium acetate, 0.1 mM DSS in 50 mM Tris-dn (▲), or 0.4 mM ATP or ADP, 1.4 mM magnesium acetate, 5 mM EDTA, 0.1 mM DSS in 50 mM Tris-dn (●).

[15] Figure 3. Double reciprocal plots of RMCK using the NMR activity assay and the IC$_{50}$ measurement of adenosine, a and b. The assays were carried out using 10 nM RMCK in 50 mM Tris-dn in D$_2$O, pH 9.0. The free Mg$^{2+}$ concentration was kept constant at 1 mM. a, The concentrations of MgATP were: ●, 0.4 mM; □, 0.6 mM; ▲, 1 mM; △, 2 mM. b, The concentrations of creatine were: ●, 5 mM; □, 7 mM; ▲, 10 mM; △, 20 mM; ·, 40 mM. The values of the Michaelis and dissociation constants were determined using multiple non-linear regression. For graphing the transformed double reciprocal of data points and the linear fit of each concentration are displayed, c, The % inhibition by adenosine is determined from the peak areas of $^1$H signal of ADP in the presence and absence of adenosine. An IC$_{50}$ value of 10.1 ± 5.5 mM and a Hill coefficient of 0.8 ± 0.3 is obtained from the fitting of % inhibition vs. adenosine concentration. The reactions were performed with 10 nM RMCK, 40 mM creatine, 0.0625 mM MgATP, and were quenched by 20 mM EDTA at four time points to determine the initial velocity.

[16] Figure 4. The $^1$H NMR spectra showing the inhibition of ATP hydrolysis in the RMCK fragment library screening. a, the reaction with 0.5 mM of Compound 98 and b, without any compound, quenched at 10 min with EDTA. The solutions also contained 10 nM RMCK, 0.4 mM MgATP, 40 mM creatine, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris-dn, pH 9.0.
Figure 5. The 1H NMR spectra of the H8 and H1' protons of cGMP and GMP resonances in the PDE2A NMR activity assay, a, the reaction with 0.2 mM cGMP and without PDE2A, quenched at 45 min with EDTA. b, the reaction with 0.2 mM cGMP and 5 nM PDE2A, quenched at 45 min with EDTA. c, the reaction with 0.2 mM GMP and 5 nM PDE2A, with EDTA added before GMP. In addition to cGMP / GMP and PDE2A, the solutions also contained 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris in D2O, pH 7.5. A total of 64 scans were recorded for each spectrum (6 min acquisition time).

Figure 6. The enzyme titration of PDE2A. The assays were carried out with 1.25, 2.5, 5, 10, 20 nM PDE2A, 0.2 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris in D2O, pH 7.5. The reaction were quenched by 20 mM EDTA at three time points for each protein concentration to determine the initial velocity. The linear fitting of the relative velocity versus enzyme concentration is shown.

Figure 7. Kinetics measurement of PDE2A using the NMR activity assay and the IC50 measurement of EHNA. a, direct fit of initial velocity as a function of cGMP concentration to the Michaelis-Menten equation. A K_M^cGMP value of 13.9 ± 2.1 µM is obtained from the data fitting. The assays were carried out using 5 nM PDE2A, 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris in D2O, pH 7.5. The reaction were quenched by 20 mM EDTA at three time points for each protein concentration to determine the initial velocity. b, calculated % inhibition as a function of EHNA concentration. An IC50 value of 15.5 ± 0.8 µM and a Hill coefficient of 1.1 ± 0.0 is obtained from the fitting. The % inhibition is determined from the initial velocity in the presence and absence of EHNA. The reaction mixtures contained 5 nM PDE2A, 0, 0.01, 0.1, 1, 4, 12, 30, 100, 500, 2000, 5000 µM EHNA, 0.2 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris, pH 7.5. The reactions were quenched by 20 mM EDTA at three time points in the linear region.

Figure 8. The 1H NMR spectra showing the inhibition of cGMP hydrolysis in the PDE2A fragment library screening. a, the reaction with 0.1 mM of
Compound 09033259 and b, without any compound, quenched at 45 min with EDTA. The solutions also contained 5 nM PDE2A, 0.2 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris, pH 7.5.

[21] **Figure 9. Simulation of competitive inhibition of MgATP by inhibitor I.** The % inhibition is plotted as a function of inhibitor concentration (a) and MgATP concentration (b). In a, the MgATP concentration is 0.4 mM and $K_M^{\text{MgATP}}$ is set to 0.33 mM. In b, the inhibitor concentration is 0.5 mM and $K_M^{\text{MgATP}}$ is set to 0.33 mM. Curves corresponding to different $K_i$ of inhibitor are labeled.

[22] **Figure 10.** The chemical shift differences ($\Delta \delta$) of adenine-derivative cofactor pairs.

[23] **Figure 11.** The chemical shift differences ($\Delta \delta$) of guanine-derivative cofactor pairs.

[24] **Figure 12.** The chemical shift differences ($\Delta \delta$) of uracil-derivative cofactor pairs.

[25] **Figure 13.** The chemical shift differences ($\Delta \delta$) of cytosine-derivative cofactor pairs.

[26] **Figure 14.** The chemical shift differences ($\Delta \delta$) of thymine-derivative cofactor pairs.

[27] **Figure 15.** The chemical shift differences ($\Delta \delta$) of hypoxanthine-derivative cofactor pairs.

[28] **Figure 16.** The chemical shift differences ($\Delta \delta$) of xanthine-derivative cofactor pairs.

[29] **Figure 17.** The chemical shift differences ($\Delta \delta$) of adenine-based cofactor (ABC) pairs.
[30] Figure 18. The chemical shift differences (Δδ) of nicotinamide-based cofactor (NBC) pairs.

[31] Figure 19. The chemical shifts of cofactors at pH 7.5.

[32] Figure 20. The chemical shifts of cofactors at pH 9.0.

[33] Figure 21. The I_{50} measurement of compound hits from PDE2A screen. The IC50 value and Hill coefficient are obtained from the fitting of % inhibition as a function of inhibitor concentration. The % inhibition is determined from the initial velocity in the presence and absence of an inhibitor. The reaction mixtures contained 5 nM PDE2A, 0, 0.02, 0.05, 0.15, 0.46, 1.37, 4.12, 12.35, 37.04, 111, 333, 1000, 2000 μM inhibitor, 0.2 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris, pH 7.5. The reactions were quenched by 20 mM EDTA at one time point in the linear region.

**DETAILED DESCRIPTION OF THE INVENTION**

[34] The invention disclosed herein describes how a variety of cofactor pairs that are involved in important enzymic reactions can be readily resolved and quantitated by proton (^1H) NMR (e.g. Figures 10-18), thus enabling the assay of the enzymes that catalyse their interconversion by an NMR assay method that directly monitors the modification of these cofactors. The structures of these cofactors, and terminology used in referring to particular NMR resonances used in their assay is indicated in Tables la-le. For example, the protons responsible for the purine H8 and pyrimidine H6 resonances are indicated on the heterocyclic ring structures at the top of the tables. The positions of protons not on these ring structures that are used in NMR assays are indicated on the R groups listed in the left hand column of each table.

[35] The present invention overcomes the limitations of NMR biochemical screening with respect to protein and substrate concentrations, labeling requirements (e.g. ^19F labeling), and limited applicability with respect to target assayed, when compared to conventional NMR HTS techniques. The present invention provides a
novel and sensitive NMR method in which the H8 signal of a purine-ring-containing substrate (e.g. a purine nucleoside) is used to monitor enzymic reactions that utilize such substrates. This allows for rapid and reliable biochemical screening at protein and substrate concentrations comparable to the concentrations utilized by standard HTS techniques.

[36] The NMR assay method of the present invention, wherein an H8 purine signal is monitored, has a number of important advantageous characteristics: i) Proton NMR spectroscopy is very sensitive; ii) Purine-ring-containing (e.g. nucleoside, nucleotide, etc.) H8 signals appear as sharp, strong singlet resonances. This property is important for compound screening because it permits the use of low substrate concentrations, thus allowing detection of medium and weak inhibitors; iii) Under the conditions developed, there are no spectral interferences from protonated solvents, buffers, or detergents typically used in enzymatic reactions; iv) The purine nucleobase H8 chemical shift is very sensitive to the chemical environment, resulting in differential chemical shifts for the starting and enzymatically transformed substrate resonances, and a signal for the substrate that is readily separated from that of the product (e.g. an ATP or GTP H8 signal is readily separated from that of ADP or GDP), thus allowing a direct comparison of their intensities; v) Modification of enzyme substrates is not required to monitor the H8 NMR signal. Thus no custom chemical synthesis is required to obtain an appropriately modified substrate compound, a process that may have to be repeated and/or adapted for different enzyme-substrate pairs. There are also no issues of where to modify the substrate such that it does not interfere with the binding to the enzyme; vi) Due to the diversity of enzymes that utilize purine nucleoside substrates, and the lack of a requirement for substrate labeling, the assay has very broad applicability.

[37] In one embodiment of the present invention, the NMR assay method has been utilized to measure the phosphorylation of creatine by rabbit muscle creatine kinase (RMCK). The enzymatic reaction was performed by incubating RMCK in the presence of creatine and ATP. The conversion of ATP to ADP generated a shift of the purine H8 signal as indicated in Figure. 1. At time zero only one signal is observed corresponding to the H8 signal of ATP. With time the H8 signal of ATP decreases and a new signal appears in the spectrum corresponding to the H8 signal of ADP. The
sum of the two integral signals at any time after the start of the reaction corresponds to the signal integral of the H8 signal of ATP the at time 0. If the enzymatic reaction is allowed to go to completion, only the H8 signal of ADP is visible in the spectrum. The same reaction performed in the presence of an inhibitor or agonist compound results in inhibition or stimulation respectively of the formation of the H8 signal of ADP, as is evident for example from the results presented in Figures 3 and 4 herein, where inhibitory compounds are monitored. In another embodiment of the present invention, the NMR assay method has also been utilized to assay the activity of the enzyme phosphodiesterase 2A, where cGMP and GMP H8 resonances are monitored.

[38] Demonstration that the NMR assay method of this invention can be readily implemented to assay creatine kinase by monitoring the purine ring H8 signal of ATP and/or ADP, or to assay the activity of phosphodiesterase 2A, by monitoring the purine ring H8 signal of cGMP and GMP, implies that the same method should be applicable to the assay of any enzyme that catalyzes conversion between the same two pairs of cofactors (e.g. most kinases). Since these two pairs of cofactors can readily be resolved and quantitated in the determination of creatine kinase and phosphodiesterase activity, that will also be the case for any other enzyme that uses these same cofactors. Additionally, it has been demonstrated herein (e.g. see Figures 10-11 and 15-18) that several other purine-H8-containing cofactor pairs can also be readily resolved and quantitated, enabling the NMR assay method of this invention to also be utilized for the assay of any enzyme that catalyzes inter-conversion between these cofactors, by monitoring the purine ring H8 resonance. The fact that ATP/ADP is one of the most difficult of all the cofactor pairs to resolve by NMR using the purine H8 resonance, and yet RMCK can readily be assayed by the NMR methods described herein, is indicative that assay of any enzyme that catalyses interconversion between any of the other cofactor pairs that are more easily resolved can readily be performed. Assay conditions for all the enzymes that utilize purine-H8-containing cofactor pairs are generally available in the art as most of these enzymes have been discovered some time ago and their properties have been well-studied. Thus no significant assay development work is required to assay them by the methods of the invention described herein.
It should be noted that only the H8 resonance provides a broadly applicable NMR assay that is capable of monitoring the activity of a broad range of enzymes that catalyze the modification of purine-ring-containing substrates. The H8 resonance was found to be suitable for monitoring enzyme assays involving all the cofactor pairs examined, except NAD/cADPR and CoA/AcetylCoA (see Figures 10-11 and 15-18). NMR proton resonances other than H8 of purine-containing cofactor pairs were also tested for their potential to be used in an NMR assay, and were only found to be useful in a few specific instances, as noted for example in Figures 10-11 and 15-18 herein. These include: (a) H1' for AMP/adenosine, cAMP/AMP, GMP/guanosine, cGMP/GMP, cGMP/GTP, ATP/SAM, SAM/SAH pairs; cAMP/ATP, IMP/inosine, IMP/GMP, IMP/XMP, inosine/adenosine, XMP/xanthosine, XMP/GMP, cADPR/ADPR, cADPR/NAD, ADPR/NAD, ADPR/NADP, NAD/NADH; NADP/NADPH, GDP/m7GDP, and GTP/m7GTP pairs; (b) H2 for adenosine/adenine, ATP/SAM, SAM/SAH, AM/adenine, inosine/hypoxanthine, IMP/hypoxantline, IMP/AMP, inosine/adenosine, hypoxanthine/adenine, cADPR/ADPR, cADPR/NAD, ADPR/NAD, NAADP/NADPH, NADP/NAADP, SAH/adenine and MTA/adenine pairs; (c) H1'' for cADPR/ADPR, cADPR/NAD, ADPR/NADP, and NADP/NAADP pairs; (d) H2'' for NAD/NADH, and NADP/NADPH pairs; (e) H4'' for NAD/NADH, NADP/NADPH, and NADP/NAADP pairs; (f) H5'' for the NADP/NAADP pair; and (g) H6'' for NAD/NADH and NADP/NADPH pairs; for all of which a \( \Delta \delta \) is readily measurable, and each member of a cofactor pair quantifiable in an NMR assay. In addition, for some purine-containing cofactor pairs, only one member of the pair was quantifiable with certain resonances, thus enabling an NMR assay to be formatted using the resonance for that cofactor (see Figures 10-11 and 15-18). These include: (a) H1' for guanosine/guanine; AMP/adenosine, adenosine/adenine, GMP/guanine, IMP/hypoxanthine, inosine/hypoxanthine, XMP/xanthine, xanthosine/xanthine, SAH/adenine and MTA/adenine pairs; (b) H2 for IMP/GMP, IMP/XMP and xanthine/hypoxanthine; (c) H1'' for GDP/GDP-man, GTP/GDP-man, NAD/NADH, NADP/NADPH, and UDP-Gal/UDP pairs; (d) H2'' for NADP/NAADP, cADPR/NAD, ADPR/NAD and ADPR/NADP pairs; (e) H4'' for cADPR/NAD, ADPR/NAD and ADPR/NADP pairs; (f) H5'' for NAD/NADH, NADP/NADPH, cADPR/NAD, ADPR/NAD and ADPR/NADP pairs; (g) H6'' for NADP/NAADP, cADPR/NAD, ADPR/NAD and ADPR/NADP pairs; (h) the methyl proton of the acyl
group of acetyl CoA for CoA/AcetylCoA; and (i) the methyl proton for \( m^7 \)GDP and \( m^7 \)GTP. Use of resonances other than H8 was not always feasible in NMR assays for a variety of reasons, including lack of resolution of cofactor pairs (i.e. substrate and product), peak splitting due to proton spin-spin interactions, overlap with peaks of resonances of other assay components etc. However, when they are feasible, as for the above resonances, they provide an alternative to purine H8 for monitoring interconversion between the above cofactors that can also be used in parallel with purine H8 to provide an additional signal verifying the degree of cofactor modification. This invention thus provides any of the methods disclosed herein that utilize the purine H8 resonance with one or more additional steps wherein one or more additional resonances (e.g. any of those indicated above) are monitored in parallel.

[40] This invention thus provides a method for identifying an inhibitor of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as an inhibitor of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of the substrate to the product resulting therefrom. In an embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the inhibitor of said enzyme. In another embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and each individual compound in said mixture so as to determine which compound in said mixture is the inhibitor of said enzyme. In another embodiment of this method the purine-ring-containing substrate is a purine nucleotide, e.g. ATP, ADP, AMP, cAMP, cADPR, GTP, GDP,
GMP, or cGMP. In a further embodiment of this method, during the course of catalysis by the enzyme for which a modulator is sought, one or more additional enzymes are included in the reaction mixture to provide a coupled enzyme reaction. In another embodiment of this method the enzyme is a kinase, a phosphotransferase, a diphosphotransferase, a nucleotidyltransferase, a phosphatidyltransferase, a glycosyl-1-phosphotransferase, a protein kinase, or a nucleotide phosphohydrolase. During the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.

[41] This invention also provides a method for identifying an agonist of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as an agonist of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of the substrate to the product resulting therefrom. In an embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the agonist of said enzyme. In another embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and each individual compound in said mixture so as to determine which compound in said mixture is the agonist of said enzyme. In another embodiment of this method the purine-ring-containing substrate is a purine nucleotide, e.g. ATP, ADP, AMP, cAMP, GTP, GDP, GMP, or cGMP. In a further
embodiment of this method, during the course of catalysis by the enzyme for which a modulator is sought, one or more additional enzymes are included in the reaction mixture to provide a coupled enzyme reaction. In another embodiment of this method the enzyme is a kinase, a phosphotransferase, a diphosphotransferase, a nucleotidyltransferase, a phosphatidyltransferase, a glycosyl-1-phosphotransferase, a protein kinase, or a nucleotide phosphohydrolase. During the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.

[42] In any of the methods of this invention the step of measuring the intensity of a signal for a substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time (typically termed step (a)), i.e. a control step for determining enzyme activity in the absence of a test compound under identical conditions (i.e. the baseline activity), can be conducted contemporaneously with the step conducted in the presence of a test compound (typically termed step (b); i.e. the experimental activity), or can be performed prior to the test assay. The control step can also be a statistical value (e.g., an average or mean) determined for a plurality of control assays. Thus, the baseline value of enzyme activity can be based upon contemporaneous or historical controls. A difference between the experimental and control activities (i.e. the effect of a test compound) can be considered to be "significant" or "statistically significant" if the difference is greater than the experimental error associated with the assay, for example. A difference can also be statistically significant if the probability of the observed difference occurring by chance (the p-value) is less than some predetermined level. As used herein a "statistically significant difference" refers, for example, to a p-value that is <0.05, preferably <0.01 and most preferably <0.001.

[43] In any of the NMR methods of the invention described herein, for the step of measuring the intensity of an NMR signal for a substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, in the presence or absence of a test compound, in order to assess the extent of the enzymic reaction, and
its potential modulation by a test compound, one may pursue any of the following options: (1) Monitor only the individual substrate NMR signal (e.g. for ATP or GTP); (2) Monitor only the individual product NMR signal (e.g. ADP or GDP, or (3) Monitor both the substrate and product NMR signals, and a ratio of signals (e.g. product NMR signal)/(substrate + product NMR signals) may, for example, be determined. Any of these options may be used in the presence or absence of one or more test compounds to monitor the enzyme reaction and identify modulators of its activity (e.g. by comparing the signal change from zero time until a time after a quantity of substrate has been converted to product, with or without test compound). Thus, for example, an inhibitor is readily identified by a decreased production of product, or a reduced loss of substrate, over a period of time. After the start of the enzyme reaction (zero time), these events may be monitored, for example, essentially continuously over the period of time, at one time point after conversion of a quantity of the substrate into the product, or at multiple time points during conversion of a quantity of the substrate into the product. The NMR signal for the resonance being monitored is determined by calculating the area under the peak (or peaks, if spin-spin coupling has occurred).

[44] This invention further provides a method for identifying an inhibitor of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the change in intensity of the purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of the enzyme after a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product resulting therefrom by the enzyme; b) measuring the change in intensity of the purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of the enzyme and a test compound after said period of time; and c) identifying the test compound as an inhibitor of the enzyme by comparing the H8 signal intensity changes measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of substrate to the product resulting therefrom. After the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.
This invention also provides a method for identifying an agonist of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the change in intensity of the purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of the enzyme after a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product resulting therefrom by the enzyme; b) measuring the change in intensity of the purine H8 signal for said substrate, and/or the product resulting therefrom, in the presence of the enzyme and a test compound after said period of time; and c) identifying the test compound as an agonist of the enzyme by comparing the H8 signal intensity changes measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of substrate to the product resulting therefrom. After the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.

This invention also provides a method for identifying an inhibitor of a kinase (e.g. RMCK, a protein kinase, or a lipid kinase), comprising: a) measuring the intensity of the purine H8 signal for ATP, and/or ADP, in the presence of the kinase over a period of time, whereby said period of time is the time length required for conversion of a quantity of the ATP into ADP by the kinase; b) measuring the intensity of the purine H8 signal for the ATP, and/or ADP, in the presence of the kinase and a test compound over said period of time; and c) identifying the test compound as an inhibitor of the kinase by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of ATP to ADP. In an embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the ATP, and/or ADP, is repeatedly measured in the presence of the kinase and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the inhibitor of the kinase enzyme. In another embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for ATP, and/or ADP resulting
therefrom, is repeatedly measured in the presence of the kinase and each individual compound in said mixture so as to determine which compound in said mixture is the inhibitor of said kinase. As an alternative to the above methods, GTP/GDP may be substituted for ATP/ADP in the above methods when the kinase can utilize GTP as a phosphate donor in the reaction.

[47] This invention also provides a method for identifying an agonist (i.e. a simulator, or activator) of a kinase (e.g. RMCK, a protein kinase, a lipid kinase), comprising: a) measuring the intensity of the purine H8 signal for ATP, and/or ADP, in the presence of the kinase over a period of time, whereby said period of time is the time length required for conversion of a quantity of the ATP into ADP by the kinase; b) measuring the intensity of the purine H8 signal for the ATP, and/or ADP, in the presence of the kinase and a test compound over said period of time; and c) identifying the test compound as an inhibitor of the kinase by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of ATP to ADP. In an embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the ATP, and/or ADP, is repeatedly measured in the presence of the kinase and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the agonist of the kinase enzyme. In another embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the ATP, and/or ADP resulting therefrom, is repeatedly measured in the presence of the kinase and each individual compound in said mixture so as to determine which compound in said mixture is the agonist of said kinase. As an alternative to the above methods, GTP/GDP may be substituted for ATP/ADP in the above methods when the kinase can utilize GTP as a phosphate donor in the reaction.

[48] For screening purposes and IC₅₀ measurements, the enzyme reactions of the methods described herein are typically stopped after an established delay that will depend on the enzyme and substrate (e.g. ATP) concentrations and Kₘ. When the substrate is a cofactor for a reaction that requires Mg²⁺ during catalysis, such as ATP or GTP, the reaction is preferably quenched by using a Mg²⁺ chelator, such as EDTA, for example; by adding, for example, an aliquot of a concentrated solution of the
chelator. This facilitates applicability of the assay universally, and means that it is not necessary to optimize the pH and/or Mg$^{2+}$ concentration just for reading the NMR signal for each enzyme assay. A further advantage can be achieved by quenching with an EDTA solution at high pH, such that the pH of the final quenched reaction mixture will be in the range pH 8 to pH 10. At this pH, in the absence of free Mg$^{2+}$, greater separation of the substrate (e.g. ATP) and product (e.g. ADP) purine H8 signals (or pyrimidine H6 signals (see below)) is achieved. Thus, for assaying an enzyme one can choose the pH and Mg$^{2+}$ concentration that is optimal for the enzymic reaction being monitored, and which for most enzymes will be well documented in the scientific literature, or the pH and Mg$^{2+}$ concentration most suitable for the test compound screen being run, and use such a chelator quench to standardize conditions for NMR signal determination. Alternative or additional chelators that may be employed include, but are not limited to, diethylenetriaminepentaacetic acid (DTPA), nitritotriacetic acid (NTA), or (S,S)-ethylendiamine-N,N'-disuccinic acid (EDDS). After the quench, the signal intensity of the purine H8 signal (or pyrimidine H6 signal) is determined. Depending on the reaction being monitored, to determine the extent of the reaction, and its modulation by an inhibitory or stimulating compound, the individual substrate H8 signal (e.g. for ATP or GTP) may be determined, the individual product H8 signal (e.g. ADP or GDP) may be determined, or a ratio of signals (e.g. product H8 signal)/(product H8 + substrate H8 signals) may, for example, be assessed in the presence or absence of one or more test compounds. Similarly, for enzyme reactions involving pyrimidine-ring-containing substrate modification (see below), the individual substrate H6 signal may be determined, the individual product H6 signal may be determined, or a ratio of signals (e.g. product H6 signal)/(product H6 + substrate H6 signals) may, for example, be assessed in the presence or absence of one or more test compounds.

[49] This invention further provides a method for identifying an inhibitor of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the change in intensity of the purine H8 signal for a substrate, and/or product resulting therefrom, in the presence of the enzyme after a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into product by the enzyme, and whereby the purine H8 signal is measured after said conversion is terminated by the addition of a chelator of
magnesium ions; b) measuring the change in intensity of the purine H8 signal for the substrate, and/or product resulting therefrom, in the presence of the enzyme and a test compound after said period of time and termination as in (a); and c) identifying the test compound as an inhibitor of the enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of substrate to product.

[50] This invention further provides a method for identifying an agonist of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the change in intensity of the purine H8 signal for a substrate, and/or product resulting therefrom, in the presence of the enzyme after a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into product by the enzyme, and whereby the purine H8 signal is measured after said conversion is terminated by the addition of a chelator of magnesium ions; b) measuring the change in intensity of the purine H8 signal for the substrate, and/or product resulting therefrom, in the presence of the enzyme and a test compound after said period of time and termination as in (a); and c) identifying the test compound as an agonist of the enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of substrate to product.

[51] This invention further provides a method for identifying an inhibitor of a kinase, comprising: a) measuring the change in intensity of the purine H8 signal for ATP, and/or ADP, in the presence of the kinase after a period of time, whereby said period of time is the time length required for conversion of a quantity of the ATP into ADP by the kinase, and whereby the purine H8 signal is measured after said conversion is terminated by the addition of a chelator of magnesium ions; b) measuring the change in intensity of the purine H8 signal for the ATP, and/or ADP, in the presence of the kinase and a test compound after said period of time and termination as in (a); and c) identifying the test compound as an inhibitor of the kinase by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of ATP to ADP. After the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a
potential modulator of the enzyme activity, it is possible to monitor just the product of
the reaction, just the substrate, or both simultaneously, to determine whether a test
compound is affecting the reaction.

[52] This invention also provides a method for identifying an agonist of a kinase,
comprising: a) measuring the change in intensity of the purine H8 signal for ATP,
and/or ADP, in the presence of the kinase after a period of time, whereby said period
of time is the time length required for conversion of a quantity of the ATP into ADP
by the kinase, and whereby the purine H8 signal is measured after said conversion is
terminated by the addition of a chelator of magnesium ions; b) measuring the change
in intensity of the purine H8 signal for the ATP, and/or ADP, in the presence of the
kinase and a test compound after said period of time and termination as in (a); and c)
identifying the test compound as an agonist of the kinase by comparing the signal
intensities measured in steps (a) and (b) to determine if the test compound has
stimulated the enzymic conversion of ATP to ADP. After the period of time referred
to above when a quantity of the substrate is converted into the product by the enzyme,
in order to monitor the enzyme reaction for the effects of a potential modulator of the
enzyme activity, it is possible to monitor just the product of the reaction, just the
substrate, or both simultaneously, to determine whether a test compound is affecting
the reaction.

[53] This invention also provides a method for identifying a modulator (e.g. an
inhibitor, an agonist) of an enzyme that catalyses the modification of a purine-ring-
containing substrate, comprising: a) measuring the intensity of a purine H8 NMR
signal for said substrate, and/or the product resulting therefrom, in the presence of
said enzyme over a period of time, whereby said period of time is the time length
required for conversion of a quantity of the substrate into the product by said enzyme;
b) measuring the intensity of a purine H8 NMR signal for said substrate, and/or the
product resulting therefrom, in the presence of said enzyme and a test compound over
said period of time; and c) identifying the test compound as a modulator of said
enzyme by comparing the signal intensities measured in steps (a) and (b) to determine
if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion
of the substrate to the product resulting therefrom. In one embodiment of this method
the substrate is a purine nucleobase, a purine nucleoside, a purine nucleoside mono,
di, or tri-phosphate, ATP, ADP, AMP, cAMP, adenine, adenosine, GTP, GDP, GMP, GDP-mannose, GDP-fucose, guanine, guanosine, cGMP, ITP, IDP, IMP, cIMP, inosine, hypoxanthine, XMP, XDP, XTP, cXMP, xanthosine, xanthine, m⁷GDP, m⁷GTP, MTA, SAH, SAM, PAP, PAPS, cADPR, ADPR, NAD, NADP, NADH, NADPH, or NAADP. In another embodiment of this method the product is a purine nucleoside, a purine nucleoside mono, di, or tri-phosphate, ATP, ADP, AMP, cAMP, adenine, adenosine, GTP, GDP, GMP, GDP-mannose, GDP-fucose, guanine, guanosine, cGMP, ITP, IDP, IMP, cIMP, inosine, hypoxanthine, XMP, XDP, XTP, cXMP, xanthosine, xanthine, m⁷GDP, m⁷GTP, MTA, SAH, SAM, PAP, PAPS, cADPR, ADPR, NAD, NADP, NADH, NADPH, or NAADP. In another embodiment the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: ATP/ADP, ADP/AMP, ATP/AMP, cAMP/AMP, ATP/cAMP, AMP/adenosine, Adenosine/adenine, AMP/adenine, GTP/GDP, GDP/GMP, GTP/GMP, cGMP/GMP, GTP/cGMP, GMP/guanosine, Guanosine/guanine, GMP/guanyne, GDP-Man/GDP, GDP-Man/GTP, GDP-Fuc/GDP, ITP/IDP, IDP/IMP, ITP/IMP, IMP/inosine, AMP / IMP, inosine/hypoxanthine, IMP/hypoxanthine, GMP/IMP, IMP/XMP, adenosine/inosine, adenine/hypoxanthine, XTP/XDP, XDP/XMP, XTP/XMP, XMP/xanthosine, XMP/GMP, XMP/xanthine, xanthosine/xanthine, guanine/xanthine, xanthine/hypoxanthine, m⁷GDP/GDP, m⁷GTP/GTP, SAH/adenine, MTA/adenine, SAM/SAH, ATP/SAM, PAPS/PAP, ADPR/cADPR, ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, and NADP/NAADP.

[54] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the hydrolysis of the substrate AMP to the product adenosine (e.g. a nucleotidase), comprising: a) measuring the intensity of the ribose H1' NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a)
and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[55] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate cAMP to the product AMP (e.g. a phosphodiesterase), comprising: a) measuring the intensity of a ribose HI' NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose HI' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[56] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate ATP to the product cAMP (e.g. an adenylyl cyclase), comprising: a) measuring the intensity of a ribose HI' NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose HI' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[57] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the hydrolysis of the substrate GMP to the product guanosine (e.g. a nucleotidase), comprising: a) measuring the intensity of a ribose HI' NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is
the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[58] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate cGMP to the product GMP (e.g. a phosphodiesterase), comprising: a) measuring the intensity of a ribose H1' NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[59] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate GDPman (i.e. GDP-mannose) to the product GDP (e.g. a mannosyltransferases), comprising: a) measuring the intensity of a mannose HI" NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a mannose HI" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.
[60] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate guanosine to the product guanine (a guanosine phosphorylase), comprising: a) measuring the intensity of a ribose HI’ NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose HI’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[61] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate ATP to the product SAM (e.g. a methionine adenosyltransferase), comprising: a) measuring the intensity of a ribose HI’ NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose HI’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[62] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate SAM to the product SAH (e.g. a methyltransferase), comprising: a) measuring the intensity of a ribose HI’ NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is
the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[63] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate adenosine to the product adenine (e.g. an adenosine phosphorylase), comprising: a) measuring the intensity of a purine H2 NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H2 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[64] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate ATP to the product SAM (e.g. a methionine adenosyltransferase), comprising: a) measuring the intensity of a purine H2 NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H2 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered
(e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[65] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate SAM to the product SAH (e.g. a methyltransferase), comprising: a) measuring the intensity of a purine H2 NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H2 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[66] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a ribose H1’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: AMP/adenosine, cAMP/AMP, GMP/guanosine, cGMP/GMP, cGMP/GTP, ATP/SAM, SAM/SAH, m7GDP/GDP, m7GTP/GTP, SAH/adenine, MTA/adenine, cAMP/ATP, IMP/inosine, IMP/GMP, IMP/XMP, inosine/adenosine, XMP/xanthosine, XMP/GMP, cADPR/ADPR, cADPR/NAD, ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, guanosine/guanine;
AMP/adenine, adenosine/adenine, GMP/guanine, IMP/hypoxanthine, inosine/hypoxanthine, XMP/xanthine, and xanthosine/xanthine.

[67] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a purine H2 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H2 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: adenosine/adenine, ATP/SAM, SAM/SAH, SAH/adenine, MTA/adenine, AMP/adenine, inosine/hypoxanthine, IMP/hypoxantine, IMP/AMP, inosine/adenosine, hypoxantine/adenine, cADPR/ADPR, cADPR/NAD, ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, NADP/NAADP, IMP/GMP, IMP/XMP and xanthine/hypoxanthine.

[68] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a sugar H1" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a sugar H1" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing
substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: cADPR/ADPR, cADPR/NAD, ADPR/NAD, ADPR/NADP, NADP/NAADP, GDP/GDP-man, GTP/GDP-man, NAD/NADH and NADP/NADPH.

[69] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a pyridine H2" " NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyridine H2" " NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: NAD/NADH, NADP/NADPH, NADP/NAADP, cADPR/NAD, ADPR/NAD and ADPR/NADP.

[70] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a pyridine H4" " NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyridine H4" " NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting
of the following cofactor pairs: NAD/NADH, NADP/NADPH, NADP/NAADP, cADPR/NAD, ADPR/NAD and ADPR/NADP.

[71] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a pyridine H5" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyridine H5" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: NADP/NAADP, NAD/NADH, NADP/NADPH, cADPR/NAD, ADPR/NAD and ADPR/NADP.

[72] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a pyridine H6" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyridine H6" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: NAD/NADH, NADP/NADPH, NADP/NAADP, cADPR/NAD, ADPR/NAD and ADPR/NADP.
[73] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of purine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the purine H8 NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of purine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: ATP/ADP, ADP/AMP, ATP/AMP, cAMP/AMP, ATP/cAMP, AMP/adenosine, adenosine/adenine, AMP/adenine, GTP/GDP: GDP/GMP, GTP/GMP, cGMP/GMP, GTP/cGMP, GMP/guanosine, guanosine/guanine, GMP/guanine, m7GDP/GDP, m7GTP/GTP, SAH/adenine, MTA/adenine, GDP-Man/GDP, GDP-Man/GTP, GDP-Fuc/GDP, ITP/IDP, IDP/IMP, ITP/IMP, IMP/inosine, AMP / IMP, inosine/hypoxanthine, IMP/hypoxanthine, GMP/IMP, IMP/XMP, adenosine/Inosine, adenine/hypoxanthine: XTP/XDP, XDP/XMP, XTP/XMP, XMP/xanthosine, XMP/GMP, XMP/xanthine, xanthosine/xanthine, guanine/xanthine, xanthine/hypoxanthine, SAM/SAH, ATP/SAM, PAPS/PAP, ADPR/cADPR, ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, and NADP/NAADP. In a further embodiment of this method the enzyme is selected from the group consisting of: a protein kinase, a lipid kinase, a phosphotransferase, a glycosyltransferase, a nucleotide phosphohydrolase and a phosphodiesterase. In a further embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[74] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of purine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the ribose H1’ NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the
pair of purine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: AMP/adenosine, cAMP/AMP, GMP/guanosine, cGMP/GMP, cGMP/GTP, ATP/SAM, SAM/SAH pairs, m\textsuperscript{7}GDP/GDP, m\textsuperscript{7}GTP/GTP, SAH/adenine, MTA/adenine, cAMP/ATP, IMP/inosine, IMP/GMP, IMP/XMP, inosine/adenosine, XMP/xanthosine, XMP/GMP, cADPR/ADPR, cADPR/NAD, ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, guanosine/guanine; AMP/adenine, adenosine/adenine, GMP/guanine, IMP/hypoxanthine, inosine/hypoxanthine, XMP/xanthine, and xanthosine/xanthine. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[75] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of purine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the purine H\textsubscript{2} NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of purine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: adenosine/adenine, ATP/SAM, SAM/SAH, SAH/adenine, MTA/adenine, AMP/adenine, inosine/hypoxanthine, IMP/hypoxantine, IMP/AMP, inosine/adenosine, hypoxantidine/adenine, cADPR/ADPR, cADPR/NAD, ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, NADP/NAADP, IMP/GMP, IMP/XMP and xanthine/hypoxanthine. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[76] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of purine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme...
for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the sugar H1" NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of purine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: cADPR/ADPR, cADPR/NAD, ADPR/NAD, ADPR/NADP, NADP/NAADP, GDP/GDP-man, GTP/GDP-man, NAD/NADH, and NADP/NADPH. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[77] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of purine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the pyridine H2" NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of purine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: NAD/NADH, NADP/NADPH, NADP/NAADP, cADPR/NAD, ADPR/NAD and ADPR/NADP. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[78] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between the pair of cofactors CoA and AcetylCoA, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the NMR signal for the the methyl proton of the acyl group of acetyl CoA of said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the
enzyme. In an embodiment of this method the steps of the method are performed in
both the presence and absence of a test compound, and the difference in the amount of
conversion of substrate to product between these two is used to assess whether the
compound is an inhibitor or an activator of the activity of the enzyme.

[79] This invention further provides a method for determining the activity of an
enzyme that catalyses the conversion between the pair of cofactors m7GTP and GTP,
comprising incubating a cofactor substrate in the presence of said enzyme for a period
of time, and measuring the amount of conversion into the cofactor product after said
period of time, wherein the intensity of the NMR signal for the the methyl proton of
the N-7 methyl group of said substrate and/or the product resulting therefrom is used
to assess the amount of conversion and thus activity of the enzyme. In an embodiment
of this method the steps of the method are performed in both the presence and absence
of a test compound, and the difference in the amount of conversion of substrate to
product between these two is used to assess whether the compound is an inhibitor or
an activator of the activity of the enzyme.

[80] This invention further provides a method for determining the activity of an
enzyme that catalyses the conversion between the pair of cofactors m7GDP and GDP,
comprising incubating a cofactor substrate in the presence of said enzyme for a period
of time, and measuring the amount of conversion into the cofactor product after said
period of time, wherein the intensity of the NMR signal for the the methyl proton of
the N-7 methyl group of said substrate and/or the product resulting therefrom is used
to assess the amount of conversion and thus activity of the enzyme. In an embodiment
of this method the steps of the method are performed in both the presence and absence
of a test compound, and the difference in the amount of conversion of substrate to
product between these two is used to assess whether the compound is an inhibitor or
an activator of the activity of the enzyme.

[81] This invention further provides a method for determining the activity of an
enzyme that catalyses the conversion between a pair of purine-ring-containing
cofactors, comprising incubating a cofactor substrate in the presence of said enzyme
for a period of time, and measuring the amount of conversion into the cofactor
product after said period of time, wherein the intensity of the pyridine H4" NMR
signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of purine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: NAD/NADH, NADP/NADPH, NADP/NAADP, cADPR/NAD, ADPR/NAD and ADPR/NADP. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[82] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of purine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the pyridine H5" NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of purine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: NADP/NAADP, NAD/NADH, NADP/NADPH, cADPR/NAD, ADPR/NAD and ADPR/NADP. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[83] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of purine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the pyridine H6" NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of purine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: NAD/NADH, NADP/NADPH,
NADP/NAADP, cADPR/NAD, ADPR/NAD and ADPR/NADP. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[84] It has been further demonstrated herein (e.g. see Figures 12-14) that several pyrimidine-H6-containing cofactor pairs can also be readily resolved and quantitated using the NMR assay method of this invention by monitoring the H6 signal of the pyrimidine ring, enabling the NMR assay method of this invention to be utilized for the assay of any enzyme that catalyzes interconversion between these cofactors. Assay conditions for all the enzymes that utilize pyrimidine-H6-containing cofactor pairs are generally available in the art as most of these enzymes have been discovered some time ago and their properties have been well-studied. Thus no significant assay development work is required to assay them in the methods of the invention described herein.

[85] It should be noted that only the H6 resonance provides a broadly applicable NMR assay that is capable of monitoring the activity of a broad range of enzymes that catalyze the modification of pyrimidine-ring-containing substrates. The H6 resonance was found to be suitable for monitoring enzyme assays involving all the cofactor pairs examined (see Figures 12-14). NMR proton resonances other than H6 of pyrimidine-containing cofactor pairs were also tested for their potential to be used in an NMR assay, and were only found to be useful in a few specific instances, as noted for example in Figures 12-14 herein. These include: (a) HI' for CTP/CDP, CDP/CMP and CMP/cytidine pairs; and (b) H5 for the CMP/cytidine, cytosine/cytidine, cytosine/uracil, and CMP/cytosine pairs; for all of which a Δδ is readily measurable, and each member of a cofactor pair quantifiable in an NMR assay. In addition, for some pyrimidine-containing cofactor pairs, only one member of the pair was quantifiable with certain resonances, thus enabling an NMR assay to be formatted using the resonance for that cofactor (see Figures 12-14). These include: (a) HI' for thymidine/thymine, CMP/cytosine, and cytidine/cytosine; (b) H5 for UMP/uracil, uridine/uracil, CTP/UTP, and cytidine/uridine pairs; and (c) HI'' for UDP/UDP-GlcNac, UDP/UDP-Gal, and UDP/UDP-GlcA pairs. Use of resonances other than H6
was not always feasible in NMR assays for a variety of reasons, including lack of resolution of cofactor pairs (i.e. substrate and product), peak splitting due to protein spin-spin interactions, overlap with peaks of resonances of other assay components etc. However, when they are feasible, as for the above resonances, they provide an alternative to pyrimidine H6 for monitoring interconversion between the above cofactors that can also be used in parallel with to pyrimidine H6 provide an additional signal verifying the degree of cofactor modification. This invention thus provides any of the methods disclosed herein that utilize the to pyrimidine H6 resonance with one or more additional steps wherein one or more additional resonances (e.g. any of those indicated above) are monitored in parallel.

[86] The present invention thus further provides additional broadly applicable NMR assay methods that overcome the limitations of NMR biochemical screening with respect to protein and substrate concentrations, or labeling requirements (e.g. 19F labeling), when compared to conventional NMR HTS techniques. These methods provide novel and sensitive NMR methods in which the H6 signal of a pyrimidine-ring-containing substrate (e.g. a pyrimidine nucleoside) is used to monitor enzymic reactions that utilize such substrates. These methods have similar advantageous characteristics to those described herein for the purine H8 NMR assay methods, although for uridine and cytidine containing substrates the NMR peaks are doublets rather than singlets.

[87] This invention thus provides a method for identifying an inhibitor of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the intensity of a pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as an inhibitor of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of the substrate to the product resulting therefrom. In an embodiment of this method, in step (b) the test
compound is contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the inhibitor of said enzyme. In another embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and each individual compound in said mixture so as to determine which compound in said mixture is the inhibitor of said enzyme. In another embodiment of this method the pyrimidine-ring-containing substrate is a pyrimidine nucleotide, e.g. CTP, CDP, CMP, cCMP, UTP, UDP, UMP, cUMP, TTP, TDP, TMP, or cTMP.

[88] In a further embodiment of this method, during the course of catalysis by the enzyme for which a modulator is sought, one or more additional enzymes are included in the reaction mixture to provide a coupled enzyme reaction. In another embodiment of this method the enzyme is a kinase (e.g. CTP-dependent galactolipid kinase), a phosphotransferase, a diphosphotransferase, a nucleotidyltransferase, a phosphatidyltransferase (e.g.CDP-diacylglycerol-inositol 3-phosphatidyltransferase), a glycosyl-1-phosphotransferase, or a nucleotide phosphohydrolase. During the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.

[89] This invention also provides a method for identifying an agonist of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the intensity of a pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as an agonist of said enzyme by comparing the signal
intensities measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of the substrate to the product resulting therefrom. In an embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the agonist of said enzyme. In another embodiment of this method, in step (b) the test compound is contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and each individual compound in said mixture so as to determine which compound in said mixture is the agonist of said enzyme. In another embodiment of this method the pyrimidine-ring-containing substrate is a pyrimidine nucleotide, e.g. CTP, CDP, CMP, cCMP, UTP, UDP, UMP, cUMP, TTP, TDP, TMP, or cTMP. In a further embodiment of this method, during the course of catalysis by the enzyme for which a modulator is sought, one or more additional enzymes are included in the reaction mixture to provide a coupled enzyme reaction. In another embodiment of this method the enzyme is a kinase (e.g. CTP-dependent galactolipid kinase), a phosphotransferase, a diphosphotransferase, a nucleotidytransferase, a phosphatidytransferase (e.g. CDP-diacylglycerol-inositol 3-phosphatidytransferase), a glycosyl-1-phosphotransferase, or a nucleotide phosphohydrolase. During the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.

[90] This invention further provides a method for identifying an inhibitor of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the change in intensity of the pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of the enzyme after a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product resulting therefrom by the enzyme; b) measuring the change in intensity of the pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of the
enzyme and a test compound after said period of time; and c) identifying the test compound as an inhibitor of the enzyme by comparing the H6 signal intensity changes measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of substrate to the product resulting therefrom. After the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.

[91] This invention also provides a method for identifying an agonist of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the change in intensity of the pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of the enzyme after a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product resulting therefrom by the enzyme; b) measuring the change in intensity of the pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of the enzyme and a test compound after said period of time; and c) identifying the test compound as an agonist of the enzyme by comparing the H6 NMR signal intensity changes measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of substrate to the product resulting therefrom. After the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.

[92] This invention also provides a method for identifying an inhibitor of a kinase (e.g. a CTP-dependent lipid kinase), comprising: a) measuring the intensity of the pyrimidine H6 signal for NTP, and/or NDP, in the presence of the kinase over a period of time, whereby said period of time is the time length required for conversion of a quantity of the NTP into NDP by the kinase; b) measuring the intensity of the pyrimidine H6 signal for the NTP, and/or NDP, in the presence of the kinase and a
test compound over said period of time; and c) identifying the test compound as an 
inhibitor of the kinase by comparing the signal intensities measured in steps (a) and 
(b) to determine if the test compound has inhibited the enzymic conversion of NTP to 
NDP. In this method NTP may be CTP, UTP or TTP; and NDP may be CDP, UDP or 
TDP. In an embodiment of this method, in step (b) the test compound is contained in 
a mixture of compounds, and the signal intensity for the NTP, and/or NDP, is 
repeatedly measured in the presence of the kinase and said mixture in the absence of 
each compound contained by said mixture so as to determine which compound in said 
mixture is the inhibitor of the kinase enzyme. In another embodiment of this method, 
in step (b) the test compound is contained in a mixture of compounds, and the signal 
intensity for the NTP, and/or NDP, resulting therefrom, is repeatedly measured in the 
presence of the kinase and each individual compound in said mixture so as to 
determine which compound in said mixture is the inhibitor of said kinase.

[93] This invention also provides a method for identifying an agonist of a kinase 
(e.g. a CTP-dependent lipid kinase), comprising: a) measuring the intensity of the 
pyrimidine H6 NMR signal for NTP, and/or NDP, in the presence of the kinase over a 
period of time, whereby said period of time is the time length required for conversion 
of a quantity of the NTP into NDP by the kinase; b) measuring the intensity of the 
pyrimidine H6 NMR signal for the NTP, and/or NDP, in the presence of the kinase 
and a test compound over said period of time; and c) identifying the test compound as 
an agonist of the kinase by comparing the signal intensities measured in steps (a) and 
(b) to determine if the test compound has stimulated the enzymic conversion of NTP 
to NDP. In this method NTP may be CTP, UTP or TTP; and NDP may be CDP, UDP 
or TDP. In an embodiment of this method, in step (b) the test compound is contained 
in a mixture of compounds, and the signal intensity for the NTP, and/or NDP, is 
repeatedly measured in the presence of the kinase and said mixture in the absence of 
each compound contained by said mixture so as to determine which compound in said 
mixture is the agonist of the kinase enzyme. In another embodiment of this method, in 
step (b) the test compound is contained in a mixture of compounds, and the signal 
intensity for the NTP, and/or NDP, resulting therefrom, is repeatedly measured in the 
presence of the kinase and each individual compound in said mixture so as to 
determine which compound in said mixture is the agonist of said kinase.
This invention further provides a method for identifying an inhibitor of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the change in intensity of the pyrimidine H6 NMR signal for a substrate, and/or product resulting therefrom, in the presence of the enzyme after a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into product by the enzyme, and whereby the pyrimidine H6 NMR signal is measured after said conversion is terminated by the addition of a chelator of magnesium ions; b) measuring the change in intensity of the pyrimidine H6 signal for the substrate, and/or product resulting therefrom, in the presence of the enzyme and a test compound after said period of time and termination as in (a); and c) identifying the test compound as an inhibitor of the enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of substrate to product.

This invention further provides a method for identifying an agonist of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the change in intensity of the pyrimidine H6 NMR signal for a substrate, and/or product resulting therefrom, in the presence of the enzyme after a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into product by the enzyme, and whereby the pyrimidine H6 NMR signal is measured after said conversion is terminated by the addition of a chelator of magnesium ions; b) measuring the change in intensity of the pyrimidine H6 signal for the substrate, and/or product resulting therefrom, in the presence of the enzyme and a test compound after said period of time and termination as in (a); and c) identifying the test compound as an agonist of the enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of substrate to product.

This invention further provides a method for identifying an inhibitor of a kinase, comprising: a) measuring the change in intensity of the pyrimidine H6 signal for NTP, and/or NDP, in the presence of the kinase after a period of time, whereby said period of time is the time length required for conversion of a quantity of the NTP into NDP by the kinase, and whereby the pyrimidine H6 NMR signal is measured after said conversion is terminated by the addition of a chelator of magnesium ions; b)
measuring the change in intensity of the pyrimidine H6 NMR signal for the NTP, and/or NDP, in the presence of the kinase and a test compound after said period of time and termination as in (a); and c) identifying the test compound as an inhibitor of the kinase by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has inhibited the enzymic conversion of NTP to NDP. In this method NTP may be CTP, UTP or TTP; and NDP may be CDP, UDP or TDP. After the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.

[97] This invention also provides a method for identifying an agonist of a kinase, comprising: a) measuring the change in intensity of the pyrimidine H6 signal for NTP, and/or NDP, in the presence of the kinase after a period of time, whereby said period of time is the time length required for conversion of a quantity of the NTP into NDP by the kinase, and whereby the pyrimidine H6 NMR signal is measured after said conversion is terminated by the addition of a chelator of magnesium ions; b) measuring the change in intensity of the pyrimidine H6 NMR signal for the NTP, and/or NDP, in the presence of the kinase and a test compound after said period of time and termination as in (a); and c) identifying the test compound as an agonist of the kinase by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of NTP to NDP. In this method NTP may be CTP, UTP or TTP; and NDP may be CDP, UDP or TDP. After the period of time referred to above when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction.

[98] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the intensity of a pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence
of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyrimidine H$^6$NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom. In one embodiment of this method the substrate is a pyrimidine containing compound, CTP, CDP, CMP, cytidine, cytosine, cCMP, CMP-Neu5Ac, UTP, UDP, UMP, uridine, uracil, cUMP, TTP, TDP, TMP, thymidine, thymine, UDP-GlcNac, UDP-GlcA, UDP-Gal, or cTMP. In another embodiment of this method the product is a pyrimidine containing compound, CTP, CDP, CMP, cytidine, cytosine, cCMP, CMP-Neu5Ac, UTP, UDP, UMP, uridine, uracil, cUMP, TTP, TDP, TMP, thymidine, thymine, UDP-GlcNac, UDP-GlcA, UDP-Gal, or cTMP. In another embodiment the pyrimidine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: UTP/UDP, UDP/UMP, UTP/UMP, UMP/Uridine, Uridine/Uracil, UMP/Uracil, UDP-GlcA/UDP, UDP-GlcNac/UDP, UDP/UDP-Gal, CTP/CDP, CDP/CMP, CMP/Cytidine, CMP/Cytosine, CTP/CMP, Cytidine/Cytosine, CMP-Neu5Ac/CMP, UTP/CTP, Uridine/Cytidine, Uracil/Cytosine, TTP/TDP, TDP/TMP, TTP/TMP, TMP/thymidine and thymidine/thymine.

This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate UDP-GlcNac to the product UDP, comprising: a) measuring the intensity of an N-acetyl glucosamine H$^1$ NMR signal for the substrate in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of an N-acetyl glucosamine H$^1$ NMR signal for said substrate in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.
[100] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate UDP-GlcA to the product UDP, comprising: a) measuring the intensity of a glucosamine H-1 NMR signal for the substrate in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a glucosamine H-1 NMR signal for said substrate in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[101] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate UDP-Gal to the product UDP, comprising: a) measuring the intensity of a galactose H-1 NMR signal for the substrate in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a galactose H-1 NMR signal for said substrate in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[102] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate CMP to the product cytosine (e.g. a nucleotidase), comprising: a) measuring the intensity of a pyrimidine H-5 NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyrimidine H-5 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a
test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[103] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate CTP to the product CDP (e.g. a kinase), comprising: a) measuring the intensity of a ribose H1’ NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[104] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate CDP to the product CMP (e.g. a phosphohydolase), comprising: a) measuring the intensity of a ribose H1’ NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[105] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the conversion of the substrate CMP to the product cytosine (e.g. a nucleotidase), comprising: a) measuring the intensity of
a ribose H1' NMR signal for the substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom.

[106] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a pyrimidine ring-containing substrate, comprising: a) measuring the intensity of a ribose H1' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the pyrimidine ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: CTP/CDP, CDP/CMP, CMP/cytidine, thymidine/thymine, CMP/cytosine, and cytidine/cytosine.

[107] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a pyrimidine ring-containing substrate, comprising: a) measuring the intensity of a pyrimidine H5 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyrimidine H5 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound.
over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the pyrimidine -ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: CMP/cytidine, cytosine/cytidine, cytosine/uracil, CMP/cytosine, UMP/uracil, uridine/uracil, CTP/UTP, and cytidine/uridine.

[108] This invention also provides a method for identifying a modulator (e.g. an inhibitor, an agonist) of an enzyme that catalyses the modification of a pyrimidine -ring-containing substrate, comprising: a) measuring the intensity of a glucosamine H1" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a glucosamine H1" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered (e.g. inhibited, stimulated) the enzymic conversion of the substrate to the product resulting therefrom; wherein the pyrimidine -ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: UDP/UDP-GlcNac and UDP/UDP-GlcA.

[109] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of pyrimidine -ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the pyrimidine H6 NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of pyrimidine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: UTP/UDP, UDP/UMP, UTP/UMP,
UMP/Uridine, Uridine/Uracil, UMP/Uracil, UDP-GlcA/UDP, UDP-GlcNac/UDP, UDP/UDP-Gal, CTP/CDP, CDP/CMP, CMP/Cytidine, CMP/Cytosine, CTP/CMP, Cytidine/Cytosine, CMP-Neu5Ac/CMP, UTP/CTP, Uridine/Cytidine, Uracil/Cytosine, TTP/TDP, TDP/TMP, TTP/TMP, TMP/thymidine, and thymidine/thymine. In a further embodiment of this method the enzyme is selected from the group consisting of: a protein kinase, a lipid kinase, a phosphotransferase, a phosphatidyltransferase, a glycosyltransferase, a nucleotide phosphohydrolase and a phosphodiesterase. In a further embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[110] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of pyrimidine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the ribose HI' NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of pyrimidine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: CTP/CDP, CDP/CMP, CMP/cytidine, thymidine/thymine, CMP/cytosine and cytidine/cytosine. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[111] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of pyrimidine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the glucosamine HI" NMR signal for said substrate and/or the product resulting therefrom is used to assess the
amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of pyrimidine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: UDP/UDP-GlcNac and UDP/UDP-GlcA. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[112] This invention further provides a method for determining the activity of an enzyme that catalyses the conversion between a pair of pyrimidine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the pyrimidine H5 NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme. In an embodiment of this method the pair of pyrimidine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: CMP/cytidine, cytosine/cytidine, cytosine/uracil, CMP/cytosine, UMP/uracil, uridine/uracil, CTP/UTP, and cytidine/uridine. In an embodiment of this method the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

[113] In an embodiment of any of the methods of this invention the purine-ring-containing or pyrimidine-ring-containing substrate is a nucleoside, e.g. a purine-ring-containing nucleotide such as ATP, ADP, AMP, cAMP, cADPR, GTP, GDP, GMP, or cGMP (wherein the H8 NMR signal is measured); or a pyrimidine-ring-containing nucleotide such as CTP, CDP CMP, cCMP, UTP, UDP, UMP, cUMP, TTP, TDP, TMP, or cTMP (wherein the H6 NMR signal is measured). In a further embodiment of any of the methods, during the course of catalysis by the enzyme for which a modulator is sought, one or more additional enzymes are included in the reaction mixture to provide a coupled enzyme reaction. For any of the methods of the invention, during the period of time when a quantity of the substrate is converted into the product by the enzyme, in order to monitor the enzyme reaction for the effects of a
potential modulator of the enzyme activity, it is possible to monitor just the product of the reaction, just the substrate, or both simultaneously, to determine whether a test compound is affecting the reaction. In another embodiment of any of the methods of the invention wherein the enzyme reaction requires Mg\(^{2+}\) as a cofactor, after conversion of a quantity of substrate to product in the steps of the method (with or without test compound), the enzyme reaction is terminated by the addition of a chelator of Mg\(^{2+}\) prior to measurement of the NMR signal and determination of whether the test compound is a modulator (e.g. an inhibitor, an agonist) of the enzyme. When metal ion is not required for the enzyme activity, the assay can be stopped by adding enzyme deactivators, known potent inhibitors of the enzyme, or protein denaturing agents, such as, for example, chaotropic agents or certain detergents (e.g. sodium dodecyl sulfate). In another embodiment of any of the methods of the invention the NMR signal is determined at a pH that is the same or different from that of the enzyme reaction by adding a quantity of buffer, acid or base prior to measuring the signal (e.g. the pH at which the signal is determined may be pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, or any pH between these values). This pH adjustment prior to NMR signal determination may be performed simultaneously with the addition of a Mg\(^{2+}\) chelating agent such as EDTA. The pH may be chosen in order to optimize the measurement of substrate and product (e.g. to maximize peak resolution). In another embodiment of any of the methods of the invention the test compound is contained in a mixture of compounds, and may be identified in a second assay by testing individual compounds.

[114] As screening methods, the methods of this invention can also be carried out in the presence of a mixture of compounds containing an inhibitor. Then, deconvolution of the active mixture is performed to identify which of the compounds in the mixture is the inhibitor. In accordance with the present invention, deconvolution may mean that the reaction is performed repeatedly in the presence of the mixture and in the absence of each one of the compounds. That is, there is no inhibition when the reaction in is carried out in the presence of the mixture minus the inhibitory compound. To confirm the inhibitory effect of one of the compounds, inhibition can be observed when the reaction is also performed in the presence of the single inhibitory compound that was identified from the mixture. Deconvolution may also be
performed by performing the reaction in the presence of each of the individual compounds in the mixture where inhibitory activity was observed.

[115] Thus, in an embodiment of any of the methods of this invention, in the step of the method wherein a test compound is added to the enzyme reaction (i.e. step (b)), the test compound may be contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, may be repeatedly measured in the presence of the enzyme and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the modulator of said enzyme.

[116] Alternatively, in an embodiment of any of the methods of this invention, in the step of the method wherein a test compound is added to the enzyme reaction (i.e. step (b)), the test compound may be contained in a mixture of compounds, and the signal intensity for the substrate, and/or the product resulting therefrom, may be repeatedly measured in the presence of the enzyme and each individual compound in said mixture so as to determine which compound in said mixture is the modulator of said enzyme.

[117] The Michaelis constant of the substrate (e.g. ATP) may be calculated first in order to optimize the substrate concentration used for the screening experiments, and for deriving the binding constant of the hits from their IC$_{50}$. For this purpose, reactions at different substrate concentrations are recorded.

[118] In accordance with the present invention, screening may be first performed at a single concentration for the compounds in the library. Compounds can be screened in small or large mixtures. For example, if only strong inhibitors with IC$_{50}$ < 5 µM will be considered, then it will be sufficient to use molecules at about a 5 µM concentration. In practice, the reliability of the method of the present invention allows the identification of a weaker inhibitor with, for example, an IC$_{50}$ in the range of 10-20 µM, even when the concentration of the screened molecules is only about 5 µM. For the characterization of the IC$_{50}$ of hits, experiments at different inhibitor concentrations are performed.
[119] The methods of the present invention represent simple and reliable enzymic assays because they are homogeneous, and can in most instances directly detect both the product and substrate via for example, their purine H8 or pyrimidine H6 signals. The NMR assay does not require i) the presence of secondary reactions performed with enzymes or specific antibodies or ii) separation and/or washing steps necessary for the readout with other methods. The method's simplicity results in reliable lead molecule identification, and quantification of the inhibitory activity of molecules. Compounds displaying only a weak inhibitory activity can also be safely selected. Small chemical changes of the weak inhibitors or the selection of similar molecules bearing the same scaffold may result in the identification of potent inhibitors.

[120] In other art recognized HTS techniques, often the real compound concentration differs from the nominal concentration. A large difference in compound concentration may results in a significant error of the derived IC50. The causes for concentration differences in other prior art HTS techniques can be ascribed to weighing errors, sample impurity, poor solubility of the compound, and chemical instability in an aqueous environment. In contrast, with the NMR method of the present invention these chemical properties can be easily measured by acquiring, in addition to the purine H8 signal, also a proton signal for the inhibitory or stimulatory compound. Therefore, the real concentration of the compound determined with H NMR allows a significantly more accurate measurement of the IC50 value.

[121] The methods of the present invention may be used for the identification of a variety of types of modulators of enzyme activity that affect the interconversion of the cofactors being monitored by NMR, e.g. inhibitors, activators, stimulators, enhancers, agonists, partial agonists, antagonists, inverse agonists, partial inverse agonists, allosteric or allotopic antagonists, allosteric or allotopic agonists, etc. For example, in the case of a protein kinase reaction, the purine H8 signal of the product ADP in the presence of an inhibitor is smaller when compared to the same signal of the reference sample (i.e. sample for which the reaction was performed in the absence of compounds to be screened). Similarly, in the case of a protein kinase reaction, the purine H8 signal of the product ADP in the presence of an agonist is larger when compared to the same signal of the reference sample (i.e. sample for which the reaction was performed in the absence of compounds to be screened).
[122] The concentration of the protein used with the NMR assay method of the invention described herein can be as low as a few nanomolar, which compares favorably with the concentration used in other HTS techniques. However, the volume necessary for each NMR sample using a 5 mm probe is about 500-550 µL. A 2- to 3-fold volume reduction may be achieved in accordance with the present invention with the use of a flow-injection probe, or a micro probe such as 1 mm or 1.7 mm probe. The high sensitivity of the purine H8 signal allows for rapid acquisition of the spectra. Further, the same spectra can be recorded even more rapidly with the use of cryogenic technology applied to the detection. Therefore, for example, under the settings of the current exemplified method, the spectra that require an acquisition time of about 6 minutes should be recorded in just 10 seconds.

[123] Bovine serum albumin (BSA), human serum albumin (HSA), other proteins, and/or non-ionic detergents, or other stabilizing agents, may be used with the method of the present invention to avoid sticking of the protein to the tube wall and/or protein aggregation [Hlady, V. et al. Curr. Opin. Biotechnol. 7, 72 77 (1996)]. However, while the enzymatic reaction in the presence of such agents becomes faster due to the fact that all the enzyme is available in solution, the IC50 of the compound can become weaker because of the compound sequestering from the solution by BSA or other proteins. Therefore, the present method performed in the presence or absence of such agents, for compounds in the hit to lead optimization phase, can provide important structural information for designing analogues with retained inhibitory activity to the target enzyme and reduced affinity to albumin.

[124] The methods of the present invention may be applied to many different types of enzymes that, for example, catalyse the modification of a purine-ring-containing or pyrimidine-ring-containing substrate, including, but not limited to the following listed enzymes that catalyse conversion between the cofactor pairs listed. Since NMR signals for the following cofactor pairs are readily resolvable and quantifiable by the NMR methods described herein (e.g. using the NMR resonances described in Figures 10-18, e.g. purine H8, pyrimidine H6), any enzyme that catalyses conversion between the following cofactor pairs, as listed below, should be capable of assay by the NMR assay methods disclosed herein, assuming there are no overriding technical issues that
would preclude its use in such an assay (e.g. if the enzyme was not soluble in an aqueous environment, and a suitable solubilizer could not be found):

[125] **ATP/ADP:** transferase, hydrolase, ligase: such as phosphotransferase (EC 2.7), ATPase (EC 3.6.3), kinesin (EC 3.6.4), **detailed examples:** nucleoside-diphosphate kinase (EC 2.7.4.6)(in presence of NTP/NDP), thymidine kinase (EC 2.7.1.21)(in presence of Thd/TMP), pyruvate kinase (EC 2.7.1.40), adenosinetriphosphatase (EC 3.6.1.3), NTPDase (EC 3.6.1.5), myosin ATPase (3.6.4.1), ATP diphosphatase (EC 3.6.1.8), ecto-ATPase (EC 3.6.1.15), CTP synthase (EC 6.3.4.2)(in presence of UTP/CDP), ATP synthase (EC 3.6.3.14).

[126] **ADP/AMP:** NDPase (EC 3.6.1.6), NTPDase (EC 3.6.1.5), adenylate kinase (EC 2.7.4.3) (ATP+AMP—2ADP), ADP-thymidine kinase (EC 2.7.1.18) (in presence of Thd/TMP), nucleoside-triphosphate—adenylate kinase (EC 2.7.4.10) (in presence of UTP/UDP).

[127] **ATP/AMP:** NTPDase (EC 3.6.1.5), ATP diphosphatase (EC 3.6.1.8), GMP synthase (EC 6.3.4.1) (in presence of XMP/GMP), GMP synthase (glutamine-hydrolysing) (EC 6.3.5.2) (in presence of ATP/AMP), phenylalanine racemase (ATP-hydrolysing) (EC 5.1.1.11).

[128] **cAMP/AMP:** cyclic nucleotide phosphodiesterases (PDE, EC 3.1.4.17), 3',5'-cyclic-AMP phosphodiesterase (EC 3.1.4.53).

[129] **ATP/cAMP:** adenylate cyclase (EC 4.6.1.1) and guanylate cyclase (EC 4.6.1.2).

[130] **AMP/adenosine:** adenosine kinase (EC 2.7.1.20) (in presence of ATP/ADP), deoxycytidine kinase (EC 2.7.1.74) (in presence of ATP/ADP), AMP—thymidine kinase (EC 2.7.1.14) (in presence of Thd/TMP), 5’-nucleotidase (EC 3.1.3.5)

[131] **Adenosine/adenine:** Purine nucleoside phosphorylase (PNPase) (EC 2.4.2.1), purine nucleosidase (EC 3.2.2.1), adenosine nucleosidase (EC 3.2.2.7), ribosylpyrimidine nucleosidase (EC 3.2.2.8)

[132] **AMP/adenine:** AMP nucleosidase (EC 3.2.2.4), adenine phosphoribosyltransferase (EC 2.4.2.7)

[133] **GTP/GDP:** GTPase (EC 3.6.5), ecto-ATPase (EC 3.6.1.15), NTPDase (EC 3.6.1.5), pyruvate kinase (EC 2.7.1.40), nucleoside diphosphate kinase (EC 2.7.4.6) (in presence of ATP/ADP)

[134] **GDP/GMP:** NDPase (EC 3.6.1.6), NTPDase (EC 3.6.1.5), ATP:GMP phosphotransferase (EC 2.7.4.8)
GTP/GMP: NTPDase (EC 3.6.1.5), ATP diphosphatase (EC 3.6.1.8), nucleoside-triphosphate diphosphatase (EC 3.6.1.19)

cGMP/GMP: cyclic nucleotide phosphodiesterases (PDE, EC 3.1.4.17)

GTP/cGMP: adenylate cyclase (EC 4.6.1.1) and guanylate cyclase (EC 4.6.1.2)

GMP/guanosine: 5'-nucleotidase (EC 3.1.3.5), inosine kinase (EC 2.7.1.73) (in presence of ATP/ADP)

Guanosine/guanine: Purine nucleoside phosphorylase (PNPase) (EC 2.4.2.1), guanosine phosphorylase (EC 2.4.2.15), purine nucleosidase (EC 3.2.2.1), ribosylpyrimidine nucleosidase (EC 3.2.2.8)

GMP/guanine: adenine phosphoribosyltransferase (EC 2.4.2.7), hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), xanthine phosphoribosyltransferase (EC 2.4.2.22)

GDP-Man/GDP: Glycosyltransferase (EC 2.4), detailed examples: glucomannan 4-beta-mannosyltransferase (EC 2.4.1.32), heteroglycan alpha-mannosyltransferase (EC 2.4.1.48)

GDP-Man/GTP: mannose-1-phosphate guanylyltransferase (EC 2.7.7.13)

GDP-Fuc/GDP: Glycosyltransferase (EC 2.4), detailed examples: glycoprotein 6-alpha-L-fucosyltransferase (EC 2.4.1.68), glycoprotein 3-alpha-L-fucosyltransferase (EC 2.4.1.214), peptide-O-fucosyltransferase (EC 2.4.1.221)

ITP/IDP: nucleoside-diphosphate kinase (EC 2.7.4.6) (in presence of ATP/ADP), NTPDase (EC 3.6.1.5)

IDP/IMP: NDPase (EC 3.6.1.6), NTPDase (EC 3.6.1.5)

ITP/IMP: ATP diphosphatase (EC 3.6.1.8), nucleoside-triphosphate diphosphatase (EC 3.6.1.19)

IMP/inosine: 5'-nucleotidase (EC 3.1.3.5), inosine kinase (EC 2.7.1.73) (in presence of ATP/ADP)

Inosine / Hypoxanthine: Purine nucleoside phosphorylase (PNPase)(EC 2.4.2.1), guanosine phosphorylase (EC 2.4.2.15), purine nucleosidase (EC 3.2.2.1), ribosylpyrimidine nucleosidase (EC 3.2.2.8), inosine nucleosidase (EC 3.2.2.2)

IMP/hypoxanthine: hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), inosinate nucleosidase (EC 3.2.2.12)

AMP / IMP: AMP deaminase (EC 3.5.4.6)

GMP/IMP in presence of NADPH/NADP+: GMP reductase (EC 1.7.1.7)
IMP/XMP in presence of NAD+/NADH: IMP dehydrogenase (EC 1.1.1.205)
Adenosine / Inosine: adenosine deaminase (EC 3.5.4.4)
Adenine / Hypoxanthine: adenine deaminase (EC 3.5.4.2)
XTP/XDP: adenylosuccinate synthase (EC 6.3.4.4), nucleotide-triphosphatase (EC 3.6.1.15), glycerol kinase (EC 2.7.1.30), mevalonate kinase (EC 2.7.1.36), polyphosphate-glucose phosphotransferase (EC 2.7.1.63), protein-synthesizing GTPase (EC 3.6.5.3), tubulin GTPase (EC 3.6.5.6), UMP/CMP kinase (EC 2.7.4.14) in presence of CMP/CDP, DNA helicase (EC 3.6.4.12), nucleoside-diphosphate kinase (EC 3.6.4.6) in presence of ATP/ADP
XDP/XMP: protein-synthesizing GTPase (EC 3.6.5.3)
XTP/XMP: nucleoside-triphosphate diphosphatase (EC 3.6.1.19)
XMP/xanthosine: 5'-nucleotidase (EC 3.1.3.5)
Xanthosine / xanthine: Purine nucleoside phosphorylase (PNPase) (EC 2.4.2.1), purine nucleosidase (EC 3.2.2.1)
XMP / xanthine: hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), xanthine phosphoribosyltransferase (EC 2.4.2.22)
XMP/GMP in presence of ATP/GMP: GMP synthase (EC 6.3.4.1), GMP synthase (glutamine-hydrolysing) (EC 6.3.5.2)
Guanine / xanthine: guanine deaminase (guanase) (EC 3.5.4.3)
Xanthine / Hypoxanthine: xanthine oxidase (EC 1.17.3.2), xanthine dehydrogenase (EC 1.17.1.4) in presence of NAD/NADH
UTP/UDP: ecto-ATPase (EC 3.6.1.15), NTPDase (EC 3.6.1.5), thymidine-triphosphatase (EC 3.6.1.39), nucleoside-diphosphate kinase (EC 2.7.4.6) in presence of ATP/ADP, nucleoside-triphosphate-adenylate kinase (EC 2.7.4.10) in presence of ADP/AMP
UDP/UMP: NDPase (EC 3.6.1.6), NTPDase (EC 3.6.1.5), UMP/CMP kinase (EC 2.7.4.14) in presence of ATP/ADP, UMP kinase (EC 2.7.4.22) in presence of ATP/ADP, nucleoside-phosphate kinase (EC 2.7.4.4) in presence of ATP/ADP
UTP/UMP: NTPDase (EC 3.6.1.5), nucleoside-triphosphate diphosphatase (EC 3.6.1.19), ATP diphosphatase (EC 3.6.1.8)
UMP/Uridine: 5'-nucleotidase (EC 3.1.3.5), uridine kinase (EC 2.7.1.48) in presence of ATP/ADP
Uridine/Uracil: uridine phosphorylase (EC 2.4.2.3), uridine nucleosidase (EC 3.2.2.3)
[169] **UMP/Uracil**: uracil phosphoribosyltransferase (EC 2.4.2.9)

[170] **UDP-GlcA/UDP**: Glycosyltransferase (EC 2.4), **detailed examples**: UDPglucuronate beta-D-glucuronosyltransferase (EC 2.4.1.17)

[171] **UDP-GlcNac/UDP**: Glycosyltransferase (EC 2.4), **detailed examples**: chitin synthase (EC 2.4.1.16), steroid N-acetylgalactosaminyltransferase (EC 2.4.1.39), lipopolysaccharide N-acetylgalactosaminyltransferase (EC 2.4.1.56)

[172] **CTP/CDP**: ecto-ATPase (EC 3.6.1.15), NTPdase (EC 3.6.1.5), nucleoside-diphosphate kinase (EC 2.7.4.6) (in presence of ATP/ADP)

[173] **CDP/CMP**: NDPase (EC 3.6.1.6), NTPdase (EC 3.6.1.5), UMP/CMP kinase (EC 2.7.4.14) (in presence of ATP/ADP)

[174] **CTP/CMP**: NTPdase (EC 3.6.1.5), ATP diphosphatase (EC 3.6.1.8)

[175] **CMP/Cytidine**: 5'-nucleotidase (EC 3.1.3.5), uridine kinase (EC 2.7.1.48) (in presence of ATP/ADP)

[176] **Cytidine/Cytosine**: pyrimidine-nucleoside phosphorylase (EC 2.4.2.2), ribosylpyrimidine nucleosidase (EC 3.2.2.8)

[177] **CMP/Cytosine**: pyrimidine-5'-nucleotide nucleosidase (EC 3.2.2.10)

[178] **CMP-Neu5Ac/CMP**: Glycosyltransferase (EC 2.4), **detailed examples**: betagalactoside alpha-2,6-sialyltransferase (EC 2.4.99.1), alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase (EC 2.4.99.3)

[179] **UTP/CTP**: CTP synthase (EC 6.3.4.2) (in presence of ATP/ADP), dCTP deaminase (EC 3.5.4.13)

[180] **Uridine/Cytidine**: cytidine deaminase (EC 3.5.4.5)

[181] **Uracil/Cytosine**: cytosine deaminase (EC 3.5.4.1)

[182] **TTP/TDP**: nucleoside-diphosphate kinase (EC 2.7.4.6) (in presence of ATP/ADP), apyrase (EC 3.6.1.5), thymidine-triphosphatase (EC 3.6.1.39)

[183] **TDP/TMP**: TMP kinase (EC 2.7.4.9) (in presence of ATP/ADP), apyrase (EC 3.6.1.5)

[184] **TTP/TMP**: deoxycytidine kinase (EC 2.7.1.74)

[185] **TMP/thymidine**: thymidine kinase (EC 2.7.1.21) (in presence of ATP/ADP), ADP-thymidine kinase (EC 2.7.1.18) (in presence of ADP/AMP), AMP—thymidine kinase (EC 2.7.1.14) (in presence of AMP/adenosine), 5'-nucleotidase (EC 3.1.3.5)

[186] **Thymidine/thymine**: nucleoside deoxyribosyltransferase (EC 2.4.2.6), thymidine phosphorylase (EC 2.4.2.4)

[187] **SAM/SAH**: methyltransferase (EC 2.1.1)
ATP/SAM: methionine adenosyltransferase (EC 2.5.1.6)
PAPS/PAP: Sulfotransferase (EC 2.8.2)
ADPR/cADPR: NAD+ nucleosidase (EC 3.2.2.5)
ADPR/NAD: NAD+ nucleosidase (EC 3.2.2.5)
cADPR/NAD: NAD+ nucleosidase (EC 3.2.2.5)
ADPR/NADP: NAD+ nucleosidase (EC 3.2.2.5)
CoA/AcetylCoA: pyruvate oxidase (CoA-acetylating) (EC 1.2.3.6), pyruvate dehydrogenase (acetyl-transferring) (EC 1.2.4.1)
NAD/NADH: oxidoreductase (EC 1), detailed examples: alcohol dehydrogenase (EC 1.1.1.1), aldehyde dehydrogenase (NAD+) (EC 1.2.1.3), dihydropyrimidine dehydrogenase (NAD+) (EC 1.3.1.1)
NADP/NADPH: oxidoreductase (EC 1), detailed examples: alcohol dehydrogenase (EC 1.1.1.1), aldehyde dehydrogenase (NAD+) (EC 1.2.1.3), dihydropyrimidine dehydrogenase (NAD+) (EC 1.3.1.1)
NADP/NAADP: NAD+ nucleosidase (EC 3.2.2.5).
SAH/adenine: adenosylhomocysteine nucleosidase (EC 3.2.2.9).
MTA/adenine: adenosylhomocysteine nucleosidase (EC 3.2.2.9), methylthioadenosine nucleosidase (EC 3.2.2.16), S-methyl-5'-thioadenosine phosphorylase (EC 2.4.2.28).
m\textsuperscript{5}GTP/GTP: mRNA (guanine-N7)-methyltransferase (EC 2.1.1.56) (in presence of SAM and SAH).
m\textsuperscript{5}GDP/GDP: mRNA (guanine-N7)-methyltransferase (EC 2.1.1.56) (in presence of SAM and SAH).
UDP-Gal/UDP: glycosyltransferase (EC 2.4.1.-), detailed examples: lactose synthase (EC 2.4.1.22), fucosylgalactoside 3-alpha-galactosyltransferase (EC 2.4.1.37), N-acetyllactosamine synthase (EC 2.4.1.90).

The methods of the present invention may be applied to many different types and subtypes of enzymes that catalyse the modification of a purine-ring-containing or pyrimidine-ring-containing substrate, including any of those that utilize ATP, GTP, ADP, or GDP, or other purine nucleoside or nucleotide substrates whose H\textsubscript{8}NMR signal is shifted as a result of enzymic catalysis. Examples of enzymes that may be used in these methods include, but are not limited to, a kinase, a phosphotransferase, a GTPase, GMP kinase, GDP-mannosyl transferase, a lipid kinase (e.g. a ceramide
kinase, a phosphatidylinositol 3-kinase, a phosphatidylinositol 4-kinase, a phosphatidylinositol-phosphate 4-kinase, a phosphatidylinositol-phosphate 5-kinase, a sphingosine kinase, a diacylglycerol kinase), an amino acid kinase, a sugar kinase, creatine kinase, aspartate kinase, hexokinase, fructokinase, galactokinase, phosphofructokinase, riboflavin kinase, shikimate kinase, thymidine kinase, ATP synthase, ADP-thymidine kinase, AMP-thymidine kinase, NAD+ kinase, glycerol kinase, pantothenate kinase, mevalonate kinase, pyruvate kinase, deoxyctydine kinase, PFP kinase, diacylglycerol kinase, phosphoinositide 3-kinase, sphingosine kinase, phosphomevalonate kinase, adenylyl cyclase, guanylyl cyclase, phosphodiesterases (e.g. the human PDE isozymes PDE1A, PDE1B, PDE1B2, PDE1C, PDE2A, PDE3A, PDE3B, PDE4A, PDE4B, PDE4B5, PDE4C, PDE4D, PDE5A, PDE6A, PDE6B, PDE6C, PDE7A, PDE7B, PDE8A, PDE8B, PDE9A, PDE10A, PDE10A2, or PDE11A), cAMP-selective phosphodiesterases (e.g. phosphodiesterases 4, 7 or 8), cGMP-selective phosphodiesterases (e.g. phosphodiesterases 5, 6 or 9), cAMP and cGMP-hydrolysing phosphodiesterases (e.g. phosphodiesterases 1, 2, 3, 10 or 11), sulfotransferases (e.g. SULT1A1, SULT1A2, SULT1A3, SULT1A4, SULT1B1, SULT1C2, SULT1C3, SULT1C4, SULT1D1P, SULT1E1, SULT2A1, SULT2B1, SULT4A1, SULT6B1), carbohydrate sulfotransferase (e.g. CHST1, CHST2, CHST3, CHST4, CHST5, CHST6, CHST7, CHST8, CHST9, CHST10, CHST11, CHST12, CHST13, or CHST14), galactose-3-O-sulfotransferase (e.g. GAL3ST1, GAL3ST2, GAL3ST3, or GAL3ST4), heparan sulfate 2-O-sulfotransferase (e.g. HS2ST1), heparan sulfate 3-O-sulfotransferase (e.g. HS3ST1, HS3ST2, HS3ST3A1, HS3ST3A2, HS3ST3B1, HS3ST3B2, HS3ST4, HS3ST5, or HS3ST6), heparan sulfate 6-O-sulfotransferase (e.g. HS6ST1, HS6ST2, or HS6ST3), N-deacetylase/N-sulfotransferase (e.g. NDST1, NDST2, NDST3, or NDST4), tyrosylprotein sulfotransferase (e.g. TPST1 or TPST2), uronyl-2-sulfotransferase (e.g. UST), estrone sulfotransferase, Chondroitin 4-sulfotransferase, adenylate kinase, nucleoside-diphosphate kinase, uridylate kinase, guanylate kinase, purine nucleoside phosphorylase, Ribose-phosphate diphosphokinase, a phosphatidylinositol transferase, N-acetylglucosamine-1-phosphate transferase, thiamine pyrophosphokinase, a guanylyl transferase, phosphoglycerate kinase, ATPase, a protein kinase, a protein-serine kinase, a protein-histidine kinase, a protein-threonine kinase, protein-tyrosine kinase, AMP nucleosidase, ADP-ribose diphosphatase, a cyclic nucleotide phosphodiesterase (PDE), kinesin, ecto-ATPase, CTP synthase,
NDPase, NTPDase, 5'-nucleotidase, methyltransferase, methionine adenosyltransferase, sulfotransferase, glycosyltransferase, GMP synthase, adenylate cyclase, adenosylhomocysteine nucleosidase, methylthioadenosine nucleosidase, S-methyl-5'-thioadenosine phosphorylase, mRNA (guanine-N7)-methyltransferase, a glycosyltransferase, lactose synthase, fucosylgalactoside 3-alpha-gal actosyltransferase, and N-acetyllactosamine synthase. The enzyme may be human, or from any other organism, e.g. rabbit (e.g. RMCK), rat, mouse, pig, bovine, plants, bacteria, fungi, viruses, etc. The enzyme may be soluble in an aqueous environment (e.g. cytoplasmic, or within an organelle), or may be bound to a biological membrane (e.g. a plasma membrane, the endoplasmic reticulum). The latter enzymes may be solubilized for assay, for example using detergents, may be assayed as a membrane suspension, using natural and/or synthetic lipids with or without detergents, or a soluble fragment or domain may be employed (e.g. a protein kinase domain). The latter is readily prepared using recombinant techniques that are routine in the arts of protein expression and purification.

[204] In a further embodiment of the present invention, the enzyme that catalyses the modification of a purine-ring-containing substrate of the present invention may be a protein kinase. Examples of protein kinases that may be used include, but are not limited to: AKT (NP 005154); AKT2 (AAA36585); AKT3 (NP 005456); PDK1 (NP 0026204); PK428 (NP 003598); CITRON-K (AAC27933); DMPK (Q09013); DMPK2 (CAA73006); MRCKb (NP 006026); p60 ROCK2 (NP 004841); ROCK1 (NP 005397); RHODK (NP 002920); BARK IA (NP 001610); BARK2 (CAA48870); GRK4 (AAC50406); GRK5 (AAA58620); GRK6 (AAA60175); GRK7 (AF063016); KIAA0303 (2224547); KIAA0561 (BAA25487); SAST (BAA76817); MNK1 (NP 003675); MNK2 (AAD21217); 138214 (CAA80911); KIAA0151 (BAA09772); T2K (AFI45705); NDR (CAAE4485); WART1 (NP 004681); WART2; PKX1 (P51817); PKCa (CAAS0597); PKAc (AAA60170); PKAcg (AAC41690); PRK2 (2695984); CAB43292 (CAB43292); EPK2 (NP 005804); PCK1 (AAA60100); PKCa (CAAS06178); PKCb (CAAS0130); PKCd (BAA01381); PKCe (CAAS46388); PKCg (CAAS78820); PKCg (AAA75571); PKCm (AAAB1701); PKCm (CAAS053384); PKCl (AAA75571); PKCz (CAAS78813); PKD (CAAS48283); PRK1 (153327); PRK2 (AAAB3346); Z25429 (CAAS80916); CGK (CAAS68810); cGMPk (BAA08297); cGMPKII (NP
006250) ; AAD30182 (AAD30182) ; MSK1 (NP 004746) ; MSK2 (NP 003933) ; p70S6K (AAA36410) ; p70S6Kb (NP 003943) ; RSK1 (CAA59427) ; RSK2 (AAA81952) ; RSK3 (AAC82497) ; MAST205 (AAC04312) ; SGK (CAA71138) ; A6 (A55922) ; EEF-2K (000418) ; BCKDK (NP 005872) ; PDK1 (Q15118) ; PDK2 (NP 002602) ; PDK3 (NP 005382) ; PDK4 (NP 002603) ; BCR (P1 1274) ; FAST (137386) ; G3955 (BAA13250) ; PK38 (BAA11492) ; AMPKα (NP 006242) ; AJ006701 CAA04119 ; AMPK2 726313 ; AMPKα2 AAA64745 ; AUF1 NP 002129 ; NIPK AB020967 ; AL049688CAB41259 ; CaMKI Q14012 ; CaMKIIb BAA19879 ; CaMKIIa BAA76812 ; CaMKIIb AAD03743 ; CaMKIId AAD20442 ; CaMKIIg B46619 ; CAMKL AAA16633 ; CAMKL1 NP 004725 ; CASK AAA64745 ; CHK2 AAC83693 ; DAPK CAA53712 ; DAPKRP1 AAC35001 ; DRAK1NP 004751 ; DRAK2 NP 004217 ; ZIP BAA24817 ; KIAA0135 BAA09484 ; KIAA0096 BAA07744 ; C-TAK1 AAC15093 ; EMK CAA66229 ; KIAA0537 BAA25463 ; KIAA0999 BAA76843 ; MAK-V AF055919 ; MARK1 CAB06294 ; MSK Q60670 ; SNRK CAA61563 ; MAPKAPK2 P49137 ; MAPKAPK3 AAD09136 ; MAPKAPK5 NP 003659 ; MLCK AAC18423 ; MLCKs AAA41625 ; Titin NP 003310 ; PHKG1 Q16816 ; PHKgT P15735 ; PSK-H1 406242 ; Trio 1353703 ; TRAD BAA76314 ; KIAA0369 BAA20824 ; CKIg2 AAB88627 ; CKIa AAC41760 ; CKId AAC50807 ; CKIe AAC41761 ; KKIAMRE AACC50918 ; CAK P50613 ; CCRK AAC98920 ; CDC2 P06493 ; CDK2 AAA35667 ; CDK3 Q00526 ; CDK4 P11802 ; CDK5 Q00535 ; CDK6Q00534 ; CDK8 CAA59754 ; CHED AAA58424 ; KIAA0904 BAA74927 ; KKIAR CAA47002 ; PCTAIRE1 S22747 ; PCTAIRE2 S22746 ; PCTAIRE3 CAA47005 ; PFTAIRE BAA74857 ; PISSLRE S49330 ; PITALRE AAA58424 ; PITSLREa2 P21 127 ; STK9 NP 003150 ; CLK1 AAA61480 ; CLK2 AAC51817 ; CLK3 AAA61484 ; CLK4 ; STY A39676 ; GSK3a AAA62432 ; GSK3b AAA66475 ; ERK1 CAA42744 ; ERK2 CAA77753 ; ERK3 Q16659 ; ERK4 P31 152 ; ERK5 Q13164 ; ERK7 AAD12719 ; JNKla AAA36131 ; JNK2 P45984 ; JNK3a AAC50101 ; NLK AAC24499 ; p38 AAA57455 ; SAPK2b AAB05036 ; SAPK3 CAA71511 ; SAPK4 NP 002745 ; KIAA0936 BAA76780 ; MAK CAA47392 ; MOK BAA81688 ; RAGE4 AAB38087 ; YAB1 ; SUDD NP 003822 ; NY-REN-64 AAD42884 ; AIE1 AAC25954 ; AIE2 AAC25955 ; AUR1 NP 004208 ; AUR2 AAC12708 ; BUB1 AAB97855 ; BUBR1 AACC12730 ; GSG2 D87326 ; CaMKIV BAA04117 ; CAMKKB AAD31507 ; CHK1 NP
001265 ; CKIIa AAA35503 ; CKIIa' AAA51548 ; COT P41279; 
DYRK2 CAA73885 ; DYRK1B NP 004705 ; DYRK3 NP 003573; DYRK4 
CAA70488 ; HIPK1 AAC63010 ; HIPK2 AAC63011 ; KIS CAA67021 ; MNB 
BAA13110 ; PKY AAC64294 ; EIF2aK 000418 ; EIF2aK Q08796 ; PEK 
AAD 19961 ; GAK BAA22623 ; IKKa AAC51671 ; IKKb AAC51860; ILK 
AAC 16892 ; IRAK AAC41949 ; IRAK2 NP 001561 ; IRAK-M AAD40879 ; 
IRE1 AAC25991 ; LIMK2 BAA08312 ; TESK1 BAA90459 ; LIMK1 
AAC13884 ; LIMK2b BAA24489 ; TESK2 CAB41970 ; LKB1 Q15831 ; DLK 
AAA67343 ; HH498 AAD29632 ; MLK1 P80192 ; MLK2 Q02779; SPRK 
AAA59859 ; MOS AAA52029 ; PIM2 AAC78506 ; KID1 BAA25182 ; PIM1 
AAA81553 ; PRK AAC50637 ; SNK NP 006613 ; PLK1 AAA56634 ; SAK 
CAA73575 ; PRP4 BAA25462 ; KSR AAC50354 ; ARaf AAA65219 ; BRaf 
AAA35609 ; Raf1 CAA27204 ; HCYP2 AF036537 ; KIAA0472 BAA32317 ; 
RICK NP 003812 ; RIP 1236943 ; RIP3 AAD39005 ; SRPK2 NP 003129 ; MSSK1 AAD01848 ; SRPK1 AAA20530 ; ACTR-II BAA54671; 
ALK6 AAC28131 ; ACTR-II AAA35504; ALK1 CAA02404; ALK2 2295238 ; 
ALK3 CAA80257 ; ALK4 CAA80258 ; ALK5 AAA16073; ALK7 AAC52919 ; 
C14 CAA50731; MISRII AAC50328 ; T-ALK CAA88759 ; TGFbRII 
BAA09332 ; TAK1 BAA11184 ; TSK1 AAA99535 ; TSK2 NA; TSK3 ; TSK4 ; 
PKUa BAA20561 ; TSL1 BAA9486 ; TTK AAA61239 ; ULK1 NP 003556 ; 
ULK2 BAA31598 ; VRK1 BAA19108; 51PKL(A) AAC29497; VRK2 
BAA19109 ; MYT1 NP 004194; ACO04918 AAD04726 ; WEE1 CAA43979; 
MPSK1 AAC28337 ; PKN rBAA36362 ; NEK1 AAD42879 ; NEK2 AAA19558 ; 
NEK3 P51956 ; NEK3a AF093416 ; NEK4 NP_003148 ; NEK5 NP_954983; 
NEK6 BAA83000 ; NRK2 NP 003148 ; MEKK3 AAB41729 ; MEKK4a 
AAB68804 ; MEKK5 AAC50894 ; NIK CAA71306 ; MEKK1 AAC97073 ; 
MEKK2 AAB03536 ; MEKK6 NP 004663 ; YSK4 AAB61531 ; MST3 
AAB82560; STE20 BAA20420; STLK2 ; STLK3 AAC72238 ; STLK4; STLK5 ; 
STLK6 ; STLK7 ; PAK1 AAA65441 ; PAK2 AAA65442 ; PAK3 AAC36097 ; 
PAK4 NP_001014831; PAK5 CAA09820 ; PAK6 CAA21067 ; ZC1 
004825 ; ZC2 BAA25477 ; ZC3 ; MINK1 NP_001020108; HPK1 AAB97983; 
BL44 AAA20968 ; KHS1 AAB48435; KHS2 NP 003609 ; GEK2 NP 005981; 
SLK BAA13195 ; SULU1 ; SULU2 BAA74904; SULU3 AAC71014; 
MST1 AAB17262 ; MST2 AAC50386 ; CDC7 AAC52080; MEKI NP 002746;
MEK2 P36507; MEK5 AAA96146; MEK6 AAC50389; MKK3 AAB40652; MKK7a AAC26142; MPK3b P46734; MPK4 AAC41719; BLK AAB33265; FGR AAA52451; FYN NP 002028; HCK PO8631; LCK P06239; LYN NP 002341; SRC P12931; YES AAA5735; BRK CAA55295; FRK NP 002022; SAD; SRM Q62270; TXK AAA74557; BMX CAA58169; BTK Q06187; ITK NP 005537; TEC NP 003206; CSK P41240; CTK P42679; ABL P00519; ARG NP 005149; SYK P43405; ZAP70 P43403; FER NP 005237; FES P07332; FAK Q05397; PYK2 NP 004094; JAK1 NP 002218; JAK2 NP 004963; JAK3 NP 000206; TYK2 NP 003322; ACK NP 005772; TNK1 AAC50427; EGFR NP 005219; HER2 NP 004439; HER3 NP 001973; HER4 NP 005226; IGF1RNP 000866; INSR P06213; IRR P14616; CSFR P07333; KIT NP 000213; PDGFRa NP 006197; PDGFRb NP 002600; FLK2 NP 004110; FLT1 NP 002010; FLT4 P35916; KDR AAB88005; FGFR1 P11362; FGFR2 NP 000132; FGFR3 NP 000133; FGFR4 NP 002002; CCK4 AAA87565; MET NP 000236; RON NP 002438; TRKA NP 002520; TRKB NP 006171; TRKC NP 002521; MER NP 006334; AXL NP 001690; TYR03 NP 006284; ZPK AAC41766; TEK NP 000450; TIE NP 005415; EphA1 NP 005223; EphA2 NP 004422; EphA3 NP 005224; EphA4 NP 004429; EphA5 P54756; EphA6 Q62413; EphA7 NP 004431; EphA8 009127; EphB1 NP 004432; EphB2 NP 004433; EphB3 NP 004434; EphB4 NP 004435; EphB5 cQ07497; EphB6 NP 004436; RYK P34925; DDR Q08345; RTK40 AFO16247; TYRO10 NP 006173; ROS NP 002935; RET P07949; ALK NP 004295; LTK P29376; ROR1 NP 005003; ROR2 NP004551; MUSK NP 005583; LMR1 NP 004911; LMR2 BAA83031; LMR3; or RTK106. In the preceding list of kinases, each kinase is denoted by the symbol approved by the HUGO Gene Nomenclature Committee, and if available, followed by an Accession Number for a representative sequence of the protein (N.B. listed kinases, with symbol, and accession number if available, are separated by semicolons). The listed kinases are human enzymes, but corresponding or homologous genes from other species that code for related kinases may also be used in the methods of the invention.

[205] In a further embodiment of the present invention, the enzyme that catalyses the modification of a purine-ring-containing substrate of the present invention may be
a lipid kinase. Examples of lipid kinases that may be used include, but are not limited
to: a ceramide kinase, a phosphatidylinositol 3-kinase, a phosphatidylinositol 4-
kinase, a phosphatidylinositol-phosphate 4-kinase, a phosphatidylinositol-phosphate
5-kinase, a sphingosine kinase, and a diacylglycerol kinase.

[206] In a further embodiment of the present invention, the enzyme that catalyses
the modification of a purine-ring-containing substrate of the present invention may be
an "ATPase", which as used herein refers to an enzyme that hydrolyzes ATP.
Examples of ATPases that may be used include, but are not limited to: proteins
comprising "molecular motors" such as myosins, kinesins, and dyneins, DNA gyrase,
DNA helicase, topoisomerase I and II, Na+-K+ ATPase, Ca2+ ATPase, F1 subunit of
ATP synthase, terminase/DNA packaging protein, recA, heat shock proteins, NSF,
katanin, SecA, 5-lipoxygenase, and actin. A "molecular motor" is a molecule that
utilizes chemical energy to produce mechanical force or movement; molecular motors
are particularly of interest in cytoskeletal systems. For further review, see, Vale and
University Press; Goldstein, 1993, Ann. Rev. Genetics 27: 319-351; Mooseker and

[207] In certain embodiments, the enzyme is a kinesin, including mitotic kinesins.
Mitotic kinesins are enzymes essential for assembly and function of the mitotic
spindle, but are not generally part of other microtubule structures, such as nerve
processes. Mitotic kinesins play essential roles during all phases of mitosis. These
enzymes are "molecular motors" that translate energy released by hydrolysis of ATP
into mechanical force which drives the directional movement of cellular cargoes
along microtubules. The catalytic domain sufficient for this task is a compact
structure of approximately 340 amino acids. During mitosis, kinesins organize
microtubules into the bipolar structure that is the mitotic spindle. Kinesins mediate
movement of chromosomes along spindle microtubules, as well as structural changes
in the mitotic spindle associated with specific phases of mitosis. Experimental
perturbation of mitotic kinesin function causes malformation or dysfunction of the
mitotic spindle, frequently resulting in cell cycle arrest. From both the biological and
enzymatic perspectives, these enzymes are attractive targets for the discovery and development of novel anti-mitotic chemotherapeutics.

Examples of kinesins that can be assayed include, but are not limited to, Kin2, chromokinesin, KiflA, KSP, CENP-E, MCAK, HSET, RabK6, Kip3D, Kifl5, K335, Q475, D679, FL1, P166, H195, FL2, E433, R494, E658, L360, K491, S553, M329, T340, S405, V465, T488, M1, M2, M3, M4, M5, M6, FL3, A2N370, A2M51 1, K519, E152.2, Q151.2, Q353, M472 and MKLPl. It is understood that unless a particular species is named, the term "kinesin" includes homologs thereof which may have different nomenclature among species. For example, the human homolog of KiflA is termed ATSV, the human homologue of Xenopus Eg5 is termed KSP, and human HSET corresponds to Chinese hamster CH02.

In a further embodiment of the present invention, the enzyme that catalyses the modification of a pyrimidine-rings-containing substrate of the present invention may be a "GTPase", which as used herein refers to an enzyme that hydrolyzes GTP. Examples of GTPases that may be used include, but are not limited to: G proteins; the Rho family GTPases: cdc42, RalA, RhoA and Rac1; Ras proteins; elongation factors including EFl.alpha., EFl.beta., gamma., EF-Tu and EF-G; septins; tubulin; ARF related GTPase; rab; SSRP receptor; ATP sulfurylase; rhodopsin; transducin; GTPase activating protein (GAP); cell division protein filamentation temperature-sensitive protein Z (FtsZ); and the cytoskeletal protein tubulin.

The enzymes that catalyse the modification of a purine-rings-containing substrate or pyrimidine-rings-containing substrate of the present invention may be wild-type or catalytically active amino acid sequence variants of wild-type proteins. These variants may be substitutional, insertional or deletional variants. Such variants are readily prepared by site-specific mutagenesis of nucleotides in the DNA encoding the protein, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. However, variant protein fragments having up to about 100-150 residues may also be prepared by in vitro synthesis using established techniques.
The enzymes used in the methods of the present invention may be prepared by any of the numerous suitable methods for recombinant protein expression, including generation of expression vectors, generation of fusion proteins, introducing expression vectors into host cells, protein expression in host cells, and protein purification methods known to those in the art. Alternatively, the enzymes may be purified from natural sources.

The selection of host cell types for the expression of enzyme proteins will depend on the protein, with both eukaryotic and prokaryotic cells finding use in the invention. Appropriate host cells include yeast, bacteria, archaeabacteria, fungi, plant, insect and animal cells, including mammalian cells. Of particular interest are Drosophila melanogaster cells, Saccharomyces cerevisiae and other yeasts, E. coli, Bacillus subtilis, SF9 cells (and other related cells for use with baculoviral expression systems), C129 cells, 293 cells, Neurospora, BHK, CHO, COS, Dictyostelium, etc.

In some embodiments, the proteins are purified for use in the NMR methods of the invention, to provide substantially pure samples. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being preferred, and at least about 90% being particularly preferred. Alternatively, the protein need not be substantially pure as long as the sample comprising the protein is substantially free of other components that can contribute to the modification of the substrates in the assay (e.g. GTP, ATP) (or, in the case of indirect assays, other components which are subsequently assayed). In some instances a cell lysate may be used for assay; for example a lysate from cells engineered to express the enzyme of interest, with identical cells not expressing the enzyme of interest (e.g. non-transfected, or non-induced) being used as a control assay. A cell or organelle membrane preparation may also be used.

The proteins may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, reverse-phase HPLC chromatography, and chromatofocusing. For example, the protein may be purified using a standard anti-protein antibody column. Ultrafiltration and

[215] As will be appreciated by those of skill in the art, the components of the NMR assay methods described herein are added with buffers and other reagents that are compatible with enzyme activity and give optimal signals (e.g. the largest H8 or H6 signal possible in the time period of the assay). The NMR assay mixtures typically will contain, for example, the substrate (s) for the enzyme, necessary cofactors (e.g., metal ions, NADH, NAPDH), buffer (s), and protein stabilizing agents (e.g. BSA, non-ionic detergents, reducing agents such as DTT). A reaction temperature is chosen that allows conversion of sufficient substrate to product to enable ready quantitation of the change in signal intensity, in a convenient time frame, and that is compatible with stability of the enzyme. If a coupled assay is to be performed, the assay solution will also generally contain the enzyme, substrate (s) and cofactors necessary for the enzymatic couple. Since the methods outlined herein also allow kinetic measurements, the incubation periods may be optimized to produce linear initial reaction velocities and to give adequate detection signals over the background, allowing enzyme specific activities to be determined. Assays may be performed using multiple time-points, with at least two data points being preferred. As will be appreciated by those in the art, the time interval can be adjusted to correlate with the biological activity of the protein. In should be noted that if a coupled assay is to be performed using the methods described herein, appropriate secondary assays may be required to determine whether a test compound, identified as a potential modulator of an enzyme that catalyses the modification of a purine-ring-containing substrate or pyrimidine-ring-containing substrate, is directly affecting that enzyme activity, and not another enzyme involved in the coupled reaction. Application of the methods of this invention to coupled enzyme systems expands the utility of these methods to enable them to identify modulators of many additional enzymes whose substrate or product cannot be directly monitored by NMR.
The NMR assay and screening methods described herein can be conducted with essentially any test compound or test agent that is compatible with the NMR methods, e.g. does not denature the enzyme, or prevent determination of the assay signal (e.g. H8 or H6 signal). Consequently, test compounds or test agents can be of a variety of general types including, but not limited to, peptides, polypeptides, antibodies or antibody fragments, inhibitory proteins; carbohydrates such as oligosaccharides and polysaccharides; lipids or phospholipids; fatty acids; steroids; or amino acid analogs. Further, the compounds or test agents can be growth factors, hormones, neurotransmitters and vasodilators, for example. Likewise, the compounds or agents can be of a variety of chemical types including, but not limited to, heterocyclic compounds, carbocyclic compounds, beta-lactams, polycarbamates, oligomeric-N-substituted glycines, benzodiazepines, thiazolidinones and imidazolidinones. Certain test compounds or test agents are "small molecules", including synthesized organic compounds. The term "small molecule" refers to a low molecular weight (i.e. less than 5000 Daltons; preferably less than 1000, and more preferably between 100 and 700 Daltons) organic compound. Test compounds can be obtained from libraries, such as natural product libraries or combinatorial libraries, for example. A number of different types of combinatorial libraries and methods for preparing such libraries have been described, including for example, PCT publications WO 93/06121, WO 95/12608, WO 95/35503, WO 94/08051 and WO 95/30642. Devices for the preparation of combinatorial libraries are also commercially available (see, e.g., 357 MPS, 390 NWS, Advanced Chem Tech, Louisville K.Y.; Symphony, Rainin, Woburn, Mass.; 433A Applied Biosystems, Foster City, Calif; 9050 Plus, Millipore, Bedford, Me.). Test compounds or test agents are added to the assay in aqueous solution, or in a solvent that is water miscible (e.g. DMSO) and becomes sufficiently diluted in the assay mixture so as to not adversely affect enzyme activity to too great an extent. Solvent controls without test compound may be performed to adjust for solvent effects on the assay.

Compound libraries may, for example, be selected from a company compound collection and/or acquired from vendors. The library may contain fragments with of low molecular weight, termed as fragment library. Filters of molecular properties (e.g. Jacoby, 2003 #17) maybe applied and compounds with undesired druggable functionalities can be removed. Compounds may be checked by liquid
chromatography-mass spectrometry for purity and identity. To ensure assay quality, preferably only compounds with the correct mass and purity higher than 85% should be used in the NMR screen. For an initial screen of a compound library, each of the reaction mixtures may contain up to 50 compounds with a concentration of nM to mM each, grouped by the chemical properties and structure similarity. Exemplary reaction mixture conditions are described herein in the experimental details section.

[218] The NMR assay and screening methods described herein can be used in conjunction with standard HTS technologies, include the use of robotic systems, in order to enable processing of many samples in a short period of time, to provide sufficient throughput for screening large compound libraries. For example, samples for assay can readily be transferred from standard plate systems (e.g. 96, 384, or 1536 well plates) to NMR tubes using commercial microfluidic systems. This permits a plurality of assay mixtures to be run in parallel and subsequently read on the NMR spectrometer. Instruments with high field strengths (e.g. 600-900 MHz) can be utilized to minimize assay time on the spectrometer, and maximize throughput.

[219] Liquid handlers for high throughput screening systems are commercially available (see, e.g., Tecan Group Ltd., Switzerland; Gilson, Middleton, Wise; Zymark Corp., Hopkinton, Mass.; Air Technical Industries, Mentor, Ohio; Beckman Instruments, Inc., Fullerton, Calif; Precision Systems, Inc., Natick, Mass., etc.). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and/or final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization.

[220] It is understood that once a test compound is identified as an enzyme modulator (e.g. an inhibitor, an agonist, or antagonist), it can be subjected to further assays to further confirm its activity. In particular, the identified agents can be entered into a computer system as lead compounds and compared to others which may have the same activity. The agents may also be subjected to in vitro and preferably in vivo assays to confirm their use in medicine as a therapeutic or diagnostic, or in the agricultural arena, and/or subjected to further chemical modification or formulation to
optimize efficacy (e.g. improved potency, better selectivity, improved solubility) or reduce toxicity.

[221] In a further embodiment of the present invention, the NMR assay may be used to test for the presence of modulators of a protein that is an activator of an enzyme that catalyses the modification of a purine-ring-containing substrate. For example, the protein may be an activator of an ATPase (or a GTPase), such as an actin filament or a microtubule; thus in this embodiment, the protein may be a protein polymer or oligomer. Alternatively, the protein can be a filament binding protein or regulatory protein. For example, the regulatory protein can be the troponin-tropomyosin complex which regulates the binding of myosin to actin. Since myosin's ATPase is activated by binding to actin, modulators of this regulatory protein complex can be identified by the methods provided herein.

[222] The invention further provides methods for identifying whether any two test proteins interact. Briefly, such an assay method is functionally similar to a yeast two-hybrid system, but relies on an increase in ATPase or GTPase activity as a result of bringing two components together as a result of a protein-protein interaction. As an example of the general approach, a system is described that uses a biological polymer binding site and a polymer stimulated ATPase, wherein the polymer may comprise, for example, a cytoskeletal protein such as tubulin or actin. It will be appreciated by those skilled in the art, however, that any two components that result in an increase in ATPase or GTPase activity as a result of association can be used (e.g. see US 7,824,880 or US 2007/0254310 for additional details).

[223] In one embodiment, a first test protein (a "bait" protein), for which an interaction is sought, is joined, usually covalently, to a biological polymer binding protein, for example a cytoskeletal binding protein (such as a microtubule binding protein) to form a first target chimera. The term "chimera" or "fusion protein" as used herein refers to a protein (polypeptide) composed of two polypeptides that, while typically unjoined in their native state, typically are joined by their respective amino and carboxyl termini through a peptide linkage to form a single continuous polypeptide. It will be appreciated that the two polypeptide components can be directly joined or joined through a peptide linker/spacer.
A second test protein (a "prey" protein) is joined, again usually covalently, to an ATPase domain that is stimulated by the cytoskeletal component to form a second target chimera. Upon combination with the cytoskeletal component, the first target chimera binds to the cytoskeletal component, and if the first and second target proteins interact, the second target chimera is brought into proximity with the cytoskeletal component, and thus the ATPase activity is stimulated and can readily be detected using the NMR methods described herein for measuring ATP or ADP levels (i.e. using an H8 signal). If there is no interaction, no increase in ATP production is observed. This assay system may also be utilized to identify modulators (e.g. inhibitors, agonists) of the interaction of two proteins that are known to interact, by monitoring the ATPase activity in the presence and absence of test compounds in the assay. In the latter case, appropriate secondary assays would of course be required to determine whether a test compound identified as a potential modulator of a protein-protein interaction was directly affecting the ATPase activity, rather than via the protein-protein interaction.

The invention thus provides an NMR assay method for identifying an agent or compound that disrupts interaction between a bait protein and a prey protein, comprising: (a) providing a reaction mixture comprising: (i) ATP; (ii) a cytoskeletal protein polymer; (iii) a bait fusion protein that comprises a cytoskeletal-protein-binding protein domain and the bait protein; and (iv) a prey fusion protein comprising an cytoskeletal-protein stimulatable-ATPase domain and the prey protein; (b) contacting the reaction mixture with a test agent or test compound; (c) incubating the reaction mixture for a period of time, wherein said period of time is that required in the absence of the test agent or compound for conversion of a quantity of ATP into ADP that is measurable by detection of the purine H8 ATP and/or ADP signal by NMR; (d) detecting a decrease in conversion of ATP into ADP, by monitoring the the purine H8 ATP and/or ADP signal, in the presence of the test agent or compound compared to the amount of conversion in a control reaction without the test agent or compound, thereby detecting an agent that disrupts interaction between the bait and prey proteins. In one embodiment the cytoskeletal protein polymer comprises tubulin or actin. In another embodiment the purine H8 NMR signal is measured after the ATP to ADP conversion is terminated by the addition of a chelator of magnesium ions.
Another application of the method of the present invention is the
determination of protein function. In view of the ready availability of high-throughput
genome sequencing, thousands of proteins have been identified. However, the precise
function of many of these proteins is unknown. The function of the protein may be
inferred from the types of substrates that can be modified. For example, if the protein
is suspected of possessing protein kinase activity, it can be incubated with a range of
potential natural or synthetic peptide or protein substrates in the presence of ATP.
Test substrates may be incubated with the enzyme individually, or multiple test
substrates may be incubated in one reaction. A reduction in signal intensity of the
purine H8 signal of the ATP, and the appearance of a new resonance at a chemical
shift characteristic of the purine H8 signal of ADP, allows the recognition of the
protein function as a phosphotransferase, with the substrate specificity providing
information on the potential physiological significance of this activity. A similar
analysis can be performed, for example, if the protein is suspected of possessing lipid
kinase activity, using a range of potential natural or synthetic lipid substrates.

The method of the present invention extends the capabilities of NMR to most
enzymatic reactions performed by enzymes that catalyses the modification of a
purine-ring-containing or pyrimidine-ring-containing substrate, such as purine-
utilizing enzymes, pyrimidine-utilizing enzymes, kinases, phosphotransferases,
diphosphotransferases, nucleotidyltransferases, phosphatidylintransferases, glycosyl-1-
phosphotransferases, protein kinases, phosphohydrolases, and other such enzymes.
The method performs well, provides a reliable array of experimental data, and is
universally applicable to this large group of enzymes. This invention will be better
understood from the Experimental Details that 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 which follow
thereafter, and are not to be considered in any way limited thereto.

**EXPERIMENTAL DETAILS**

[228] **Introduction**
[229] Herein is exemplified a direct and highly sensitive NMR screening assay method, based on the use of a 400 MHz $^1$H NMR spectrometer to resolve and quantify the NMR signals of natural substrate and product pairs such as adenosine-5'-triphosphate (ATP) and adenosine-5'-diphosphate (ADP) or guanosine-5'-triphosphate (GTP) and guanosine-5'-diphosphate (GDP). This method has a low resource requirement, with minimum usage of proteins and easy set-up, enabling its ready implementation in both small and large discovery laboratories. It can potentially be applied, for example, to all purine-utilizing and pyrimidine-utilizing enzymes, including kinases, ATPases, GTPases, phosphodiesterases, and sulfotransferases, which are all highly pursued pharmaceutical targets, across a variety of therapeutic areas (e.g. cardiovascular, central nervous system, hematology, inflammation, metabolic disorders, and oncology), for the discovery of small molecule inhibitors interacting with the active and/or allosteric binding sites. The $^1$H NMR-based activity assay is exemplified using the well characterized creatine kinase system {McLeish, 2005 #4}, and phosphodiesterase 2A. We also describe experimental procedures for the implementation of the assay and its application for kinetic characterization and inhibitor screening for enzymes that utilize a range of important cofactors.

[230] **A. Cofactors survey**

[231] **Al. Materials and Methods**

[232] Cofactors were generally purchased from Sigma-Aldrich (St. Louis, MO). 2'-Deoxythymidine-5'-'diphosphate trisodium salt was obtained from Wonda Science (Montreal, QC, Canada), and xanthosine-5'-'diphosphate triethylammonium salt was purchased from Jena Bioscience GmbH (Jena, Germany). Stock solutions of cofactors were prepared in 100 mM in 200 mM Tris in $D_2O$, pH 7.5, whenever possible. A few of the cofactors that were not soluble at 100 mM in the above aqueous buffer were dissolved in DMSO-$d_6$, or 200 mM Tris in $D_2O$, pH 13. pH values were measured using a Mettler Toledo MP220 pH meter, and corrected for the glass electrode solvent isotope artifact {Glasoe, 1960 #1}. An aliquot (6 µL) of stock solutions was added into 0.6 mL of assay mixture to achieve the final concentration of 1 mM for each cofactor tested.
[233] For the determination of chemical shifts of individual cofactor samples, the final assay solution consisted of 1 mM cofactor, 0.1 mM 3-(trimethylsilyl)-l-propanesulfonic acid (DSS) in 200 mM Tris in D$_2$O at pH 7.5 or 9.0, respectively. For chemical shift differences between cofactor pairs, solutions also contained 1 mM of cofactor in 200 mM Tris in D$_2$O at pH 7.5 or 9.0, with 0.1 mM of DSS (i.e. 4,4-dimethyl-4-silapentane-1-sulfonic acid, or 3-(trimethylsilyl)-l-propanesulfonic acid). The chemical shifts measured are consistent and reproducible under such conditions that no tangible differences beyond experimental fluctuations have been observed between individual cofactor and cofactor pair samples. The data shown were calculated from two repeated trials. For M$^7$GTP and M$^7$GDP, H$_2$O was used instead of D$_2$O in chemical shifts measurement because of fast solvent exchange on H8 proton {Tomasz, 1970 #60} and the disappearance of peak in D$_2$O.

[234] NMR experiments were performed at 25°C on a Bruker DPX 400-MHz spectrometer equipped with a 5 mm QNP probe and a BACS120 autosampler. The spectrometer was controlled using TopSpin (Bruker BioSpin, v 1.3 pi 10), and each dataset was collected under automation using the ICON-NMR software (Bruker BioSpin, v 4.0.7 build 1). Some 2D NMR experiments were performed at 25°C on a Varian Mercury 400-MHz spectrometer equipped with a 5 mm Inverse Detection probe. The spectrometer was controlled using VNMR (Agilent Technologies, v 6.1C). The H experiment with optional water suppression {Hwang, 1995 #2} comprised 65536 data points with a spectral width of 8278.15 Hz and a scan number of 128. For a few of compounds that H spectra could not give sufficient data for H peak assignment, additional 1H-1H COSY, 1H-13C HMQC, and/or 1H-13C HMBC experiments were performed. All data were processed using ACD/Labs NMR Manager (Advanced Chemistry Development, Inc., Canada). The chemical shifts data were batch-processed using the ACD/Labs group macro, including zero-filling to 2 times of time domain points, an exponential window function with a line broadening of 0.5 Hz, Fourier transform, phase, baseline correction, and peak picking.

[235] A2. Results
The resonances for 55 cofactors (see Table 1) in the aromatic region at pH 7.5 and pH 9.0 were surveyed, assigned and summarized in Figures 10-20. The cofactors are grouped based on their nucleotide core structures, shown as adenine-, guanine-, uracil-, cytosine-, hypoxanthine-, xanthine-derivatives, and adenine-based cofactors (ABC), and nicotinamide-based cofactors (NBC) (see Tables 1a-e for structures). The peak assignments are obtained from H experiments, and/or 1H-1H COSY, 1H-13C HMQC, 1H-13C HMBC experiments whenever necessary. Among those 55 cofactors, chemical shifts differences were measured for 64 pairs (Figures 10-18) for which corresponding enzymes can be identified that catalyze the cofactor conversion (see list on pages 37-41). Generally, all the cofactor pairs tested give a resolution of chemical shift difference (8 - 6088 ppb) sufficient to monitor the substrate/product conversion, and thus the NMR assay is fully capable of monitoring any of these conversions. For purine-based nucleotide cofactors, adenine-, guanine-, hypoxanthine-, xanthine-derivatives, and other cofactors derived from adenosine, the H8 proton of the purine ring is the preferred probe signal because of its singlet multiplicity and wide applicability among those cofactor pairs, and is the Δδ listed for purines in Figures 10-18, unless noted otherwise. Occasionally, protons other than H8 can also be used as the probe signal such as H1’ of AMP/adenosine, H2 of adenosine/adenine, H1’ of adenosine in adenosine/adenine, and H2″, H4″, H6″ of NAD/NADH. Those protons can work as alternatives to H8 when H8 is overlapping with compound signals in an inhibition screen. There are two cofactor pairs (NAD/cADPR, CoA/AcetylCoA) where the H8 protons are overlapping, but in this case other protons can be used as the probe signal. For pyrimidine-based cofactors, uracil-, cytosine-, thymine-derivatives, the H6 proton of the pyrimidine ring is generally used as the probe signal, and is the Δδ listed for pyrimidines in Figures 10-18, unless noted otherwise, but other protons also work in some cases, such as H5 of uracil/uridine, H1″ of UDP-GlcNac in UDP-GlcNac/UDP, and H1’ of CTP/CDP.

For most cofactor pairs, larger chemical shift differences were obtained at pH 9.0 than at pH 7.5. For ATP/ADP and GTP/GDP pairs, the small chemical shifts at pH 7.5 make the measurement at 400 MHz NMR somewhat difficult, but the differences improve sufficiently at pH 9.0 to facilitate very accurate resolution and quantification. For ITP/IDP and XTP/XDP, sufficient resolution at 400 MHz can only be achieved at a pH higher than 9.4 and 10.0, respectively. In an end-point enzyme...
assay, the final pH can be increased by using EDTA at high pH as the reaction quencher to raise the final assay pH and thus the chemical shift difference for the NMR measurement. For a real time assay experiment, a 600 MHz or higher NMR spectrometer is recommended for the measurement at lower pH for those cofactor pairs with difficult peak separations.

[238] This study has demonstrated (e.g. Figures 10-18) that many cofactor pairs can be readily resolved and quantitated by NMR, by using for example purine H8 or pyrimidine H6 resonances, thus enabling the assay of enzymes that catalyse their interconversion by an NMR assay method, by monitoring these resonances.
### Table 1a: Chemical Structures of Phosphate-based Nucleotide Cofactors Applicable to the NMR Assay

<table>
<thead>
<tr>
<th>R</th>
<th>Adenine</th>
<th>Guanine</th>
<th>Uracil</th>
<th>Cytosine</th>
<th>Hypoxanthine</th>
<th>Xanthine</th>
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<tbody>
<tr>
<td>H</td>
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<td>Guanosine</td>
<td>Uridine</td>
<td>Cytidine</td>
<td>Inosine</td>
<td>Xanthosine</td>
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### Table 1b: Chemical Structures of Thymidine-based Cofactors

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</tr>
<tr>
<td>R</td>
<td>PAP</td>
</tr>
<tr>
<td>---</td>
<td>-----</td>
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<tr>
<td><img src="image1" alt="Chemical Structure of PAP" /></td>
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</table>
Table 1e: Chemical Structures of Nicotinamide-based Nucleotide Cofactors Applicable to the NMR Assay

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Table 1d: The Chemical Structure of cADPR Applicable to the NMR Assay

<table>
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</table>
Table If: Chemical Structures of $\text{m}^7\text{GTP}$ and $\text{m}^7\text{GDP}$ Applicable to the NMR Assay

<table>
<thead>
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<th>$R$</th>
<th></th>
</tr>
</thead>
<tbody>
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<td><img src="image1" alt="Chemical Structure" /></td>
<td>$\text{m}^7\text{GDP}$</td>
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<tr>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>$\text{m}^7\text{GTP}$</td>
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</table>
B. NMR assay

[239] Bl. Introduction

[240] The application of the NMR assay to the characterization of enzyme kinetics, enzyme inhibition and compound screen was demonstrate using two very representative cofactor pairs: ATP/ADP and cGMP/GMP. ATP/ADP is one of the most common cofactor pairs, associated with a wide range of enzymes and biological processes, and represents a relatively "difficult" cofactor pair to assay by NMR due to the relatively small chemical shift difference between ATP and ADP (see Figures 19-20). cGMP/GMP represents an example of a guanine-derivative pair, where either H8 or H1' protons can be used as the probe signal. Two enzymes utilizing these cofactor pairs have been chosen to demonstrate the ready and broad applicability of the NMR assay to the assay of enzymes that catalyze interconversion of the cofactor pairs that are resolvable and quantifiable by NMR: rabbit muscle creatine kinase (RMCK) that catalyses a bisubstrate reaction including ATP/ADP conversion, and cyclic nucleotide phosphodiesterase 2A (PDE2A) that catalyses the single-substrate cGMP—»GMP conversion. The data thus obtained demonstrates how a proven ability to resolve and quantify a cofactor pair by NMR can be utilized for assay of an enzyme that catalyzes interconversion of that cofactor pair.

[241] Creatine kinase (CK; EC 2.7.3.2), found in all vertebrates, is a well-characterized small molecule kinase which catalyzes the reversible reaction of creatine and ATP into phosphocreatine and ADP {Morrison, 1965 #3; McLeish, 2005 #4}. Creatine and phosphocreatine analogues were shown to have antitumor activity both in vitro and in vivo against a broad spectrum of solid tumors characterized by high levels of CK expression, establishing the CK system as a promising target for anticancer chemotherapy drug design{Wyss, 2000 #5; Bergnes, 1996 #6}. Rabbit muscle creatine kinase (RMCK) represents one CK isozyme member that has been studied most extensively {McLeish, 2005 #4; Cleland, 1967 #7}. At pH 8.0 and above, RMCK operates by a rapid equilibrium random bimolecular, bimolecular mechanism optimized for the forward (creatine phosphorylation) reaction {Morrison, 1965 #3; McLeish, 2005 #4}. We describe the experimental procedures of the H
Similarly, another enzyme system exemplified is a cyclic nucleotide phosphodiesterase (PDE) system, which has been well characterized and is of considerable pharmaceutical interest [Martins, 1982 #8; Manallack, 2005 #9]. PDEs catalyze the conversion of nucleoside 3',5'-monophosphates to nucleoside 5'-monophosphates. One isoform, PDE2A (EC 3.1.4.17), is a cGMP stimulated PDE, which is characterized by an increased hydrolysis of cyclic nucleotide stimulated by low levels of cGMP, and can use either cAMP or cGMP as a substrate [Martins, 1982 #8; Beavo, 2006 #10]. PDE2A is expressed in a wide variety of tissues and cell types and involved in regulating many different biochemical processes, such as the cross-talk between cGMP and cAMP pathways, cAMP and PKA phosphorylation of Ca^{2+}-channels in the heart, and cGMP in neurons, long-term memory, and barrier function of endothelial cells under inflammatory conditions [Beavo, 2006 #10]. Selective inhibitors of PDE2A have been reported including erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) [Podzuweit, 1995 #11] and BAY 60-7550 [Hendrix, 2004 #12]. These PDE2A inhibitors have been found to enhance neuronal plasticity and improve memory functions of rats [Hendrix, 2004 #12; Beavo, 2006 #10], though they have so far served primarily as research tool compounds, and have not yet achieved clinical proof of concept. However, there are ongoing efforts and a growing interest in investigating PDE2 inhibitors for treating disorders of endothelial permeability, or learning and memory [Reneerkens, 2009 #13], in therapeutic areas of sepsis and acute respiratory distress syndrome [Seybold, 2005 #14], and also as an attractive anti-cancer agent because of the importance of PDE2A in angiogenesis [Schermuly, 2010 #15]. Experimental procedures for an NMR-based activity assay and its application to kinetic characterization and inhibitor screening for PDE2A using the guanine-based cofactor cGMP are described.
RMCK, as a lyophilized, ATPase-free powder was purchased from Roche (Catalogue No. 10127566001). Stock solution of 150 µM RMCK in 50 mM Tris-dn, pH 9.0 was prepared and stored in aliquots at -20°C. The pH value was corrected for the glass electrode solvent isotope artifact [Glasoe, 1960 #1].

Recombinant full-length human PDE2A as 0.1 µg/µl solution was purchased from SigalChem (Catalogue No. P90-30G). The stock solution of PDE2A was stored in aliquots at -80°C.

The fragment library (MW 150 - 250) used in the inhibitor screen was selected randomly from the proprietary compound library of OSI Pharmaceuticals, Inc. Stock solutions of 100 or 200 mM of compounds were prepared in DMSO-d₆. An aliquot of stock solution was added into each 0.6 ml of reaction mixture to achieve the final concentration of 0.1 or 0.5 mM for each compound.

**NMR experiments**

NMR experiments were performed at 25°C on a Bruker DPX 400-MHz spectrometer equipped with a 5 mm BBI probe and a BACS120 autosampler. The spectrometer was controlled using TopSpin (Bruker BioSpin, v 1.3 pi 10), and each data set was collected under automation using the ICON-NMR software (Bruker BioSpin, v 4.0.7 build 1). The H experiment with water suppression [Hwang, 1995 #24] comprised 65536 data points for chemical shift measurement and 32768 data points in all other experiments, with a spectral width of 8278.15 Hz. A scan number from 64 to 8000 was used for samples containing <0.2 mM of substrate and 64 scans were collected for samples with >0.2 mM of substrate resulting a measurement time of 6 min. All data were processed using ACD/Labs NMR Manager (Advanced Chemistry Development, Inc., Canada). The data were batch-processed using the ACD/Labs group macro, including zero-filling to 2 times of time domain points, an exponential window function with a line broadening of 0.5 Hz, Fourier transform, phase, baseline correction, and peak fitting.

Measurements of ¹H chemical shifts difference of cofactor pairs as a function of pH and metal ion.
[251] For free ATP or ADP samples, the solution contained 0.4 mM ATP or ADP, 0.1 mM DSS in 50 mM Tris-dn in D₂O at different pH. For MgATP or MgADP samples, each mixture contained 0.4 mM ATP or ADP, 40 mM magnesium acetate, 0.1 mM DSS, 50mM Tris-dn in D₂O at different pH. For samples with magnesium ion and EDTA, each mixture contained 0.4 mM ATP or ADP, 1.4 mM magnesium acetate, 5 mM EDTA, 0.1 mM DSS, 50mM Tris-dn in D₂O at different pH. The pH of ATP and the corresponding ADP samples were adjusted to the same value (± 0.02 pH unit) using Tris-dn or deuterium chloride.

[252] For free cGMP or GMP samples, the solution contained 1 mM cGMP or GMP, 0.1 mM DSS in 200 mM Tris in D₂O at pH 7.5 or 9.0. For MgGMP samples, each mixture contained 1 mM cGMP or GMP, 40 mM magnesium chloride, 0.1 mM DSS, 200 mM Tris-dn in D₂O at pH 7.5.

[253] The pH was measured using a Mettler Toledo MP220 pH meter, and corrected for the glass electrode solvent isotope artifact {Glasoe, 1960 #1}. The errors are calculated from two repeated trials.

[254] Characterization of enzyme kinetics

[255] The specific assay conditions for enzyme kinetics measurements are optimized into the NMR format from the literature methods of the specific enzyme system. Generally a 0.5 - 0.6 ml assay mixtures contain variable concentration (µM - mM) of substrate, an appropriate concentration of metal ion if required for the enzyme activity, 0.02% Triton X-100, 0.1 mM 3-(trimethylsilyl) propanoic acid (TMSP) in 50 mM Tris in D₂O, at optimal pH for the enzyme activity. The enzyme concentration used depends on the kₗₐₜ and substrate concentration, and needs to be optimized for enzyme inhibition using enzyme titration. The concentration of enzyme used is comparable to that used in a biochemical assay, and generally in the low nM range. The NMR assay is performed in either real time or end-point format, and is triggered by adding substrate. For the real time experiment, the assay takes place in the 5 mm NMR tube in the NMR spectrometer, with user-defined time intervals for automatic data acquisition. The end-point experiments can be performed in 96 well plate or
individual tubes, and after a certain period of time within the linear range of enzyme activity (1 - 30 min typically), the reaction is stopped by adding EDTA for reactions that require a metal ion, or by adding enzyme deactivators, known potent inhibitors, or denaturing agents such as detergents when a metal ion is not required for the specific enzyme activity. Control experiments of quenched samples after a few days were compared with data of fresh sample, to confirm that the quencher was sufficient to stop the reaction. The assay mixtures were transferred from plate to NMR tubes using a multichannel pipet. The concentrations of substrate and product were determined based on qualification of the corresponding peak areas, and the initial velocity data were analyzed by data fitting software.

[256] The kinetic analysis of RMCK was performed by monitoring the forward reaction MgATP + creatine → MgADP + phosphocreatine + H⁺ [Morrison, 1965 #3]. Each 0.6 ml of reaction mixtures contained 10 nM RMCK, 5, 7, 10, 20, 40 mM creatine, 0.4, 0.6, 1, 2 mM MgATP, 1 mM excess magnesium acetate, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris-dn in D₂O, pH 9.0. The concentrations of total magnesium and total nucleotide to give the required concentrations of MgATP while maintaining the concentration of Mg²⁺ at 1 mM were calculated as described by Morrison and coworkers [Morrison, 1961 #16; Morrison, 1965 #3] using the apparent stability constants of MgATP and MgADP. The reactions were quenched by 20 mM EDTA at three time points within the linear region of the kinetics. The initial velocity data from the ATP/ADP H₈ peak areas were analyzed by multiple non-linear regression using Grafit (Erithacus Software Ltd. UK). For graphing the transformed double reciprocal of data points and the linear fit of each concentration are displayed.

[257] The kinetic analysis of PDE2A was performed by monitoring the reaction cGMP + H₂0 → GMP [Martins, 1982 #8]. In the enzyme titration, each 0.6 ml of reaction mixtures contained 1.25, 2.5, 5, 10, 20 nM PDE2A, 0.2 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris in D₂O, pH 7.5. In the kinetics measurement, each 0.6 ml of reaction mixtures contained 5 nM PDE2A, 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris in D₂O, pH 7.5. The reactions were quenched by 20 mM EDTA at three time points within the linear
region of the kinetics. The initial velocity data from the cGMP/GMP H8 peak areas were analyzed by GraphPad Prism (GraphPad Software, Inc.).

[258] **Determination of IC$_{50}$ of inhibitor**

[259] The NMR assay can be applied to determine the IC$_{50}$ of an enzyme inhibitor. A 0.5 - 0.6 ml assay mixture consists of enzyme, µM - mM substrate, variable concentration (nM - mM) of inhibitor, metal ion if required for the enzyme activity, 0.02% Triton X-100, 0.1 mM TMSP in 50 mM Tris in D$_2$O, at optimal pH for the enzyme activity. The assay protocol and data analysis is the same as in the section above for the enzyme kinetics. The data is fitted to \( \frac{V_i}{V_0} = \frac{1}{1 + ([I]/IC_{50})^h} \), where \( V_i \) and \( V_0 \) is the velocity with and without inhibitor, respectively, and \( h \) is the Hill coefficient. \( \frac{IC_{50}}{K_M} = \frac{[I]^h}{K_s} \) for competitive inhibitor is calculated using where \( K_M \) is determined from the section above.

[260] To determine the IC$_{50}$ of adenosine, a known RMCK inhibitor, the reaction mixtures contained 10 nM RMCK, 0, 1, 2, 4, 7, 10, 14, 20 mM adenosine, 40 mM creatine, 0.0625 mM MgATP, 1 mM excess magnesium acetate, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris-dn, pH 9.0. The reactions were quenched by 20 mM EDTA at four time points in the linear region.

[261] The characterization of EHNA, a known PDE2A inhibitor, was performed in reaction mixtures contained 5 nM PDE2A, 0, 0.01, 0.1, 1, 4, 12, 30, 100, 500, 2000, 5000 µM EHNA, 0.2 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris, pH 7.5. The reactions were quenched by 20 mM EDTA at three time points in the linear region.

[262] **Screen of compound libraries**

[263] Libraries may be selected from a company compound collection and/or acquired from outside vendors. The library may contain fragments of low molecular weight. Filters of molecular properties (e.g. Jacoby, 2003 #17) may be applied and
compounds with undesired druggable functionalities can be removed. Compounds should be checked by liquid chromatography-mass spectrometry for purity and identity. To ensure assay quality, preferably only compounds with the correct mass and purity higher than 85% should be used in the NMR screen.

[264] For an initial screen of a compound library, each of the reaction mixtures may contain up to 50 compounds with a concentration of nM to mM each, grouped by the chemical properties and structure similarity. In the examples described herein, reaction mixtures containing up to ten compounds were used. The 0.5 - 0.6 ml reaction solution contains enzyme, µM - mM substrate, nM - mM compound, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris in D2O, at pH optimal for the enzyme activity. Compounds were incubated for a certain period (generally 15 min) in the assay medium containing enzyme before the reaction is initiated by the addition of substrate. The reactions were quenched by EDTA after a period of time that was in the linear region of enzyme activity, or mid-point of the reaction {Wu, 2003 #18}. Control experiments in the absence of any compound and negative controls in the absence of enzyme were carried out for the same time interval. A control experiment with a known enzyme inhibitor was also performed. The screen was performed in 96 well plate and transferred to NMR tubes for data acquisition. The % inhibition was determined from peak areas of substrate and product signal in the NMR H spectra with and without compound:

\[ \% \text{Inhibition} = \left( \frac{V_o - V_i}{V_o} \right) \times 100\% \]

where \( V_o \) and \( V_i \) is the velocity in the absence and presence of compounds, respectively

[265] Multiple repeated trials were performed for each mixture to obtain a standard deviation of % inhibition. Compound mixtures were ranked by their % inhibition, and an arbitrary cutoff value was defined to deconvolute mixtures with % inhibition higher than that value. Enzyme assays were performed under similar conditions as the initial exemplified screens but with single compounds in each reaction. The solubility of compounds can be checked by the relative intensity of compound peaks in the H NMR spectra compared to substrate, product and TMSP signals. Multiple repeated trials should be performed for each fragment deconvolution, and the standard deviation of % inhibition is calculated from the trials.
In the RMCK screen, each reaction mixture contained 3 - 8 compounds with a concentration of 0.5 mM each. The 0.6 ml reaction solution contained 10 nM RMCK, 0.5 mM compound, 40 mM creatine, 0.4 mM MgATP, 1 mM excess magnesium acetate, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris-dn, pH 9.0. The reactions were quenched by 20 mM EDTA at 10 min and 20 min. The % inhibition was determined from H8 peak areas of ATP and ADP signal in the NMR spectra. Compound mixtures with a % inhibition higher than 40% underwent deconvolution.

In the PDE2A screen, each reaction mixture contained 7 - 10 compounds with a concentration of 0.1 mM each. The 0.6 ml reaction solution contained 5 nM PDE2A, 0.1 mM compound, 0.2 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris, pH 7.5. The reactions were quenched by 20 mM EDTA at 45 min. The % inhibition was determined from H8 or H1 peak areas of cGMP and GMP signal in the NMR spectra. Compound mixtures with a % inhibition higher than 60% underwent deconvolution.

**B3. Results**

**Case 1 (RMCK):**

The NMR activity assay

The impact of pH and magnesium ions, a cofactor necessary for kinase activity, on the proton chemical shifts of ATP and ADP has been well characterized {Jardetzky, 1960 #19; Wang, 1996 #20; Scheller, 1983 #21}. The focus of this investigation is not on individual ATP or ADP chemical shifts as reported by others, but on the differentiation of ATP and ADP peaks under enzymatic assay conditions, in order to develop a method to accurately monitor the ATP ↔ ADP conversion by NMR spectroscopy. The H8 proton on the heterocyclic purine ring was used as the probe signal, and its chemical shift difference in ATP and ADP (\(\Delta\delta = \delta_{\text{ATP}} - \delta_{\text{ADP}}\)) to assess the peak separation. The H8 signal is the best resolved peak between ATP and ADP, and can be very easily quantified due to its singlet NMR
multiplicity and location in a lower field region (∼8.5 ppm) that is separated from buffer and solvent resonances (Figure 1). Before an assay format compatible with the RMCK enzymatic profile could be established, the effect of pH and Mg$^{2+}$ on the H8 $\Delta\delta$ between ATP and ADP was first systematically investigated.

[272] A gradual increase of $\Delta\delta$ is observed with increasing pH in the range from pH 6 to 10, which represents a typical pH range for most enzymes with ATP hydrolytic activities (Figure 2). Both ATP and ADP H8 resonances are downfield shifted with increasing pH (about 0.02 - 0.03 ppm in H chemical shifts). Studies showed that this pH-dependent shifts may be due to increased population of the anti conformation in 5'-adenine nucleotides as a result of increased phosphate deprotonation{Wang, 1996 #20}. The ATP H8 is shifted more than ADP, giving better separation at higher pH with the observed $\Delta\delta$ more than 10 ppb at a pH higher than 9. Over the entire pH range tested $\Delta\delta$ is at least 7 ppb, a resolution sufficient for accurate quantification.

[273] The effect of Mg$^{2+}$-ion on $\Delta\delta$ was also tested. In the presence of saturating amount of Mg$^{2+}$ in the pH 6-10 range, the order of chemical shifts for ATP and ADP is reversed, resulting in negative values of $\Delta\delta$ (Figure 2). Particularly, upfield shifts of the H8 peaks are observed for both ATP and ADP upon binding with Mg$^{2+}$, consistent with the study of Wang et al. {Wang, 1996 #20}. Such upfield shifts have been suggested to be related to the indefinite non-cooperative base stacking that is enhanced by the presence of Mg$^{2+}$ {Scheller, 1983 #21; Wang, 1996 #20}. The value of $\Delta\delta$ remains generally unchanged with the dominant formation of MgATP and MgADP complexes, and stays around 10.5 ppb apart, independent of pH changes (Figure 2).

[274] However, the H8 peaks may not be resolved when mixed populations of Mg-chelated and non-chelated ATP and ADP are present in the presence of non-saturating levels of Mg$^{2+}$-ions. In this case, the opposite $\Delta\delta$ for Mg-chelated and non-chelated species as discussed above may cancel out. Additionally, this phenomenon makes it hard to predict $\Delta\delta$ values when an enzyme is present in solution. Many kinases can bind more than one Mg$^{2+}$-ion that surrounds ATP and the enzyme kinetics are changed upon binding of the second Mg$^{2+}${Adams, 2001 #22; Morrison, 1965 #3},
therefore the saturating concentration of Mg$^{2+}$ may not be desired for optimal kinase activity under specific assay conditions. In order to avoid this potential issue, a general assay process was developed that uses a non-saturating amount of Mg$^{2+}$ in the enzyme-catalyzed reaction stage, which is then totally removed through a metal scavenger, ethylenediaminetetraacetic acid (EDTA), just prior to NMR quantification. As expected, $\Delta \delta$ with the presence of Mg$^{2+}$ and EDTA tracks back to the initial profile of $\Delta \delta$ in the absence of Mg$^{2+}$ (Figure 2). Hence, EDTA is the preferred quencher for this NMR activity assay, in comparison to other quenchers such as protein denaturants and enzyme deactivators, especially for situations where non-saturating Mg$^{2+}$ ions are used. EDTA also brings additional benefit as it has been found to increase $\Delta \delta$ for better peak resolution. In practice, a stock solution of EDTA can be prepared in high pH as EDTA is highly soluble in basic condition, so the addition of EDTA with a final high concentration relatively to the buffer capacity can increase the solution pH and achieve better separation of ATP and ADP peaks for NMR measurements.

[275] Based on the observations discussed above, the NMR activity assay was carried out using the following process: the reaction was performed with RMCK, creatine, MgATP, with or without inhibitors, incubated for a certain time and quenched with EDTA. The assay conditions have been optimized for MgATP concentration in the range of 0.0625 - 2 mM, which is close to or higher than the $K_M$ of ATP for kinases (typically $K_M$ ranges from 1µM to 1mM) [Knight, 2005 #23]. This condition is also in line with the common practice that inhibitor screens are usually performed with the ATP concentration identical to or higher than its $K_M$. Under this range of ATP concentration, the $\Delta \delta$ studies with H8 chemical shifts have been found quite reproducible for RMCK and should be readily applicable to all other kinases, and all other enzymes that utilize purine nucleotides as substrates. The concentration of RMCK (10 nM) used is so low compared to that of MgATP that RMCK has no effect on H8 peak separation. pH 9.0 was chosen for RMCK activity assay, not only because of the good separation of ATP and ADP peaks at this pH but also because this is the optimal pH for the forward reaction (ATP hydrolysis) of RMCK. The peak areas of ATP and ADP in the NMR spectrum were measured to determine initial velocity or percentage of ATP conversion.
[276] **Determination of kinetic constants of RMCK.**

[277] In order to properly set up the ATP concentration used in the inhibitor screen {Fersht, 1985 #24}, the kinetics of the forward reaction catalyzed by RMCK were studied at pH 9.0 using the NMR activity assay. An initial velocity pattern was determined with varied MgATP and creatine concentrations, and the linear fit of each concentration are shown to see how the lines converge (Figure 3). The values of the Michaelis and dissociation constants in terms of each substrate with various enzyme forms were determined using multiple non-linear regression. Synergism in the binding of the substrates is observed, as shown in a decreased $K_M^{MgATP} \ (0.33 \pm 0.06 \ \text{mM})$ as compared to $K_D^{MgATP} \ (1.08 \pm 0.26 \ \text{mM})$, and a decreased $K_M^{CT} \ (6.7 \pm 1.1 \ \text{mM})$ as compared to $K_D^{CT} \ (21.8 \pm 5.6 \ \text{mM})$. It has been suggested that the synergism may be associated with substrate induced conformational changes within the tertiary complex {Chen, 2000 #25}. The kinetics obtained from the NMR measurement agree well with those reported using other types of enzyme assays {Maggio, 1977 #26; Morrison, 1965 #3}.

[278] **Determination of IC$_{50}$ of adenosine.**

[279] The inhibition of RMCK by adenosine, which is a known ATP competitive inhibitor {Noda, 1960 #27}, was characterized using the NMR activity assay to demonstrate the application of this method in determining the IC$_{50}$ value of inhibitor. At 0.0625 mM of MgATP, the IC$_{50}$ value of adenosine (IC$_{50}$ = 10.1 ± 5.5 mM, Hill coefficient = 0.8 ± 0.3) was obtained through titration (Figure 3). The inhibition constant (Ki = 8.5 mM) was calculated based on the measured IC$_{50}$ value and agrees well with the reported Ki of 7 mM in the literature {Noda, 1960 #27}.

[280] **RMCK inhibition screen of fragment library.**

[281] A fragment library containing 178 compounds was randomly selected from an OSI Pharmaceuticals compound collection using the following criteria: molecule weight from 150 to 250 Da, synthesized in house, and availability of more than 20 mg in powder. The resulting list was manually examined by chemists to eliminate compounds that are unstable or contain unwanted functionality. Compounds were
then requested and checked by liquid chromatography-mass spectrometry. 141 compounds passed quality control with the correct mass and purity higher than 85% and were used in the NMR screen. They were grouped into 27 mixtures with 3 - 8 compounds in each group, based on their chemical properties and structure similarity. The NMR activity assay was performed on 27 fragment mixtures, as well as a positive control without any fragment, and a negative control without RMCK. No ADP was formed in the negative control, and about 20% of ATP was converted to ADP in the positive control (Figure 4). The % inhibition of the mixtures was calculated (Table 2), and 11 mixtures with % inhibition higher than 40% were further tested individually. Out of 56 fragments from those mixtures, 12 fragments were confirmed to have % inhibition higher than 40% (Table 2). It is worth noting that there are three additional compounds which show high inhibition (40%>, 43%, and 54% inhibition, respectively), but are not included in Table 2 because their corresponding relative integrals of proton signals comparing to those of ATP/ADP and 3-(Trimethylsilyl) propanoic acid (TMSP) are much lower than expected. Further studies found their NOE cross peaks in assay solution without RMCK have the same phase as the diagonal peaks, suggesting that they form aggregates and cause false positive inhibition [Feng, 2005 #28]. Only one compound, out of 141 fragments screened, has partially overlapping signals with the ADP resonance in the H spectrum. However, its % inhibition can still be calculated by quantifying the non-overlapping ATP signal and internal reference (100 μM TMSP).

[282] Case 2 (PDE2A):

[283] The NMR activity assay

[284] At pH 7.5 and pH 9.0, the aromatic H resonances of cGMP and GMP are well resolved with large chemical shifts differences in cGMP and GMP (Δδ = |δ_{cGMP} - δ_{GMP}|) (Figures 5 and 11). Either the H8 proton on the purine ring (Δδ ≥ 3.106 ppb at pH 7.5 and 9.0) or the H1’ proton on the sugar moiety (Δδ ≥ 59.5 ppb at pH 7.5 and 9.0) can be used as the probe signal, though the H8 signal is preferred whenever possible because the singlet multiplicity of H8 in cGMP and GMP give a better signal intensity than the doublet of H1’ in GMP.
With saturating concentration of $\text{Mg}^{2+}$, the value of $\Delta \delta$ remains large with the dominant formation of Mg-cGMP and Mg-GMP complexes, with values of 326.0 ppb for H8 and 54.5 ppb for H1'.

Because of the well resolved cGMP and GMP resonances in either the free state or the $\text{Mg}^{2+}$-complexed state, the NMR assay can readily be carried out in real time to monitor the Mg-cGMP/Mg-GMP peaks, or in an end-point assay that uses EDTA to quench the reaction and then quantifies the cGMP/GMP resonances. The data presented here are from end-point experiments because it allows more flexibility in setting up the time frame of the assay and data acquisition.

An enzyme titration was performed to determine the PDE2A concentration to be used in the assay, in order to ensure that any diminution of reaction velocity quantitatively correlated with the formation of the enzyme-inhibitor complex (Copeland, 2005 #29). Within the enzyme concentrations tested from 1.25 nM to 20 nM, the initial velocity tracks linearly with PDE2A concentrations (Figure 6). A 5 nM concentration of PDE2A was selected as it falls in the linear region of the enzyme titration, and also provides an appropriate assay time interval (~45 min to reach mid-point).

**Determination of kinetic constants of PDE2A.**

The $K_M$ of cGMP was determined to demonstrate the application of the NMR assay to the characterization of enzyme kinetics. An initial velocity pattern was determined with varied cGMP concentrations (Figure 7). The value of the Michaelis constant ($K_M^{\text{cGMP}} = 13.9 \pm 2.1 \mu\text{M}$) obtained from data fitting agrees well with the literature value of 10 $\mu\text{M}$ (Martins, 1982 #8).

**Determination of IC$_{50}$ of EHNA.**

The application of the NMR assay to enzyme inhibition was demonstrated by determining the IC$_{50}$ value of EHNA, a cGMP competitive PDE2A
inhibitor {Podzuweit, 1995 #11}. At 0.2 mM of cGMP, the IC₅₀ value of EHNA was obtained through titration (Figure 7). The inhibition constant (Ki = 1.0 µM) was calculated, based on the fitted IC₅₀ value (15.5 ± 0.8 µM, Hill coefficient = 1.1 ± 0.0) and agrees well with the reported ¾ of ~ 1 µM in the literature {Manallack, 2005 #9; Rosman, 1997 #30; Podzuweit, 1995 #11}.


[293] A fragment library was designed based on the combination of most common core structures and side chains in drugs, drug candidates {Moore, 1999 #31; Roughley, 2011 #32; Fesik, 2000 #33} and in OSFs proprietary compound collection. Filters of molecular properties {Jacoby, 2003 #17} were applied and the resulting list was searched to establish compound availability from OSI’s company library, or from outside vendors. The available compound list was manually examined by chemists to eliminate compounds that are unstable or contain unwanted functionality. Compounds were then assembled and checked by liquid chromatography-mass spectrometry for purity and identity. 385 compounds passed quality control with the correct mass or UV purity higher than 85%, and were used in the NMR screen. They were grouped into 39 mixtures with 7 - 10 compounds in each group, based on their chemical properties and structure similarity. The NMR activity assay was performed on 39 fragment mixtures in the 96 well plate (2 ml), including positive controls without any fragment, and a negative control without PDE2A. No GMP was formed in the negative control, and about 50% of cGMP was converted to GMP in the positive control (Figure 8). The % inhibition of the mixtures was calculated (Table 3), and seven mixtures with % inhibition higher than 60% were further tested individually. Out of 66 fragments from those mixtures, 6 fragments were confirmed to have % inhibition higher than 50% (Table 3). Among 385 fragments screened, seven compounds had overlapping or partially overlapping signals with cGMP H8 and with GMP H8 resonance, respectively. The % inhibition of those compounds were calculated by quantifying the cGMP and GMP H1’ resonances, which have chemical shifts of ~ 6 ppm lying in a region free of compound signals. Two out of seven mixtures that have shown higher than 60% of inhibition did not show any hits during deconvolution. Further studies have found four compounds in the two mixtures that probably precipitate out during the assay, resulting in abnormally low abundances of
$^1$H peak integration signals. NOESY experiments subsequently performed did not show any expected NOEs, suggesting that these false positives were possibly due to low order compound aggregation [Feng, 2005 #28]. Finally, it's worthy to note that one compound showed -90% inhibition but did not show any expected compound signals in the assay mixture as well as in its DMSO stock solution, indicating that false positive inhibition could arise from enzyme deactivation or/and minor impurities in the sample. Further studies found that the identify of the sample could not be confirmed by LC/MS analysis, although its UV purity is higher than 85%. This is another example demonstrating the additional capability of this NMR assay for eliminating false positives that may not be easily confirmed by other methods.

[294] Determination of IC$_{50}$ of inhibitor

[295] The IC$_{50}$ measurements of fragment hits were performed in reaction mixtures contained 5 nM PDE2A, 0, 0.02, 0.05, 0.15, 0.46, 1.37, 4.12, 12.35, 37.04, 111.333, 1000 µM compound, 0.2 mM cGMP, 2.5 mM magnesium chloride, 0.02% Triton X-100, 0.1 mM TMSP, and 50 mM Tris, pH 7.5. For Compounds MFCD07778360, 03791 187, 00661583, and 08236767, an additional compound concentration of 2000 µM was used. The reactions were quenched by 20 mM EDTA at one time point in the linear region.

[296] Results

[297] PDE2A inhibition screen of a fragment library

[298] The IC$_{50}$ values of six hits were determined at 0.2 mM of cGMP with serial dilution of compounds. Two compounds with highest inhibition (MFCD07369990 and MFCD09033259) showed IC$_{50}$ values of 6.6 and 4.4 µM, respectively. However, their corresponding hill coefficients identified were 2.4, a value higher than one indicating positive co-operativity probably due to the possibilities of nonspecific mechanism of inhibition, very tight binding or irreversible inhibition [Copeland, 2005 #57]. The other four hits showed IC$_{50}$ values from 29 to 134 µM with hill slopes close to 1, and can be used as starting points to expand analogs to be screened next to identify stronger inhibitors.
Finally, it's worthy to note that one compound showed -85% inhibition but did not show any expected compound signals in the assay mixture as well as in its DMSO stock solution, indicating that false positive inhibition may arise from enzyme deactivation or/and minor impurities in the sample. An IC$_{50}$ of 16.4 ± 5.1 μM and a Hill slope of 1.1 ± 0.2 was obtained for this compound at 0.2 mM of cGMP concentration. The IC$_{50}$ data did not show any warning sign or deviation from the typical IC$_{50}$ curve shape of a true inhibitor. The identity of this sample could not be confirmed by a QC LC/MS analysis, although a large UV peak of more than 85% was observed. To further clarify this discrepancy and confirm that the observed inhibition was not from the expected structure, the same compound was ordered from a different supplier and tested for NMR and PDE2A inhibition. For this new material its NMR spectra in either aqueous or DMSO solution matched with its structure, yet no inhibition on the PDE2A activity was observed. This is another example demonstrating the additional capability of this NMR assay for eliminating false positives that may not be easily confirmed by other methods.
Table 2. Compound hits from RMCK screen using the NMR activity assay\textsuperscript{a}

<table>
<thead>
<tr>
<th>Structure</th>
<th>Comp. No.</th>
<th>% Inhibition\textsuperscript{b}</th>
<th>Initial group</th>
<th>% Inhibition of mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure" /></td>
<td>27</td>
<td>52 ± 8</td>
<td>M7</td>
<td>63</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure" /></td>
<td>31</td>
<td>45 ± 4</td>
<td>M7</td>
<td>63</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure" /></td>
<td>48</td>
<td>56 ± 5</td>
<td>M10</td>
<td>60</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure" /></td>
<td>49</td>
<td>53 ± 6</td>
<td>M10</td>
<td>60</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure" /></td>
<td>50</td>
<td>57 ± 6</td>
<td>M11</td>
<td>64</td>
</tr>
<tr>
<td><img src="image6.png" alt="Structure" /></td>
<td>53</td>
<td>49 ± 5</td>
<td>M11</td>
<td>64</td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure" /></td>
<td>75</td>
<td>56 ± 2</td>
<td>M16</td>
<td>66</td>
</tr>
<tr>
<td><img src="image8.png" alt="Structure" /></td>
<td>76</td>
<td>66 ± 6</td>
<td>M16</td>
<td>66</td>
</tr>
<tr>
<td><img src="image9.png" alt="Structure" /></td>
<td>98</td>
<td>76 ± 6</td>
<td>M20</td>
<td>68</td>
</tr>
<tr>
<td><img src="image10.png" alt="Structure" /></td>
<td>100</td>
<td>63 ± 7</td>
<td>M21</td>
<td>66</td>
</tr>
<tr>
<td><img src="image11.png" alt="Structure" /></td>
<td>118</td>
<td>75 ± 7</td>
<td>M24</td>
<td>64</td>
</tr>
<tr>
<td><img src="image12.png" alt="Structure" /></td>
<td>130</td>
<td>43 ± 2</td>
<td>M27</td>
<td>42</td>
</tr>
<tr>
<td><img src="image13.png" alt="Structure" /></td>
<td>Ref 1\textsuperscript{c}</td>
<td>82 ± 6</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td><img src="image14.png" alt="Structure" /></td>
<td>Ref 2\textsuperscript{d}</td>
<td>42 ± 4</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} The compound, MgATP and RMCK concentrations were 0.5 mM, 0.4 mM and 10 mM in the assay, respectively. The % inhibition was calculated as described in the Experimental Section. Only fragment hits with % inhibition higher than 40% are listed.

\textsuperscript{b} The errors are standard deviation of % inhibition from 3 repeated trials.

\textsuperscript{c} A reported RMCK inhibitor that exhibits 35% inhibition at 0.0275 mM against 0.4 mM MgATP using pH-stat method [Bretonnet, 2007 #44]. The compound concentration tested here was 0.25 mM.

\textsuperscript{d} A reported RMCK inhibitor that exhibit 20% inhibition at 0.0275 mM against 0.4 mM MgATP using pH-stat method [Bretonnet, 2007 #44]. The compound concentration was 0.1 mM.
Table 3. Compound hits from PDE2A screen using the NMR activity assay*

<table>
<thead>
<tr>
<th>Structure</th>
<th>Comp. No.</th>
<th>% Inhibitionb</th>
<th>Initial mixture</th>
<th>% Inhibition of mixture</th>
<th>IC₅₀ (µM)d</th>
<th>Hill Coefficientd</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure 1" /></td>
<td>07778360</td>
<td>56 ± 15</td>
<td>M6</td>
<td>77 ± 6</td>
<td>134.3 ± 26.0</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td><img src="image2" alt="Structure 2" /></td>
<td>03791187</td>
<td>57 ± 10</td>
<td>M13</td>
<td>71 ± 10</td>
<td>66.7 ± 10.8</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td><img src="image3" alt="Structure 3" /></td>
<td>07369990</td>
<td>94 ± 2</td>
<td>M14</td>
<td>97 ± 5</td>
<td>6.6 ± 1.0</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td><img src="image4" alt="Structure 4" /></td>
<td>09033259</td>
<td>96 ± 4</td>
<td>M14</td>
<td>97 ± 5</td>
<td>4.4 ± 0.4</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td><img src="image5" alt="Structure 5" /></td>
<td>00661583</td>
<td>86 ± 4</td>
<td>M17</td>
<td>86 ± 7</td>
<td>29.2 ± 6.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td><img src="image6" alt="Structure 6" /></td>
<td>08236767</td>
<td>76 ± 12</td>
<td>M38</td>
<td>84 ± 6</td>
<td>41.9 ± 7.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td><img src="image7" alt="Structure 7" /></td>
<td>EHNAc</td>
<td>50 ± 8</td>
<td>EHNA</td>
<td>57 ± 10</td>
<td>15.5 ± 0.8</td>
<td>1.1 ± 0.0</td>
</tr>
</tbody>
</table>

a. Compound, cGMP and PDE2A concentrations were 0.1 mM, 0.2 mM and 5 nM in the assay, respectively. The % inhibition was calculated as described in the Experimental Section. Only fragment hits with % inhibition higher than 50% are listed.
b. The errors are standard deviation of % inhibition from 3 repeated trials.
c. A reported PDE2A inhibitor {Podzuweit, 1995 #11} was tested using the NMR assay (Figure. 9b) at 15 µM in the presence of 0.2 nM cGMP.
d. IC₅₀ and hill coefficient of hits were obtained from serial dilution of inhibitors. The errors are from data fitting.
Discussion

[300] The 1H NMR activity assay described in this study allows for generic, rapid and reliable biochemical screening of enzyme systems catalyzing cofactor conversions. It is demonstrated to be a superior method to other reported NMR biophysical and biochemical screens, due to its high throughput, low resource requirement, and broad application to numerous drug targets. Its consumption of protein is in the low nanomolar (nM) range, comparable to traditional HTS, and about 2-5 orders of magnitude lower than that used by NMR biophysical methods, but it offers significantly higher throughput than other NMR screens. The studies demonstrated here were performed in a conventional NMR automation lab with relatively standard equipment (400 MHz, 5mm conventional probe, BACS autosampler, 600 μl sample volume). By supplementing this with a liquid handler, a higher magnetic field NMR instrument, and a cryo-microprobe, this method should be comparable with HTS in terms of the total protein consumption, and running time in seconds for each queue sample of 30μl in contrast to a typical biophysical STD experiment that requires about 2 hour of acquisition time per sample under the presence of ~5 μM kinase [McCoy, 2005 #34]. Another advantage of this 1H NMR based activity assay over other biophysical NMR screens is that the inhibitory activity of a compound on the enzyme activity can be directly confirmed during the assay, while in other biophysical NMR screens hits may arise from non-specific binding rather than inhibition of enzyme activity. The greatest benefit of the 1H NMR activity assay disclosed herein resides in it's unexpected but extremely useful ability to directly monitor the NMR resonances of a variety of cofactors, all of which are key substrates for a wide range of enzymes. Hence, this NMR assay can be used, for example, as a universal screen for inhibitors of all purine- and pyrimidine-utilizing enzymes, including kinases, ATPases, GTPases, sulfotransferases, and PDEs, all of which are of great pharmaceutical interest. This broad potential for application makes the 1H NMR activity assay more attractive for drug discovery than other NMR biochemical screens, such as those that are dependent on artificial labelling of non-natural substrates (e.g. 19F-labeling) or are specific for only one type of compound or reaction (e.g. 1H NMR of nicotinic acid adenine dinucleotide){Dalvit, 2003 #35; Dalvit, 2004 #36; Stockman, 2008 #37; Stockman, 2007 #38}.
A general concern for $^1$H NMR screens is the issue of resonance overlap. The studies described herein focus on the proton resonances of cofactors in the aromatic region of $^1$H spectra, free from peaks of common solvents, buffers, and detergents. It is possible that substrate and product signals will overlap with compound peaks in screen mixtures. However, in addition to the purine H8 and pyrimidine ring H6, other protons can be sometimes be used as the probe signal (see Figures 10-20), providing alternative means for detection that can help to minimize any potential problem of signal overlap. With the use of an internal reference (100 µM TMSP in this study), quantitative data can be obtained with either substrate or product peak along with the reference peaks. Thus quantification becomes impossible only on the rare occasion when a compound overlaps with all substrate and product resonances. In the first case study, of RMCK, there was only 1 compound partially overlapping with the ADP resonance out of 141 fragments in the screen. However the % inhibition could still be calculated from the non-overlapping ATP peak and internal reference (100 µM TMSP). In the second case study, of PDE2A, a screen of 385 fragments, only seven compounds had overlapping signals with cGMP H8 and GMP H8 resonances. The % inhibition of those compounds could still be calculated by quantifying the cGMP and GMP H1’ resonances, which have chemical shifts of ~ 6 ppm, lying in a region free of compound signals.

The $^1$H NMR activity assay also has a number of other advantages over traditional biochemical assays {Avila, 2006 #39; Rowlands, 2004 #40; Wigle, 2007 #41; Rominger, 2007 #42}. Biochemical assays frequently require the presence of a secondary reporting reaction, and many operate in rather complex multi-component systems, and are thus susceptible to a high rate of false positives resulting from interference with the fluorimetric or colorimetric detection. In addition, the low solubility and aggregation states of a compound, especially in high concentration screening for fragment based drug discovery, can present a major cause of false positives {Rishton, 1997 #43; Feng, 2005 #28}. The $^1$H NMR activity assay directly detects substrate and product signals and does not need any product derivation for detection, making this method the simplest of assay formats, with minimal possibility of interference or artifact. The concentration of compounds can be obtained from the same $^1$H spectrum, providing essential information to derive accurate inhibition data. In the case of RMCK, there are three compounds showing inhibition but their $^1$H peak
integrations are much lower than expected, and their NOESY spectra point to compound aggregation. By contrast, false positives from compound aggregation are not as easily recognizable in a traditional biochemical assay [Feng, 2005 #28]. Additionally, the NMR activity assay does not require significant assay development, and thus may be extremely useful in academic settings and smaller organizations, where HTS capability is not as readily available, and development of biochemical assay protocols may be challenging.

[303] As an intrinsic biochemical assay, the NMR based activity assay has limitations in the affinity range of the inhibitors that it can detect. From the equation

\[ I = \frac{7C_{50}}{\sqrt{K_M}} \text{ th} \]

\[ K_I = \frac{7C_{50}}{\sqrt{K_M}} \] the Michaelis constant \( K_M \), the substrates concentration, and the compound concentration used in the screen limit the \( \frac{3}{4} \) range that can be measured.

For the RMCK screen in this study, for example, a cutoff of 40% inhibition at 0.5 mM of compound concentration sets the high assay limit of \( IC_{50} \) range up to 0.75 mM, and the weakest ATP-competitive fragment that can be detected would have a \( \frac{3}{4} \) of 0.34 mM, and the weakest creatine-competitive fragment, with a \( \frac{3}{4} \) of 0.11 mM. For the PDE2A screen, a cutoff of 50% inhibition at 0.1 mM of compound concentration sets the high assay limit of \( IC_{50} \) range up to 0.1 mM, and the weakest cGMP-competitive fragment that can be detected would have a \( \frac{3}{4} \) of 6.5 \( \mu \)M. More details of predictions of RMCK inhibition are illustrated in Figure 9. From the simulation plot of % inhibition of RMCK as a function of the concentration of the inhibitor, it shows that the % inhibition is most sensitive to detect small \( \frac{3}{4} \) when the inhibitor concentration is low, and a higher inhibitor concentration is needed in order to detect weak binders (Figure 9a). As indicated in the % inhibition as a function of the concentration of substrate, a very low substrate concentration is required to detect weaker competitive binders (Figure 9b), but it will be compromised with longer NMR acquisition time. Simulations such as those shown in Figure 9 should preferably be performed to determine an appropriate substrate and inhibitor concentration before a screen for inhibitors takes place.

[304] In conclusion, utilizing cofactor proton resonance to directly monitor the substrate/product conversion, the NMR activity assay described here can be applied to screen lead-like compounds, as well as smaller molecules in fragment
based drug discovery, for a wide range of enzymes. The broad application, high throughput, cost-effectiveness of samples, and simple implementation of this method make it an extremely versatile tool for compound library screening in both small and large drug discovery laboratories.

References


[366] **Abbreviations**

[367] PAPS, 3'-phosphoadenosine-5'-phosphosulfate; SAM, S-adenosyl-L-methionine; GDP-mannose or GDP-man, guanosine diphosphate-mannose; UDP-GlcNac, uridine diphosphate N-acetylglucosamine; ATP, adenosine-5'-triphosphate; ADP, adenosine-5'-diphosphate; AMP, adenosine-5'-monophosphate; cAMP, 3'-5'-cyclic adenosine monophosphate; ADPR, ADP-ribose; NAD, Nicotinamide adenine dinucleotide; GTP, guanosine-5'-triphosphate; GDP, guanosine-5'-diphosphate; GMP, guanosine-5'-monophosphate; cGMP, 3',5'-cyclic guanosine monophosphate; SAH, S-adenosyl-L-homocysteine; PAP, 3'-phosphoadenosine-5'-monophosphate; GDP-fucose, guanosine-5'-diphosphate L-fucose; ITP, inosine 5'-triphosphate; IDP, inosine 5'-diphosphate; IMP, inosine 5'-monophosphate; cIMP, 3'-5'-cyclic inosine monophosphate; XDP, xanthosine 5'-diphosphate XTP, xanthosine 5'-triphosphate; XMP, xanthosine 5'-monophosphate; cXMP, 3',5'-cyclic xanthosine monophosphate; cADPR, cyclic ADP Ribose; NADP, nicotinamide adenine dinucleotide phosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; UDP-GlcA, UDP-glucuronate; CTP, cytidine 5'-triphosphate; CDp, cytidine 5'-diphosphate CMP; cytidine 5'-monophosphate; cCMP, 3',5'-cyclic cytidine monophosphate; UTP, uridine 5'-triphosphate; UDP, uridine 5'-diphosphate; UMP, uridine 5'-monophosphate; cUMP, 3',5'-cyclic uridine monophosphate; TTP, thymidine 5'-triphosphate; TDP, thymidine 5'-diphosphate; TMP, thymidine 5'-monophosphate; cTMP, 3',5'-cyclic thymidine monophosphate; CMP-Neu5Ac, cytidine 5'-monophosphate-N-acetylneuraminic acid; CoA, coenzyme A; AcetylCoA, acetyl coenzyme A; WaterLOGSY, Water-Ligand Observed via Gradient Spectroscopy; NOESY, Nuclear Overhauser effect spectroscopy; COSY, Correlation spectroscopy; HMQC, Heteronuclear multiple-quantum correlation spectroscopy; HMBC,
heteronuclear multiple-bond correlation spectroscopy; NMR, nuclear magnetic resonance; STD, Saturation Transfer Difference; RMCK, rabbit muscle creatine kinase; PDE, phosphodiesterase; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; TMSP, 3-(trimethylsilyl) propanoic acid; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid, a.k.a. 3-(trimethylsilyl)-l-propanesulfonic acid; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine, a.k.a. 3-(6-aminopurin-9-yl)nonan-2-ol hydrochloride; MTA, 5'-methylthioadenosine; m\(^7\)GTP, 7-methylguanosine 5'-triphosphate; m\(^7\)GDP, 7-methylguanosine 5'-diphosphate; UDP-Gal, UDP-galactose.

[368] **Incorporation by Reference**

[369] All patents, published patent applications and other references disclosed herein are hereby expressly incorporated herein by reference.

[370] **Equivalents**

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.
WHAT IS CLAIMED IS:

1. A method for identifying a modulator of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a purine H8 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H8 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the substrate to the product resulting therefrom.

2. The method of claim 1, wherein the modulator is an enzyme inhibitor.

3. The method of claim 1, wherein the modulator is an enzyme agonist.

4. The method according to claim 1, wherein said enzyme is selected from the group consisting of: a kinase, a phosphotransferase, a GTPase, GMP kinase, GDP-mannosyltransferase, a lipid kinase, a ceramide kinase, a phosphatidylinositol 3-kinase, a phosphatidylinositol 4-kinase, a phosphatidylinositol-phosphate 4-kinase, a phosphatidylinositol-phosphate 5-kinase, a sphingosine kinase, a diacylglycerol kinase, an amino acid kinase, a sugar kinase, creatine kinase, aspartate kinase, hexokinase, fructokinase, galactokinase, phosphofructokinase, riboflavin kinase, shikimate kinase, thymidine kinase, ATP synthase, ADP-thymidine kinase, AMP-thymidine kinase, NAD+ kinase, glycerol kinase, pantothenate kinase, mevalonate kinase, pyruvate kinase, deoxyctydine kinase, PFP kinase, diacylglycerol kinase, phosphoinositide 3-kinase, sphingosine kinase, phosphomevalonate kinase, adenyl cyclase, guanylyl cyclase, phosphodiesterases, the human PDE isozymes PDE1A, PDE1B, PDE1B2, PDE1C, PDE2A, PDE3A, PDE3B, PDE4A, PDE4B, PDE4B5, PDE4C, PDE4D, PDE5A, PDE6A, PDE6B, PDE6C, PDE7A, PDE7B, PDE8A, PDE8B, PDE9A, PDE10A, PDE10A2, and PDE11A, cAMP-selective phosphodiesterases, phosphodiesterases 4, 7 or 8, cGMP-selective...
phosphodiesterases, phosphodiesterases 5, 6 or 9, cAMP and cGMP-hydrolyzing phosphodiesterases, phosphodiesterases 1, 2, 3, 10 or 11, sulfotransferases, sulfotransferases SULT1A1, SULT1A2, SULT1A3, SULT1A4, SULT1B1, SULT1C2, SULT1C3, SULT1C4, SULT1D1P, SULT1E1, SULT2A1, SULT2B1, SULT4A1, or SULT6B1, carbohydrate sulfotransferase, carbohydrate sulfotransferase CHST1, CHST2, CHST3, CHST4, CHST5, CHST6, CHST7, CHST8, CHST9, CHST10, CHST11, CHST12, CHST13, or CHST14, galactose-3-O-sulfotransferase, galactose-3-O-sulfotransferase GAL3ST1, GAL3ST2, GAL3ST3, or GAL3ST4, heparan sulfate 2-O-sulfotransferase, heparan sulfate 2-O-sulfotransferase HS2ST1, heparan sulfate 3-O-sulfotransferase, heparan sulfate 3-O-sulfotransferase HS3ST1, HS3ST2, HS3ST3A1, HS3ST3A2, HS3ST3B1, HS3ST3B2, HS3ST4, HS3ST5, or HS3ST6, heparan sulfate 6-O-sulfotransferase, heparan sulfate 6-O-sulfotransferase HS6ST1, HS6ST2, or HS6ST3, N-deacetylase/N-sulfotransferase, N-deacetylase/N-sulfotransferase NDST1, NDST2, NDST3, or NDST4, tyrosylprotein sulfotransferase, tyrosylprotein sulfotransferase TPST1 or TPST2, uronyl-2-sulfotransferase, uronyl-2-sulfotransferase UST, estrone sulfotransferase, chondroitin 4-sulfotransferase, adenylylate kinase, nucleoside-diphosphate kinase, uridylate kinase, guanylate kinase, purine nucleoside phosphorylase, Ribose-phosphate diphosphokinase, a phosphatidylinositoltransferase, N-acetylgalactosamine-1-phosphate transferase, thiamine pyrophosphokinase, a guanylyl transferase, phosphoglycerate kinase, ATPase, a protein kinase, a protein-serine kinase, a protein-histidine kinase, a protein-threonine kinase, protein-tyrosine kinase, AMP nucleosidase, ADP-ribose diphosphatase, a cyclic nucleotide phosphodiesterase (PDE), kinesin, ecto-ATPase, CTP synthase, NDPase, NTPDase, 5'-nucleotidase, methyltransferase, methionine adenosyltransferase, sulfotransferase, glycosyltransferase, adenosylhomocysteine nucleosidase, methylthioadenosine nucleosidase, S-methyl-5'-thioadenosine phosphorylase, mRNA (guanine-N7)-methyltransferase, GMP synthase, and adenylyl cyclase.

5. The method of claim 1, wherein the substrate is ATP, ADP, AMP, cAMP, adenine, adenosine, GTP, GDP, GMP, GDP-mannose, GDP-fucose, guanine, guanosine, m^2GDP, m^2GTP, MTA, cGMP, ITP, IDP, IMP, cIMP, inosine, hypoxanthine, XMP, XDP, XTP, cXMP, xanthosine, xanthine, SAH, SAM, PAP, PAPS, cADPR, ADPR, NAD, NADP, NADH, NADPH, or NAADP.
6. The method of claim 1, wherein the product is ATP, ADP, AMP, cAMP, adenine, adenosine, GTP, GDP, GMP, GDP-mannose, GDP-fucose, guanine, guanosine, m^7GDP, m^7GTP, MTA, cGMP, ITP, IDP, IMP, cIMP, inosine, hypoxanthine, XMP, XDP, XTP, cXMP, xanthosine, xanthine, SAH, SAM, PAP, PAPS, cADPR, ADPR, NAD, NADP, NADH, NADPH, or NAADP.

7. The method of claim 1, wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: ATP/ADP, ADP/AMP, ATP/AMP, cAMP/AMP, ATP/cAMP, AMP/adenosine, Adenosine/adenine, AMP/adenine, GTP/GDP, GDP/GMP, GTP/GMP, cGMP/GMP, GTP/cGMP, GMP/guanosine, Guanosine/guanine, GDP/guanine, GDP-Man/GDP, GDP-Man/GTP, GDP-Fuc/GDP, ITP/IDP, IDP/IMP, ITP/IMP, IMP/inosine, AMP / IMP, inosine/hypoxanthine, IMP/hypoxanthine, GMP/IMP, IMP/XMP, adenosine/inosine, adenine/hypoxanthine, XTP/XDP, XDP/XMP, XTP/XMP, XMP/xanthosine, XMP/GMP, XMP/xanthine, xanthosine/xanthine, guanine/xanthine, xanthine/hypoxanthine, m^7GDP/GDP, m^7GTP/GTP, SAH/adenine, MTA/adenine, SAM/SAH, ATP/SAM, PAPS/PAP, ADPR/cADPPv, ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, and NADP/NAADP.

8. The method of claim 1, wherein in step (b), the test compound is contained in a mixture of compounds, and wherein the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the modulator of said enzyme.

9. The method of claim 1, wherein in step (b), the test compound is contained in a mixture of compounds, and wherein the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and each individual compound in said mixture so as to determine which compound in said mixture is the modulator of said enzyme.
10. The method of claim 1, wherein during the course of catalysis by the enzyme for which a modulator is sought, one or more additional enzymes are included in the reaction mixture to provide a coupled enzyme reaction.

11. The method of claim 1, wherein the enzyme that catalyses the modification of a purine-ring-containing substrate is a protein kinase selected from the group consisting of: AKT; AKT2; AKT3; PDK1; PK428; CITRON-K; DMPK; DMPK2; MRCKb; p160 ROCK2; ROCK1; RHODK; BARK1A; BARK2; GRK4; GRK5; GRK6; GRK7; KIAA0303; KIAA0561; SAST; MNK1; MNK2; 138214; KIAA0151; T2K; TEST1; NDR; WART1; WART2; PKXI; PKACa; PKACb; PKACg; PRKY; CAB43292; EPK2; EPK2; PCK1; PKCa; PKCb; PKCd; PKCe; PKCg; PKCi; PKCm; PKCt; PKCz; PKD; PRK1; PRK2; Z25429; CGK; cGMPk; cGMPKII; AAD30182; MSK1; MSK2; p70S6K; p70S6Kb; RSK1; RSK2; RSK3; MAST205; SGK; A6; EEF-2K; BCKDK; PDK1; PDK2; PDK3; PDK4; BCR; FAST; GS3955; PK38; AMPKal; AJ006701; AMPK2; AMPKa2; AUF1; NIPK; U13258; AL049688; CaMKI; CaMKIb2; CaMKIIa; CaMKIIb; CaMKIIg; CAMKL; CAMKL1; CASK; CHK2; DAPK; DAPKR1P1; DRAK1; DRAK2; ZIP; KIAA0135; KIAA0096; C-TAK1; EMK; KIAA0537; KIAA0999; MAK-V; MARK1; MSK; SNRK; MAPKAPK2; MAPKAPK3; MAPKAPK5; MLCK; MLCKs; Titin; PHKG1; PHKgT; PSK-H1; Trio; TRAD; KIAA0369; CKIg2; CKIa; CKId; CKIe; KKIAMRE; CAK; CCRK; CDC2; CDK2; CDK3; CDK4; CDK5; CDK6; CDK8; CHED; KIAA0904; KKLARE; PCTAIRE1; PCTAIRE2; PCTAIRE3; PFTAIRE; PISSLRE; PITALRE; PITSLREa2; STK9; CLK1; CLK2; CLK3; CLK4; STY; GSK3a; GSK3b; ERK1; ERK2; ERK3; ERK4; ERK5; ERK7; XNKla; XNK2; XNK3a; NLK; p38; SAPK2b; SAPK3; SAPK4; KIAA0936; MAK; MOK; RAGE4; YAB1; SUDD; NY-REN-64; AIE1; AIE2; AUR1; AUR2; BUB1; BUBR1; GSG2; CaMKIV; CAMKKB; CHK1; CKIIa; CKIIa'; COT; DYRK2; DYRK1B; DYRK3; DYRK4; HIPK1; HIPK2; KIS; MNB; PKY; EIF2aK; EIF2aK; PEK; GAK; IKKa; IKKb; ILK; IRAK; IRAK2; IRAK-M; IRE1; LIMK2; TESK1; LIMK1; LIMK2b; TESK2; LKB1; DLK; HH498; MLK1; MLK2; SPRK; MOS; PIM2; KID1; PIM1; PRK; SNK; PLK1; SAK; PRP4; KSR; ARaf; BRaf; Raf1; HCYP2; KIAA0472; RICK; RIP; RIP3; SRPK2;
12. A method for identifying an inhibitor of a kinase, comprising: a) measuring the change in intensity of the purine H8 NMR signal for ATP, and/or ADP, in the presence of the kinase after a period of time, whereby said period of time is the time length required for conversion of a quantity of the ATP into ADP by the kinase, and whereby the purine H8 NMR signal is measured after said conversion is terminated by the addition of a chelator of magnesium ions; b) measuring the change in intensity of the purine H8 NMR signal for the ATP, and/or ADP, in the presence of the kinase and a test compound after said period of time and termination as in (a); and c) identifying the test compound as an inhibitor of the kinase by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has inhibited the enzymatic conversion of ATP to ADP.

13. A method for identifying an agonist of a kinase, comprising: a) measuring the change in intensity of the purine H8 NMR signal for ATP, and/or ADP, in the
presence of the kinase after a period of time, whereby said period of time is the time length required for conversion of a quantity of the ATP into ADP by the kinase, and whereby the purine H8 NMR signal is measured after said conversion is terminated by the addition of a chelator of magnesium ions; b) measuring the change in intensity of the purine H8 NMR signal for the ATP, and/or ADP, in the presence of the kinase and a test compound after said period of time and termination as in (a); and c) identifying the test compound as an agonist of the kinase by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has stimulated the enzymic conversion of ATP to ADP.

14. The method of claim 12 or 13, wherein the kinase is a protein kinase.

15. The method of claim 12 or 13, wherein the kinase is a lipid kinase.

16. A method for identifying a modulator of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the intensity of a pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyrimidine H6 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the substrate to the product resulting therefrom.

17. The method of claim 16, wherein the modulator is an enzyme inhibitor.

18. The method of claim 16, wherein the modulator is an enzyme agonist.

19. The method according to claim 16, wherein said enzyme is selected from the group consisting of: a kinase, CTP-dependent galactolipid kinase, a phosphotransferase, a diphosphotransferase, a nucleotidyltransferase, a...
phosphatidyltransferase, CDP-diacylglycerol-inositol 3-phosphatidyltransferase, a glycosyl-1-phosphotransferase, and a nucleotide phosphohydrolase.

20. The method of claim 15, wherein the substrate is a pyrimidine containing compound, CTP, CDP, CMP, cytidine, cytosine, cCMP, CMP-Neu5Ac, UTP, UDP, UMP, uridine, uracil, cUMP, TTP, TDP, TMP, thymidine, thymine, UDP-GlcNac, UDP-GlcA, UDP-Gal, or cTMP.

21. The method of claim 16, wherein the product is a pyrimidine containing compound, CTP, CDP, CMP, cytidine, cytosine, cCMP, CMP-Neu5Ac, UTP, UDP, UMP, uridine, uracil, cUMP, TTP, TDP, TMP, thymidine, thymine, UDP-GlcNac, UDP-GlcA, UDP-Gal, or cTMP.

22. The method of claim 16, wherein the pyrimidine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: UTP/UDP, UDP/UMP, UTP/UMP, UMP/Uridine, Uridine/Uracil, UMP/Uracil, UDP-GlcA/UDP, UDP-GlcNac/UDP, UDP-Gal/UDP, CTP/CDP, CDP/CMP, CMP/Cytidine, CMP/Cytosine, CTP/CMP, Cytidine/Cytosine, CMP-Neu5Ac/CMP, UTP/CTP, Uridine/Cytidine, Uracil/Cytosine, TTP/TDP, TDP/TMP, TTP/TMP, TMP/thymidine, and thymidine/thymine.

23. The method of claim 16, wherein in step (b), the test compound is contained in a mixture of compounds, and wherein the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the modulator of said enzyme.

24. The method of claim 16, wherein in step (b), the test compound is contained in a mixture of compounds, and wherein the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and each individual compound in said mixture so as to determine which compound in said mixture is the modulator of said enzyme.
25. The method of claim 16, wherein during the course of catalysis by the enzyme for which a modulator is sought, one or more additional enzymes are included in the reaction mixture to provide a coupled enzyme reaction.

26. A method for identifying a modulator of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a ribose H1' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1' NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: AMP/adenosine, cAMP/AMP, GMP/guanosine, cGMP/GMP, cGMP/GTP, ATP/SAM, SAM/SAH pairs, cAMP/ATP, IMP/inosine, IMP/GMP, IMP/XMP, inosine/adenosine, XMP/xanthosine, XMP/GMP, cADPR/ADPR, cADPR/NAD, ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, guanosine/guanine; AMP/adenine, adenosine/adenine, GMP/guanine, IMP/hypoxanthine, inosine/hypoxanthine, XMP/xanthine, and xanthosine/xanthine.

27. A method for identifying a modulator of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a purine H2 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a purine H2 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the
substrate to the product resulting therefrom; wherein the purine-ring-containing
substrate and resulting product are a cofactor pair selected from the group consisting
of the following cofactor pairs: adenosine/adenine, ATP/SAM, SAM/SAH,
AMP/adenine, inosine/hypoxanthine, IMP/hypoxantine, IMP/AMP,
inosine/adenosine, hypoxanthine/adenine, cADPR/ADPR, cADPR/NAD,
ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, NADP/NAADP,
IMP/GMP, IMP/XMP and xanthine/hypoxanthine.

28. A method for identifying a modulator of an enzyme that catalyses the
modification of a purine-ring-containing substrate, comprising: a) measuring the
intensity of a sugar H1" NMR signal for said substrate, and/or the product resulting
therefrom, in the presence of said enzyme over a period of time, whereby said period
of time is the time length required for conversion of a quantity of the substrate into the
product by said enzyme; b) measuring the intensity of a sugar H1" NMR signal for
said substrate, and/or the product resulting therefrom, in the presence of said enzyme
and a test compound over said period of time; and c) identifying the test compound as
a modulator of said enzyme by comparing the signal intensities measured in steps (a)
and (b) to determine if the test compound has altered the enzymic conversion of the
substrate to the product resulting therefrom; wherein the purine-ring-containing
substrate and resulting product are a cofactor pair selected from the group consisting
of the following cofactor pairs: cADPR/ADPR, cADPR/NAD, ADPR/NAD,
ADPR/NADP, NADP/NAADP, GDP/GDP-man, GTP/GDP-man, NAD/NADH, and
NADP/NADPH.

29. A method for identifying a modulator of an enzyme that catalyses the
modification of a purine-ring-containing substrate, comprising: a) measuring the
intensity of a pyridine H2" NMR signal for said substrate, and/or the product
resulting therefrom, in the presence of said enzyme over a period of time, whereby
said period of time is the time length required for conversion of a quantity of the
substrate into the product by said enzyme; b) measuring the intensity of a pyridine
H2"NMR signal for said substrate, and/or the product resulting therefrom, in the
presence of said enzyme and a test compound over said period of time; and c)
identifying the test compound as a modulator of said enzyme by comparing the signal
intensities measured in steps (a) and (b) to determine if the test compound has altered
the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: NAD/NADH, NADP/NADPH, NADP/NAADP, cADPR/NAD, ADPR/NAD, and ADPR/NADP.

30. A method for identifying a modulator of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a pyridine H4" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyridine H4" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: NAD/NADH, NADP/NADPH, NADP/NAADP, cADPR/NAD, ADPR/NAD, and ADPR/NADP.

31. A method for identifying a modulator of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a pyridine H5" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyridine H5" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: NAD/NADH, NADP/NADPH, NADP/NAADP, cADPR/NAD, ADPR/NAD, and ADPR/NADP.
32. A method for identifying a modulator of an enzyme that catalyses the modification of a purine-ring-containing substrate, comprising: a) measuring the intensity of a pyridine H6’’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyridine H6’’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the substrate to the product resulting therefrom; wherein the purine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: NAD/NADH, NADP/NADPH, NADP/NAADP, cADPR/NAD, ADPR/NAD, and ADPR/NADP.

33. A method for identifying a modulator of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the intensity of a ribose H1’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a ribose H1’ NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the substrate to the product resulting therefrom; wherein the pyrimidine-ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: CTP/CDP, CDP/CMP, CMP/cytidine, thymidine/thymine, CMP/cytosine, and cytidine/cytosine.

34. A method for identifying a modulator of an enzyme that catalyses the modification of a pyrimidine-ring-containing substrate, comprising: a) measuring the intensity of a pyrimidine H5 NMR signal for said substrate, and/or the product
resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a pyrimidine H5 NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the substrate to the product resulting therefrom; wherein the pyrimidine -ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: CMP/cytidine, cytosine/cytidine, cytosine/uracil, CMP/cytosine, UMP/uracil, uridine/uracil, CTP/UTP, and cytidine/uridine.

35. A method for identifying a modulator of an enzyme that catalyses the modification of a pyrimidine -ring-containing substrate, comprising: a) measuring the intensity of a glucosamine H1" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme over a period of time, whereby said period of time is the time length required for conversion of a quantity of the substrate into the product by said enzyme; b) measuring the intensity of a glucosamine H1" NMR signal for said substrate, and/or the product resulting therefrom, in the presence of said enzyme and a test compound over said period of time; and c) identifying the test compound as a modulator of said enzyme by comparing the signal intensities measured in steps (a) and (b) to determine if the test compound has altered the enzymic conversion of the substrate to the product resulting therefrom; wherein the pyrimidine -ring-containing substrate and resulting product are a cofactor pair selected from the group consisting of the following cofactor pairs: UDP/UDP-GlcNac and UDP/UDP-GlcA.

36. The method of any of claims 26-35, wherein the modulator is an enzyme inhibitor.

37. The method of any of claims 26-35, wherein the modulator is an enzyme agonist.

38. The method of any of claims 26-35, wherein in step (b), the test compound is contained in a mixture of compounds, and wherein the signal intensity for the
substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and said mixture in the absence of each compound contained by said mixture so as to determine which compound in said mixture is the modulator of said enzyme.

39. The method of any of claims 26-35, wherein in step (b), the test compound is contained in a mixture of compounds, and wherein the signal intensity for the substrate, and/or the product resulting therefrom, is repeatedly measured in the presence of the enzyme and each individual compound in said mixture so as to determine which compound in said mixture is the modulator of said enzyme.

40. The method of any of claims 26-35, wherein during the course of catalysis by the enzyme for which a modulator is sought, one or more additional enzymes are included in the reaction mixture to provide a coupled enzyme reaction.

41. A method for determining the activity of an enzyme that catalyses the conversion between a pair of purine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the purine H8 NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme.

42. The method of claim 41, wherein the pair of purine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: ATP/ADP, ADP/AMP, ATP/AMP, cAMP/AMP, ATP/cAMP, AMP/adenosine, Adenosine/adenine, AMP/adenine, GTP/GDP, GDP/GMP, GTP/GMP, cGMP/GMP, GTP/cGMP, GMP/guanosine, Guanosine/guanine, GMP/guanine, m^7GDP/GDP, m^7GTP/GTP, SAH/adenine, MTA/adenine, GDP-Man/GDP, GDP-Man/GTP, GDP-Fuc/GDP, ITP/IDP, IDP/IMP, ITP/IMP, IMP/inosine, AMP / IMP, inosine/hypoxanthine, IMP/hypoxanthine, GMP/IMP, IMP/XMP, adenosine/inosine, adenine/hypoxanthine, XTP/XDP, XDP/XMP, XTP/XMP, XMP/xanthosine, XMP/GMP, XMP/xanthine, xanthosine/xanthine, guanine/xanthine, xanthine/hypoxanthine, SAM/SAH, ATP/SAM, PAPS/PAP, ADPR/cADPR, ADPR/NAD, ADPR/NADP, NAD/NADH, NADP/NADPH, and NADP/NAADP.
43. The method according to claim 41, wherein the enzyme is selected from the group consisting of: a protein kinase, a lipid kinase, a phosphotransferase, a glycosyltransferase, a nucleotide phosphohydrolase and a phosphodiesterase.

44. The method of claim 41, wherein the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

45. A method for determining the activity of an enzyme that catalyses the conversion between a pair of pyrimidine-ring-containing cofactors, comprising incubating a cofactor substrate in the presence of said enzyme for a period of time, and measuring the amount of conversion into the cofactor product after said period of time, wherein the intensity of the pyrimidine H6 NMR signal for said substrate and/or the product resulting therefrom is used to assess the amount of conversion and thus activity of the enzyme.

46. The method of claim 45, wherein the pair of pyrimidine-ring-containing cofactors are selected from the group consisting of the following cofactor pairs: UTP/UDP, UDP/UMP, UTP/UMP, UMP/Uridine, Uridine/Uracil, UMP/Uracil, UDP-GlcA/UDP, UDP-Gal/UDP, UDP-GlcNac/UDP, CTP/CDP, CDP/CMP, CMP/Cytidine, CMP/Cytosine, CTP/CMP, Cytidine/Cytosine, CMP-Neu5Ac/CMP, UTP/CTP, Uridine/Cytidine, Uracil/Cytosine, TTP/TDP, TDP/TMP, TTP/TMP, TMP/thymidine, and thymidine/thymine.

47. The method according to claim 45, wherein the enzyme is selected from the group consisting of: a protein kinase, a lipid kinase, a phosphotransferase, a phosphatidylintransferase, a glycosyltransferase, a nucleotide phosphohydrolase and a phosphodiesterase.

48. The method of claim 45, wherein the steps of the method are performed in both the presence and absence of a test compound, and the difference in the amount of
conversion of substrate to product between these two is used to assess whether the compound is an inhibitor or an activator of the activity of the enzyme.

49. A method for identifying an agent that disrupts interaction between a bait protein and a prey protein, comprising:
(a) providing a reaction mixture comprising: (i) ATP; (ii) a cytoskeletal protein polymer; (iii) a bait fusion protein that comprises a cytoskeletal-protein-binding protein domain and the bait protein; and (iv) a prey fusion protein comprising an cytoskeletal-protein stimulatable-ATPase domain and the prey protein;
(b) contacting the reaction mixture with a test agent;
(c) incubating the reaction mixture for a period of time, wherein said period of time is that required in the absence of the test agent for conversion of a quantity of ATP into ADP that is measurable by detection of the purine H8 ATP and/or ADP signal by NMR;
(d) detecting a decrease in conversion of ATP into ADP, by monitoring the the purine H8 ATP and/or ADP signal, in the presence of the test agent compared to the amount of conversion in a control reaction without the test agent, thereby detecting an agent that disrupts interaction between the bait and prey proteins.

50. The method of claim 49, wherein the cytoskeletal protein polymer comprises tubulin or actin.

51. The method of claim 49, wherein the purine H8 NMR signal is measured after the ATP to ADP conversion is terminated by the addition of a chelator of magnesium ions.
Figure 1

a

b

C

Chemical Shift (ppm)
Figure 4

a
Compound 98
ATP
70% inhibition
ADP

Compound 98

b
ATP
ADP

Chemical Shift (ppm)
Figure 6

![Graph showing the relationship between Relative Velocity and [PDE2A] (nM). The graph is a linear regression with data points indicated by black circles. The x-axis represents [PDE2A] (nM) ranging from 0 to 25, and the y-axis represents Relative Velocity ranging from 0 to 100.]
Figure 7A

[Graph showing the relationship between [cGMP] (µM) and v (µM/min).]

Figure 7B

[Graph showing the relationship between lg[EHNA] (M) and % Inhibition.]
**Figure 8**

91% inhibition

<table>
<thead>
<tr>
<th>Compound</th>
<th>09033259</th>
</tr>
</thead>
</table>

H8 of GMP  | H8 of cGMP | H1' of cGMP | H1' of GMP

Chemical Shift (ppm)

8.2  | 8.1  | 8.0  | 7.9  | 7.8  |
6.5  | 6.4  | 6.3  | 6.2  | 6.1  | 6.0  | 5.9  | 5.8  |
The chemical shift differences (Δδ) of adenine-derivative cofactor pairs.

<table>
<thead>
<tr>
<th>Δδ_{1H} (ppb)</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Adenosine</th>
<th>Adenine</th>
<th>c-AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td></td>
<td>9.6 ± 0.5</td>
<td>53.4 ± 0.6</td>
<td></td>
<td>274.2 ± 0.8; 68.5 ± 0.4</td>
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<tr>
<td>ADP</td>
<td></td>
<td>60.5 ± 1.4</td>
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<tr>
<td>AMP</td>
<td></td>
<td>256.3 ± 0.6; 66.1 ± 0.4</td>
<td>407.6 ± 0.3; 24.5 ± 1.6; *</td>
<td>327.6 ± 0.2; 75.1 ± 1.0</td>
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</tr>
<tr>
<td>Adenosine</td>
<td></td>
<td></td>
<td></td>
<td>151.3 ± 0.8; 18.1 ± 1.7; *</td>
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<tr>
<td>Adenine</td>
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<td></td>
</tr>
<tr>
<td>c-AMP</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a. Measured at pH 7.5 unless described otherwise. The Δδ values at pH 9.0 are larger than those at pH 7.5
b. Shown Δδ at pH 9.0. The value at pH 7.5 is 7.2 ± 1.5 ppb. The assay performed on a 400 MHz NMR provides better separation and accurate quantification of ATP/ADP peaks at pH 9.0 or higher. If lower pH needs to be used, 600 MHz or higher NMR spectrometer is recommended for real time measurement, and EDTA at high pH is the preferred reaction quencher for end point measurement.
c. Shown Δδ of H1'
d. Shown Δδ of H2
e. H1' of substrate can be also used as the probe signal
### Figure 11

The chemical shift differences ($\Delta\delta$) of guanine-derivative cofactor pairs.

<table>
<thead>
<tr>
<th>$\Delta\delta_{\text{ppb}}$</th>
<th>GTP</th>
<th>GDP</th>
<th>GMP</th>
<th>Guanosine</th>
<th>Guanine</th>
<th>c-GMP</th>
<th>GDP-man</th>
<th>GDP-fuc</th>
<th>$\text{m}^{\prime}\text{GTP}$</th>
<th>$\text{m}^{\prime}\text{GDP}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>8.1 ± 0.8°</td>
<td>59.8 ± 0.8</td>
<td></td>
<td>Guanosine</td>
<td>Guanine</td>
<td>250.8 ± 0.0; 59.5 ± 0.5°</td>
<td>32.7 ± 0.4°</td>
<td>37.7 ± 0.6</td>
<td>943.8 ± 0.1g; 125.5 ± 0.0°</td>
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<tr>
<td>GDP</td>
<td>64.4 ± 1.6</td>
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<td></td>
<td>28.1 ± 0.4°</td>
<td></td>
<td>33.1 ± 0.3</td>
<td>960.7 ± 1.6°; 121.9 ± 0.5°</td>
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<tr>
<td>GMP</td>
<td></td>
<td>208.7 ± 1.6; 29.9 ± 0.3°</td>
<td>329.9 ± 1.5°; 310.6 ± 0.8; 59.5 ± 0.2°</td>
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<tr>
<td>Guanosine</td>
<td>121.2 ± 0.1°</td>
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<tr>
<td>Guanine</td>
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<tr>
<td>c-GMP</td>
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<td>GDP-man</td>
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<td>GDP-fuc</td>
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<tr>
<td>$\text{m}^{\prime}\text{GTP}$</td>
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<tr>
<td>$\text{m}^{\prime}\text{GDP}$</td>
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</tr>
</tbody>
</table>

a. Measured at pH 7.5 unless described otherwise. The $\Delta\delta$ values at pH 9.0 is larger than those at pH 7.5 unless described otherwise
b. Shown $\Delta\delta$ at pH 9.0. The value at pH 7.5 is 4.6 ± 0.8 ppb. The assay performed on a 400 MHz NMR provides better separation and accurate quantification of GTP/GDP peaks at pH 9.0 or higher. If lower pH needs to be used, 600 MHz or higher NMR spectrometer is recommended for real time measurement, and EDTA at high pH is the preferred reaction quencher for end point measurement.

c. Shown $\Delta\delta$ of H1°
d. H1° of substrate or product can be also used as the probe signal
e. Guanine is highly insoluble at both pH 7.5 and 9.0. The solution concentration is approximately 50 μM from NMR integration
f. $\Delta\delta$ at pH 9.0 is 110.0 ± 0.1 for H8
g. Shown $\Delta\delta$ of all protons in 200 mM Tris in H2O. The H8 proton rapidly exchanges with the solvent and the peak is not observed in 200 mM Tris in D2O.
h. The methyl proton of $\text{m}^{\prime}\text{GTP}/\text{m}^{\prime}\text{GDP}$ can be also used as the probe signal.
i. $\Delta\delta$ at pH 9.0 are 908.4 ± 0.1 and 124.3 ± 1.6 for H8 and H1°, respectively.
j. $\Delta\delta$ at pH 9.0 are 923.5 ± 0.8 and 122.0 ± 2.9 for H8 and H1°, respectively.
### Figure 12

The chemical shift differences ($\Delta\delta$) of uracil-derivative cofactor pairs.

<table>
<thead>
<tr>
<th>$\Delta\delta_{\text{H6}}$ (ppb)$^a$</th>
<th>UTP</th>
<th>UDP</th>
<th>UMP</th>
<th>Uridine</th>
<th>Uracil</th>
<th>UDP-GlcNac</th>
<th>UDP-GlcA</th>
<th>UDP-Gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTP</td>
<td>11.4 ± 0.5$^b$</td>
<td>122.1 ± 0.1</td>
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<tr>
<td>UDP</td>
<td>110.7 ± 0.4</td>
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<td></td>
<td>38.7 ± 1.5$^c$</td>
<td>40.3 ± 0.4$^c$</td>
<td>42.2 ± 0.2$^c$</td>
<td></td>
</tr>
<tr>
<td>UMP</td>
<td></td>
<td>227.9 ± 0.4</td>
<td></td>
<td>573.8 ± 0.6$^d,e$</td>
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<tr>
<td>Uridine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>345.9 ± 1.0$^d,e$</td>
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<tr>
<td>Uracil</td>
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<tr>
<td>UDP-GlcNac</td>
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<tr>
<td>UDP-GlcA</td>
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<tr>
<td>UDP-Gal</td>
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<td></td>
</tr>
</tbody>
</table>

- a. Shown $\Delta\delta$ at pH 7.5 which is smaller than $\Delta\delta$ at pH 9.0 unless described otherwise
- b. $\Delta\delta$ at pH 9.0 is 9.8 ± 0.5 for H6
- c. H1" of substrate can be also used as the probe signal
- d. H5 of uracil can be also used as the probe signal
- e. $\Delta\delta$ at pH 9.0 is 322.3 ± 0.6 for H6
### Figure 13

The chemical shift differences ($\Delta\delta$) of cytosine-derivative cofactor pairs.

<table>
<thead>
<tr>
<th>$\Delta\delta_{1H}$ (ppb)$^a$</th>
<th>CTP</th>
<th>CDP</th>
<th>CMP</th>
<th>Cytidine</th>
<th>Cytosine</th>
<th>CMP-Neu5Ac</th>
<th>UTP</th>
<th>Uridine</th>
<th>Uracil</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTP</td>
<td>7.2 ± 0.4; 25.8 ± 0.7$^b$</td>
<td></td>
<td>91.6 ± 3.6</td>
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<tr>
<td>CDP</td>
<td></td>
<td>84.4 ± 3.2; 26.8 ± 0.3$^b$</td>
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<tr>
<td>CMP</td>
<td></td>
<td></td>
<td>229.8 ± 0.9; 71.8 ± 0.4$^c$, 93.6 ± 2.4$^b$</td>
<td>575.1 ± 1.2; 155.8 ± 0.2$^c$, $^d$</td>
<td>107.3 ± 1.9</td>
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</tr>
<tr>
<td>Cytidine</td>
<td></td>
<td></td>
<td></td>
<td>354.2 ± 0.3; 84.0 ± 0.5$^c$, $^d$, $^e$</td>
<td></td>
<td></td>
<td></td>
<td>32.2 ± 0.8; $^g$</td>
<td></td>
</tr>
<tr>
<td>Cytosine</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>31.5 ± 0.5; 175.9 ± 0.4$^e$</td>
</tr>
<tr>
<td>CMP-Neu5Ac</td>
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<td></td>
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<tr>
<td>UTP</td>
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<tr>
<td>Uridine</td>
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<tr>
<td>Uracil</td>
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<td></td>
</tr>
</tbody>
</table>

a. Shown $\Delta\delta$ at pH 7.5 which is smaller than $\Delta\delta$ at pH 9.0 unless described otherwise. Shown $\Delta\delta$ of protons other than H8 for blue boxes
b. Shown $\Delta\delta$ of H1'
c. Shown $\Delta\delta$ of H5
d. H1' of substrate can be also used as the probe signal
e. $\Delta\delta$ at pH 9.0 are 348.4 ± 0.4, and 83.7 ± 1.4 for H6 and H5, respectively
f. Shown $\Delta\delta$ at pH 9.0. The value at pH 7.5 is 1.7 ± 0.5
g. H5 of CTP or Cytidine can be used as the probe signal
h. $\Delta\delta$ at pH 9.0 is 10.7 ± 0.1 for H6
<table>
<thead>
<tr>
<th></th>
<th>Thymine</th>
<th>Thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta \delta$ (ppb)$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTP</td>
<td>9.8 ± 0.3</td>
<td>58.2 ± 0.8</td>
</tr>
<tr>
<td>TDP</td>
<td>48.4 ± 0.5</td>
<td>142.0 ± 0.8</td>
</tr>
<tr>
<td>Thymidine</td>
<td>280.4 ± 0.4,$^b$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The $\delta$ at pH 7.5 is shown and it is smaller than $\delta$ at pH 9.0 unless described otherwise.

$^b$ H1 of substrate can be also used as the probe signal.

$^c$ $\Delta \delta$ at pH 9.0 is 277.1 ± 1.1 for H8.

---

**Figure 14**

The chemical shift differences ($\Delta \delta$) of thymine-derivative cofactor pairs.
The chemical shift differences (Δδ) of hypoxanthine-derivative cofactor pairs.

<table>
<thead>
<tr>
<th>Δδ_{H8} (ppb) a</th>
<th>ITP</th>
<th>IDP</th>
<th>IMP</th>
<th>Inosine</th>
<th>Hypoxanthine</th>
<th>AMP</th>
<th>GMP</th>
<th>XMP</th>
<th>Adenosine</th>
<th>Adenine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP</td>
<td>5.2 ± 0.4 b</td>
<td>61.3 ± 0.6</td>
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<tr>
<td>IDP</td>
<td>62.3 ± 0.6</td>
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<td></td>
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</tr>
<tr>
<td>IMP</td>
<td>225.1 ± 0.1; 49.5 ± 1.6 c</td>
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<tr>
<td></td>
<td>359.8 ± 0.1; 41.0 ± 0.1 d, i</td>
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<td></td>
<td>30.8 ± 0.8; 33.6 ± 0.1 d</td>
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<tr>
<td></td>
<td>357.5 ± 1.3; 206.9 ± 1.9; e, g</td>
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<tr>
<td></td>
<td>432.3 ± 0.9; 237.5 ± 0.0; e, h</td>
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<tr>
<td>Inosine</td>
<td>134.7 ± 0.0; 43.4 ± 0.8 d, i</td>
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<td></td>
<td>24.7 ± 1.0; 24.5 ± 0.9; j</td>
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<tr>
<td>Hypoxanthine</td>
<td>17.1 ± 0.9; 50.0 ± 1.6 d</td>
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<td>AMP</td>
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<td>Adenosine</td>
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</tbody>
</table>

a. The Δδ at pH 7.5 is shown unless described otherwise. The Δδ at pH 9.0 is larger than values at pH 7.5 unless described otherwise.
b. Shown Δδ at pH 9.0. The value at pH 7.5 is 1.0 ± 0.4 ppb. Although the assay performed on a 400 MHz NMR at pH 9.0 could not provide sufficient resolution to separate ITP and IDP H8 peaks, Δδ increases at higher pH (7.7 ± 0.2 ppb at pH 9.42) and allows for accurate quantification of ITP/IDP peaks. Higher pH can be achieved in end-point experiment by adding reaction quencher at high pH such as EDTA. If lower pH needs to be used, 600 MHz or higher NMR spectrometer is recommended for real time measurement.
c. Shown Δδ of H1’.
d. Shown Δδ of H2.
e. H2 of substrate can be also used as the probe signal.
f. H1’ of substrate can be also used as the probe signal.
g. Δδ at pH 9.0 are 348.9 ± 0.1 and 199.4 ± 2.4 for H8 and H1’, respectively.
h. Δδ at pH 9.0 are 411.3 ± 0.7 and 225.0 ± 0.2 for H8 and H1’, respectively.
i. H8 and H2 of hypoxanthine at pH 9.0 are overlapping.
j. Δδ at pH 9.0 is 6.3 ± 0.4 for H1’, and is 46.0 ± 0.7 for H8. H8 can be used at pH 9.0 as the probe signal.
The chemical shift differences (Δδ) of xanthine-derivative cofactor pairs.

<table>
<thead>
<tr>
<th>Δδ_{H8} (ppb)</th>
<th>XTP</th>
<th>XDP</th>
<th>XMP</th>
<th>Xanthosine</th>
<th>Xanthine</th>
<th>GMP</th>
<th>Guanine</th>
<th>Hypoxanthine</th>
</tr>
</thead>
<tbody>
<tr>
<td>XTP</td>
<td>7.1 ± 0.6(^b)</td>
<td>38.7 ± 0.8</td>
<td></td>
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</tr>
<tr>
<td>XDP</td>
<td></td>
<td>44.8 ± 1.9</td>
<td></td>
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<tr>
<td>XMP</td>
<td></td>
<td></td>
<td>261.3 ± 0.5; 56.7 ± 2.0(^c)</td>
<td>221.4 ± 3.2; (^d)</td>
<td>74.8 ± 2.3; 30.7 ± 2.0(^f)</td>
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</tr>
<tr>
<td>Xanthosine</td>
<td></td>
<td></td>
<td></td>
<td>39.9 ± 2.7; (^d)</td>
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<tr>
<td>Guanine</td>
<td></td>
<td></td>
<td></td>
<td>33.7 ± 2.4(^a)</td>
<td>293.8 ± 2.1; (^g)</td>
<td></td>
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</tr>
<tr>
<td>GMP</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Hypoxanthine</td>
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</tr>
</tbody>
</table>

\(^a\) The Δδ at pH 7.5 is shown unless described otherwise. The Δδ at pH 9.0 is larger than values at pH 7.5.
\(^b\) Shown Δδ at pH 9.0. The value at pH 7.5 is 6.1 ± 1.1 ppb. Although the assay performed on a 400 MHz NMR at pH 9.0 could not provide sufficient resolution to separate XTP and XDP H8 peaks, Δδ increases at higher pH (9.5 ± 0.2 ppb at pH 10.0) and allows for accurate quantification of XTP/XDP peaks. Higher pH can be achieved in endpoint experiment by adding reaction quencher at high pH such as EDTA. If lower pH needs to be used, 600 MHz or higher NMR spectrometer is recommended for real time measurement.
\(^c\) Shown Δδ of H1'
\(^d\) H1' of substrate can be also used as the probe signal
\(^e\) Guanine is highly insoluble at both pH 7.5 and 9.0. The solution concentration is approximately 50 μM from NMR integration.
\(^f\) Δδ at pH 9.0 are 62.5 ± 0.6 and 25.6 ± 2.2 for H8 and H1', respectively
\(^g\) H2 of substrate can be also used as the probe signal
### Figure 17

The chemical shift differences (Δδ) of adenine-based cofactor (ABC) pairs.

<table>
<thead>
<tr>
<th>ΔδH8 (ppb)</th>
<th>PAPS</th>
<th>PAP</th>
<th>SAM</th>
<th>SAH</th>
<th>ATP</th>
<th>Adenine</th>
<th>MTA</th>
<th>cADPR</th>
<th>ADPR</th>
<th>NAD</th>
<th>NADP</th>
<th>CoA</th>
<th>Acetyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAPS</strong></td>
<td>61.2 ± 1.5°</td>
<td></td>
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<tr>
<td><strong>PAP</strong></td>
<td></td>
<td>66.0 ± 0.5°; 20.9 ± 0.8; 26.1 ± 0.1;</td>
<td>239.2 ± 0.2; 20.1 ± 1.1; 30.7 ± 0.3;</td>
<td>181.1 ± 1.6; 27.2 ± 1.8c; g</td>
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<td><strong>SAM</strong></td>
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<tr>
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<td><strong>Adenine</strong></td>
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<tr>
<td><strong>CoA</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>Acetyl-CoA</strong></td>
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</tr>
</tbody>
</table>

a. Shown Δδ at pH 7.5 which is smaller than Δδ at pH 9.0 unless described otherwise. Light shaded boxes show Δδ of protons other than H8. b. Sample needs to run freshly because of rapid decomposition of SAM and PAPS at room temperature. c. Shown Δδ of H2. d. Shown Δδ of H1°. e. Δδ at pH 9.0 are 18.4 ± 0.9 and 21.2 ± 0.5 for H2 and H1°, respectively. f. Δδ at pH 9.0 is 19.1 ± 0.8 for H2. g. The H1° proton of SAH or MTA can be also used as the probe signal. h. Shown Δδ of H1°. i. Δδ at pH 9.0 is 584.2 ± 1.4 and 798.4 ± 2.8 for H2 and H1°, respectively. j. The H2°, H4*, H5*, H6° protons of NAD or NADP can be also used as the probe signal. k. Δδ at pH 9.0 are 206.9 ± 2.3, 677.9 ± 1.7, 19.5 ± 1.0, 34.3 ± 0.4 for H8, H2, H1°, and H1°, respectively. l. Δδ at pH 9.0 are 93.7 ± 0.3, 106.0± 0.1 and 764.1 ± 2.4 for H2, H1° and H1°, respectively. m. Δδ at pH 9.0 is 711.6 ± 2.1 for H1°. n. The methyl proton of AcetylCoA at 2.3387 ppm can be used as the probe signal. o. Sample at high pH needs to run freshly because of rapid hydrolysis of CoA and AcetylCoA in alkaline solutions.
Figure 18

The chemical shift differences (Δδ) of nicotinamide-based cofactor (NBC) pairs.

<table>
<thead>
<tr>
<th>ΔδNHB (ppb)*</th>
<th>NAD</th>
<th>NADH</th>
<th>NADP</th>
<th>NADPH</th>
<th>NAADP</th>
</tr>
</thead>
</table>
|              |      | 51.4 ± 0.3;  
| NAD          | 67.7 ± 0.5; 89.8 ± 0.2; 2384.3 ± 0.7; 6088.2 ± 0.2; 3161.6 ± 0.6 |      |       |        |        |
| NADH         | 53.9 ± 0.2; 93.3 ± 0.0; 105.4 ± 2.3; 2351.9 ± 1.3; 6027.6 ± 0.5 | 16.8 ± 0.1; 30.2 ± 0.6; 28.2 ± 1.2; 81.4 ± 1.2; 146.4 ± 0.4 |       |        |        |
| NADP         |      |       | 3135.3 ± 1.0 |        |        |
| NADPH        |      |       |        |        |        |
| NAADP        |      |       |        |        |        |

a. Shown Δδ at pH 7.5 which is smaller than Δδ at pH 9.0 unless described otherwise. b. Shown Δδ of H2. c. Shown Δδ of H1'. d. Shown Δδ of H2''. e. Shown Δδ of H4''. f. Shown Δδ of H6''. g. The H1'' and H5'' protons of NAD and NADP can be also used as the probe signal. h. Δδ at pH 9.0 are 66.0 ± 0.8, 87.8 ± 0.5 for H2 and H1', respectively. i. Δδ at pH 9.0 are 2350.7 ± 0.1, 6022.9 ± 0.7 for H2'' and H4'', respectively. j. Shown Δδ of H1''. k. Shown Δδ of H5''. l. The H2'' of NADP and H6'' of NAADP can also be used as the probe signal. m. Δδ at pH 9.0 are 25.2 ± 0.9 and 80.0 ± 0.2 for H1'' and H4'', respectively.
<table>
<thead>
<tr>
<th>Cofactor</th>
<th>H8</th>
<th>H2</th>
<th>H1•</th>
<th>CH3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>8.5420 ± 0.0002</td>
<td>8.5348 ± 0.0018</td>
<td>8.5953 ± 0.0004</td>
<td>8.3390 ± 0.0001</td>
</tr>
<tr>
<td>ADP</td>
<td>8.2600 ± 0.0007</td>
<td>8.2588 ± 0.0006</td>
<td>8.2566 ± 0.0002</td>
<td>8.2502 ± 0.0004</td>
</tr>
<tr>
<td>AMP</td>
<td>6.1379 ± 0.0002</td>
<td>6.1390 ± 0.0001</td>
<td>6.1312 ± 0.0004</td>
<td>6.0652 ± 0.0008</td>
</tr>
<tr>
<td>GTP</td>
<td>8.1474 ± 0.0002</td>
<td>8.1428 ± 0.0006</td>
<td>8.2071 ± 0.0010</td>
<td>7.9984 ± 0.0006</td>
</tr>
<tr>
<td>GDP</td>
<td>7.9772 ± 0.0002</td>
<td>7.9886 ± 0.0002</td>
<td>8.0993 ± 0.0001</td>
<td>7.8715 ± 0.0002</td>
</tr>
<tr>
<td>GMP</td>
<td>5.9802 ± 0.0002</td>
<td>5.9652 ± 0.0002</td>
<td>5.9596 ± 0.0005</td>
<td>5.9062 ± 0.0005</td>
</tr>
<tr>
<td>UTP</td>
<td>7.7356 ± 0.0001</td>
<td>7.7258 ± 0.0001</td>
<td>7.7840 ± 0.0006</td>
<td>7.6420 ± 0.0002</td>
</tr>
<tr>
<td>UDP</td>
<td>6.3327 ± 0.0015</td>
<td>6.3238 ± 0.0007</td>
<td>6.3319 ± 0.0011</td>
<td>6.2882 ± 0.0000</td>
</tr>
<tr>
<td>CTP</td>
<td>8.0533 ± 0.0001</td>
<td>8.0410 ± 0.0002</td>
<td>8.0508 ± 0.0003</td>
<td>7.8960 ± 0.0007</td>
</tr>
<tr>
<td>CDP</td>
<td>6.8264 ± 0.0019</td>
<td>6.8252 ± 0.0001</td>
<td>6.8230 ± 0.0010</td>
<td>6.8225 ± 0.0006</td>
</tr>
<tr>
<td>IDP</td>
<td>6.1420 ± 0.0005</td>
<td>6.1449 ± 0.0005</td>
<td>6.1391 ± 0.0015</td>
<td>6.0896 ± 0.0001</td>
</tr>
<tr>
<td>XTP</td>
<td>6.0943 ± 0.0004</td>
<td>6.0876 ± 0.0006</td>
<td>6.1323 ± 0.0013</td>
<td>6.0846 ± 0.0005</td>
</tr>
<tr>
<td>NAD</td>
<td>8.4208 ± 0.0001</td>
<td>8.4722 ± 0.0001</td>
<td>8.4224 ± 0.0004</td>
<td>8.4760 ± 0.0001</td>
</tr>
<tr>
<td>NADP</td>
<td>8.1691 ± 0.0003</td>
<td>8.2368 ± 0.0008</td>
<td>8.1477 ± 0.0001</td>
<td>8.2410 ± 0.0001</td>
</tr>
<tr>
<td>c-AMP</td>
<td>8.2687 ± 0.0006</td>
<td>8.2570 ± 0.0002</td>
<td>8.2321 ± 0.0013</td>
<td>8.2580 ± 0.0002</td>
</tr>
<tr>
<td>c-MGP</td>
<td>7.9866 ± 0.0002</td>
<td>8.1147 ± 0.0002</td>
<td>7.9499 ± 0.0013</td>
<td>7.9483 ± 0.0001</td>
</tr>
<tr>
<td>GDP-man</td>
<td>5.9735 ± 0.0002</td>
<td>5.9840 ± 0.0002</td>
<td>6.1168 ± 0.0011</td>
<td>5.9782 ± 0.0004</td>
</tr>
<tr>
<td>GDP-fucose</td>
<td>5.5026 ± 0.0000</td>
<td>5.5267 ± 0.0000</td>
<td>5.6132 ± 0.0003</td>
<td>5.6313 ± 0.0001</td>
</tr>
<tr>
<td>mGTP</td>
<td>9.1028 ± 0.0009</td>
<td>6.0566 ± 0.0004</td>
<td>1.4270 ± 0.0001</td>
<td>4.1059 ± 0.0001</td>
</tr>
<tr>
<td>m’GDP</td>
<td>7.1095 ± 0.0001</td>
<td>8.1097 ± 0.0004</td>
<td>9.0911 ± 0.0003</td>
<td>6.0577 ± 0.0003</td>
</tr>
</tbody>
</table>

*The chemical shifts of cofactors at pH 7.5.*
### Figure 20: The chemical shifts of cofactors at pH 9.0.

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Adenosine</th>
<th>Adenine</th>
<th>c-AMP</th>
<th>m'GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>H8</td>
<td>8.5501 ± 0.0002</td>
<td>8.5405 ± 0.0007</td>
<td>8.6128 ± 0.0008</td>
<td>8.3533 ± 0.0006</td>
<td>8.1744 ± 0.0003</td>
<td>8.2680 ± 0.0006</td>
<td>H8 9.0585 ± 0.0004</td>
</tr>
<tr>
<td>H2</td>
<td>8.2585 ± 0.0003</td>
<td>8.2571 ± 0.0001</td>
<td>8.2552 ± 0.0005</td>
<td>8.2460 ± 0.0004</td>
<td>8.2245 ± 0.0002</td>
<td>8.2571 ± 0.0001</td>
<td>H1 9.0521 ± 0.0015</td>
</tr>
<tr>
<td>H1'</td>
<td>6.1356 ± 0.0000</td>
<td>6.1375 ± 0.0005</td>
<td>6.1298 ± 0.0002</td>
<td>6.0623 ± 0.0002</td>
<td>6.2047 ± 0.0009</td>
<td>6.0401 ± 0.0001</td>
<td>CH3 4.1010 ± 0.0001</td>
</tr>
<tr>
<td>GTP</td>
<td>8.1431 ± 0.0004</td>
<td>8.1351 ± 0.0004</td>
<td>8.2083 ± 0.0004</td>
<td>7.9785 ± 0.0004</td>
<td>7.6865 ± 0.0003</td>
<td>7.8879 ± 0.0002</td>
<td>8.1087 ± 0.0001</td>
</tr>
<tr>
<td>GDP</td>
<td>5.9263 ± 0.0007</td>
<td>5.9300 ± 0.0014</td>
<td>5.9267 ± 0.0002</td>
<td>5.8970 ± 0.0009</td>
<td>5.9897 ± 0.0002</td>
<td>5.9810 ± 0.0002</td>
<td>5.5014 ± 0.0003</td>
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<tr>
<td>GMP</td>
<td>5.9873 ± 0.0002</td>
<td>5.9566 ± 0.0005</td>
<td>5.9905 ± 0.0005</td>
<td>5.9061 ± 0.0008</td>
<td>5.9768 ± 0.0005</td>
<td>5.9810 ± 0.0006</td>
<td>5.6302 ± 0.0001</td>
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<tr>
<td>UDP</td>
<td>7.9750 ± 0.0003</td>
<td>7.9848 ± 0.0002</td>
<td>8.1057 ± 0.0006</td>
<td>7.8480 ± 0.0001</td>
<td>7.5258 ± 0.0004</td>
<td>7.9423 ± 0.0004</td>
<td>7.9410 ± 0.0004</td>
</tr>
<tr>
<td>CTP</td>
<td>5.9609 ± 0.0007</td>
<td>5.9533 ± 0.0007</td>
<td>5.9704 ± 0.0002</td>
<td>5.8770 ± 0.0007</td>
<td>5.7823 ± 0.0008</td>
<td>5.9629 ± 0.0005</td>
<td>5.9649 ± 0.0005</td>
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<tr>
<td>UTP</td>
<td>5.9837 ± 0.0002</td>
<td>5.9566 ± 0.0005</td>
<td>5.9905 ± 0.0005</td>
<td>5.9061 ± 0.0008</td>
<td>5.9768 ± 0.0005</td>
<td>5.9879 ± 0.0009</td>
<td>5.9825 ± 0.0001</td>
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<tr>
<td>UDP-GlcNac</td>
<td>7.9879 ± 0.0003</td>
<td>7.9980 ± 0.0002</td>
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<td>7.8373 ± 0.0002</td>
<td>7.4890 ± 0.0002</td>
<td>7.9671 ± 0.0002</td>
<td>7.9384 ± 0.0001</td>
</tr>
<tr>
<td>UDP-GlcA</td>
<td>6.1312 ± 0.0013</td>
<td>6.1181 ± 0.0002</td>
<td>6.1276 ± 0.0002</td>
<td>6.0447 ± 0.0005</td>
<td>5.9610 ± 0.0009</td>
<td>6.1151 ± 0.0005</td>
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</tr>
<tr>
<td>UDP-Gal</td>
<td>6.0325 ± 0.0019</td>
<td>6.3218 ± 0.0002</td>
<td>6.3310 ± 0.0019</td>
<td>6.2876 ± 0.0004</td>
<td>6.5198 ± 0.0004</td>
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<tr>
<td>CTP</td>
<td>5.9898 ± 0.0009</td>
<td>5.9600 ± 0.0010</td>
<td>5.9653 ± 0.0002</td>
<td>5.8929 ± 0.0004</td>
<td>5.8986 ± 0.0004</td>
<td>5.8986 ± 0.0004</td>
<td>5.8986 ± 0.0004</td>
</tr>
<tr>
<td>CDP</td>
<td>5.9898 ± 0.0009</td>
<td>5.9600 ± 0.0010</td>
<td>5.9653 ± 0.0002</td>
<td>5.8929 ± 0.0004</td>
<td>5.8986 ± 0.0004</td>
<td>5.8986 ± 0.0004</td>
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</tr>
<tr>
<td>CMP</td>
<td>6.1312 ± 0.0013</td>
<td>6.1336 ± 0.0003</td>
<td>6.1261 ± 0.0022</td>
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Figure 21: The IC50 measurement of compound hits from PDE2A screen.
INTERNATIONAL SEARCH REPORT

PCT/US2012/065893

A. CLASSIFICATION OF SUBJECT MATTER

C12Q1/00 C12Q1/30 C12Q1/48

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)

C12Q

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)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C. X See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search

23 January 2013

Date of mailing of the international search report

06/02/2013

Name and mailing address of the ISA:

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

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Van Bohemen, Charles
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