

**Supporting Information for**  
**Phosphorus NMR and its application to metabolomics**

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## Materials and Methods

### Chemicals

Deuterium oxide (99.9% D) was obtained from Sigma Aldrich (Milwaukee, WI). 3-(trimethylsilyl) propionic-2,2,3,3-d<sub>4</sub> acid sodium salt (98% D, TMSP) was purchased from Cambridge Isotopes (Andover, MA). Potassium phosphate dibasic salt (anhydrous, 99.1% pure) and monobasic salt (crystal, 99.8% pure) were purchased from Fisher Scientific (Fair Lawn, NJ). All compounds used to obtain reference spectra were obtained from Sigma Aldrich (Milwaukee, WI). <sup>15</sup>N-labeled compounds were purchased from Cambridge Isotopes (Andover, MA). <sup>13</sup>C-labeled and phosphorylated compounds were purchased from Sigma Aldrich (Milwaukee, WI). A 2 M MgCl<sub>2</sub> and 500 mM EDTA solution was prepared in Nanopure water by sonicating at room temperature until completely dissolved. The solutions were stored at 4 °C until needed.

### Preparation of NMR samples for individual metabolites and metabolite mixtures

Stock solutions for the 38 metabolites listed in **Table S1** were prepared by weighing-out the required amount of each compound and dissolving the compound into 2 mL of Nanopure water to produce a final concentration of 2 mM. An adenosine analog mixture comprising the three compounds listed in **Table S3** was prepared by combining ~330 µL from each stock solution to a final volume of ~ 1 mL and a final concentration of 0.67 mM for each metabolite. A sugar phosphate mixture comprising the seven compounds listed in **Table S3** was prepared by combining ~140 µL from each stock solution to a final volume of ~ 1 mL and a final concentration of 0.29 mM for each metabolite. Each sample was stored at -20°C until needed. If required, fresh samples were prepared after a week of storage.

To prepare the NMR samples, each of the 38 individual metabolite solutions and the 2 metabolite mixtures (adenosine analogs and sugar phosphates) were thawed to room temperature on ice. 45  $\mu\text{L}$  of  $\text{D}_2\text{O}$  was added as the lock solvent to 450  $\mu\text{L}$  from each of the metabolite or mixture solutions. Two replicate samples were prepared for each individual metabolite, and three replicate samples were prepared for the two metabolite mixtures. The individual metabolite samples were manually adjusted to a pH of 4 by adding, as needed, 1 M NaOD or 1 M DCL. A second set of NMR samples was prepared for each individual metabolite without adjusting the sample pH. The three replicate samples for each metabolite mixture were manually adjusted to either a pH of 4, 7 or 12 by adding, as needed, 1 M NaOD or 1 M DCL. 500  $\mu\text{L}$  of each sample was then transferred to a 5 mm NMR tube for data collection. A total of 82 NMR samples were prepared - 76 individual metabolite samples (two different pHs of 38 metabolites) and 6 metabolite mixture samples (three different pHs of 2 mixtures).

### **Preparation of NMR samples of metabolite mixtures for stability testing**

~2 mL of the adenosine analogs and sugar phosphate mixtures were prepared as described above. Each mixture was then split into seven aliquots of ~300  $\mu\text{L}$  each to test the impact of three different metabolome extraction protocols on compound stability. One aliquot from each of the adenosine analogs and sugar phosphate mixtures was manually adjusted to a pH of 4 by the addition, as needed, of 1 M NaOD or 1 M DCL. The samples were transferred to a 5 mm NMR tube for data collection. An additional aliquot from each mixture was then subjected to either being sonicated in a water bath for five minutes, sonicated for five rounds of 10 second burst using a wand tip, or bead beating in Lysing Matrix B using a FastPrep (1200 beats per minute for five rounds of 30

second burst). The aliquots were then manually adjusted to a pH of 4 by the addition, as needed, of 1 M NaOD or 1 M DCl, and transferred to a 5 mm NMR tube for data collection.

The remaining three aliquots from each mixture were diluted with an equal volume of methanol (~ 300  $\mu$ L) to mimic a common 1:1 water: methanol extraction method. Similar to the first set of three aliquots, one aliquot from each mixture was then subjected to either being sonicated in a water bath, sonicated with a wand tip, or bead beating with a FastPrep. Methanol was removed using a SpeedVac Plus SC110A system, and the samples were then lyophilized using a FreeZone freeze dryer. The dried samples were dissolved in 550  $\mu$ L of 90:10 Nanopure water: D<sub>2</sub>O, and the pH was manually adjusted to 4 by adding, as needed, 1 M NaOD or 1 M DCL. The samples were then transferred to a 5 mm NMR tubes for data collection. A total of 14 NMR samples were prepared, 7 replicates each of the adenosine analogs and sugar phosphate mixtures corresponding to the different extraction techniques.

### **Preparation of NMR samples from bacterial cell lysates**

*E. coli* (strain MG1655) was cultured in M9 minimal media containing <sup>15</sup>NH<sub>4</sub>Cl as the sole nitrogen source and <sup>13</sup>C<sub>6</sub> glucose as the sole carbon source. *Saccharomyces pombe* cells were grown in a YES media for 12 hours without the addition of <sup>13</sup>C or <sup>15</sup>N labeled nutrients to the cell culture media. *Mycobacterium smegmatis* cells were grown in 7H9 media for 18 hours also without the addition of <sup>13</sup>C or <sup>15</sup>N labeled nutrients to the cell culture media. Triplicate 50 mL cell cultures were grown aerobically until a final O.D.<sub>600</sub> of 1.5 per mL. *E. coli* cells were harvested at the 12-hour time point upon reaching stationary phase. *S. pombe* were also harvested at the 12-hour time point, while *M. smegmatis* cells were harvested at 18 hours. Each cell culture was centrifuged at

5000 rpm for 20 min at 4°C to pellet the cell suspension. Pellets were resuspended in 1.5 mL of 1:1 water:methanol or 1:1 methanol:chloroform solution. *E. coli* cells were mechanically lysed by sonication in a water bath for five minutes. *S. pombe* and *M. smegmatis* cells were suspended in 1 mL of 1:1 water:methanol solvent and then lysed using Lysing Matrix B beads in a FastPrep. Cell suspension were beaten for 5 rounds of 30 seconds burst at 1200 beats per minute. The lysed cells were then centrifuged at 13000 rpm for 20 min at 4°C, and 1 mL of the supernatant was transferred to a microcentrifuge tube. The metabolome cell extraction protocol was repeated, and the two supernatants were combined. The samples were kept on ice during the entire extraction process. Methanol was removed using a SpeedVac Plus SC110A system (Savant, Thermo Scientific, Asheville, NC), and the samples were then lyophilized using a FreeZone freeze dryer (Labconco, Kansas City, MO). The *E. coli*, *S. pombe* and *M. smegmatis* dried cell lysates were dissolved in 550  $\mu$ L of 90:10 Nanopure water: D<sub>2</sub>O. The samples were then manually adjusted to a pH of 4 by adding, as needed, 1 M NaOD or 1 M DCL. The samples were centrifuged at 13000 rpm to pellet any remaining protein or cell debris. 500  $\mu$ L of the cell lysate samples was transferred to a 5 mm NMR tube for data collection. A total of 9 NMR samples were prepared, 3 each for *E. coli*, *S. pombe* and *M. smegmatis*.

### **NMR data acquisition to identify optimal conditions**

A total of 9 1D <sup>31</sup>P, 9 2D <sup>1</sup>H-<sup>31</sup>P HSQC TOCSY and 9 2D <sup>1</sup>H-<sup>31</sup>P HMBC experiments were collected to optimize sample preparation and experimental parameters. All NMR spectra were collected with a Bruker AVANCE III-HD 700 MHz spectrometer equipped with a 5 mm quadruple resonance QCI-P cryoprobe (<sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N, and <sup>31</sup>P) with *z*-axis gradients. A SampleJet automated sample changer system with Bruker ICON-NMR software was used to automate the NMR data

collection. Each 1D  $^{31}\text{P}$  spectrum was acquired with 64K data points and a spectral width of 80 ppm (22727 Hz) centered at 0 ppm. A total of 256 scans and 4 dummy scans were collected with a 2 second recycle delay. 2D  $^1\text{H}$ - $^{31}\text{P}$  HMBC and 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC TOCSY spectra were acquired with a  $^1\text{H}$  spectral width of 13 ppm (9090 Hz) centered at 4.7 ppm and  $^{31}\text{P}$  spectral width of 80 ppm (22727 Hz) centered at 0 ppm. The 2D spectra were acquired with 16 scans, 4 dummy scans, a 2 second recycle delay, 2048 points in the  $^1\text{H}$  dimension and 256 points in the  $^{31}\text{P}$  dimension. All spectra were collected at 298 K. After the spectra acquisition was optimized then a set of 1D  $^{31}\text{P}$ , 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC TOCSY and 2D  $^1\text{H}$ - $^{31}\text{P}$  HMBC were collected at 277 K. The data were Fourier transformed, phased, and reconstructed using Topspin 3.6.

#### **NMR data acquisition for phosphorylated-metabolite standards**

1D  $^{31}\text{P}$ , 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC TOCSY and 2D  $^1\text{H}$ - $^{31}\text{P}$  HMBC experiments were collected for 38 common phosphorylated-metabolites. All NMR spectra were acquired with a Bruker AVANCE III-HD 700 MHz spectrometer equipped with a 5 mm quadruple resonance QCI-P cryoprobe ( $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ , and  $^{31}\text{P}$ ) with  $z$ -axis gradients. A SampleJet automated sample changer system with Bruker ICON-NMR software was used to automate the NMR data collection. Each 1D  $^{31}\text{P}$  spectrum was collected with a spectral width of 100 ppm (28409 Hz) centered at 0 ppm. The spectra were acquired with 128 scans, 4 dummy scans, and a 2 second recycle delay. 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC TOCSY were collected with a  $^1\text{H}$  spectral width of 13 ppm (9090 Hz) centered at 4.7 ppm and a  $^{31}\text{P}$  spectral width of 100 ppm (28409 Hz) centered at 0 ppm. The spectra were acquired with 16 scans, 4 dummy scans, and a 2 second recycle delay. The data were Fourier transformed, phased, and reconstructed using Topspin 3.6.

### **NMR data acquisition for cell lysates**

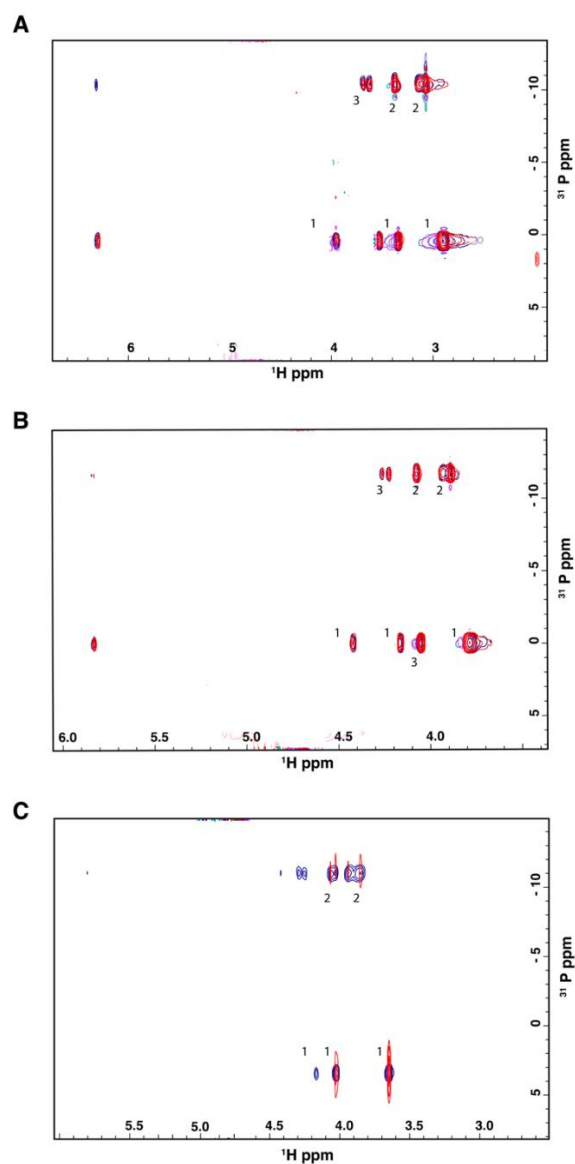
1D  $^{31}\text{P}$ , 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC TOCSY and 2D  $^1\text{H}$ - $^{31}\text{P}$  HMBC spectra were collected for each of the three cell types *E. coli*, *S. pombe*, and *M. smegmatis* cell lysates. All NMR spectra were acquired with a Bruker AVANCE III-HD 700 MHz spectrometer equipped with a 5 mm quadruple resonance QCI-P cryoprobe ( $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ , and  $^{31}\text{P}$ ) with  $z$ -axis gradients. A SampleJet automated sample changer system with Bruker ICON-NMR software was used to automate the NMR data collection. For the *E. coli* cell lysates 1D  $^{31}\text{P}$ , 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC TOCSY and 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC experiments were conducted at 277.15 K, while the 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra were collected at 298K. The  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were acquired with a  $^1\text{H}$  spectral width of 11160 Hz, a  $^{15}\text{N}$  spectral width of 10646 Hz and a 2 second recycling delay. The 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra were acquired with  $^1\text{H}$  spectral width of 9090 Hz,  $^{13}\text{C}$  spectral width of 29059 Hz, and a 2 second recycling delay. The 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC-TOCSY and 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC were collected with uniform sampling and the 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were collected using non-uniform sampling at 25% sampling sparsity.<sup>1</sup> The data were Fourier transformed, phased, and reconstructed using Topspin 3.6.

### **Metabolite assignments for $^{13}\text{C}$ , $^{15}\text{N}$ and $^{31}\text{P}$ NMR spectra**

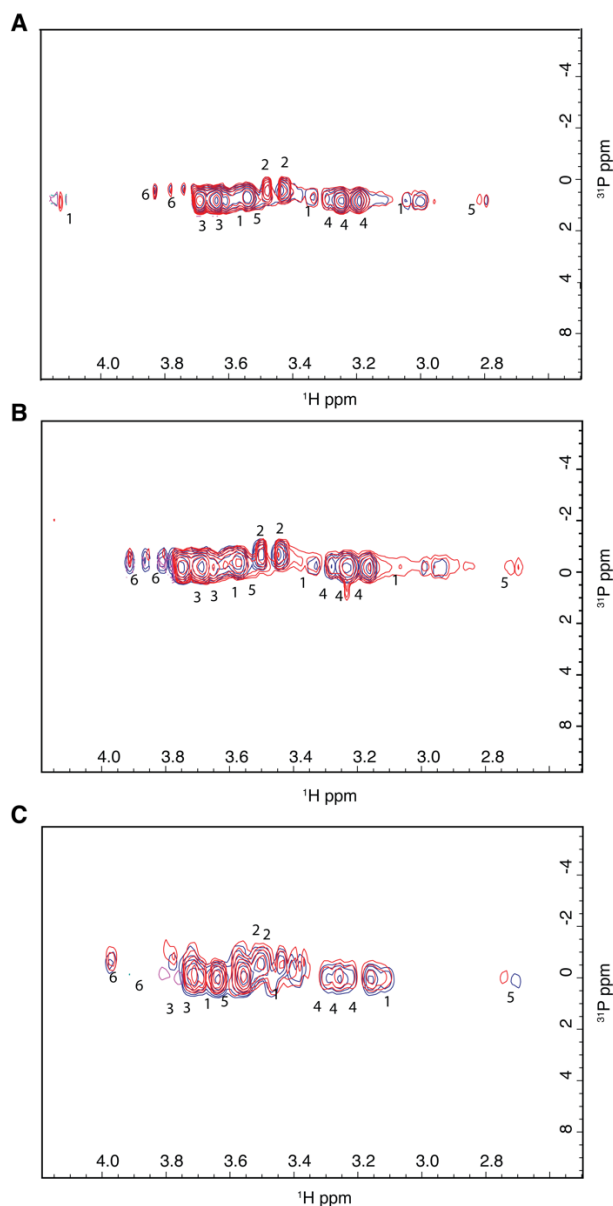
Our data set of 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC reference spectra<sup>2</sup> were used to annotate the NMR spectra obtained from the *E. coli* cell lysates. Similarly, our data set of 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC-TOCSY reference spectra were used to annotate the NMR spectra obtained from the *E. coli*, *M. smegmatis* and *S. pombe* cell lysates. 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra were assigned using the Human Metabolome Database (<http://www.hmdb.ca/>),<sup>3</sup> BioMagResBank (<http://www.bmrb.wisc.edu/metabolomics/>),<sup>4</sup>



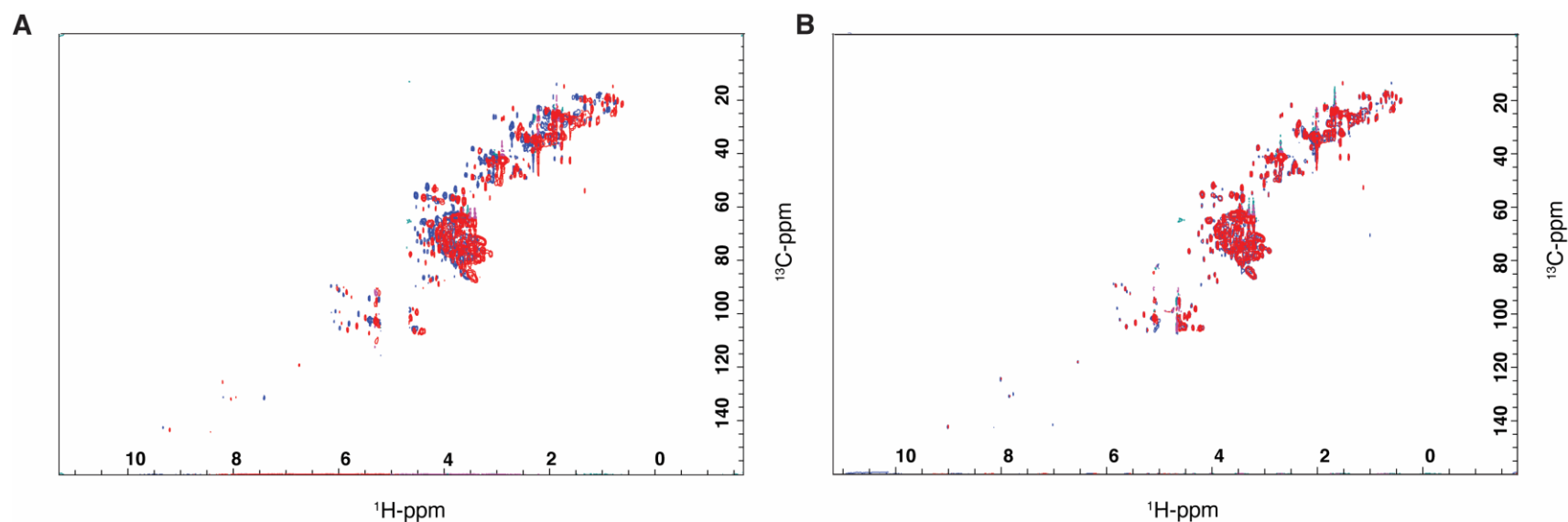
and SpinAssign from RIKEN (<http://dmar.riken.jp/spinassign/>)<sup>5</sup> with a 0.08 and 0.25 ppm error range for  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift, respectively.



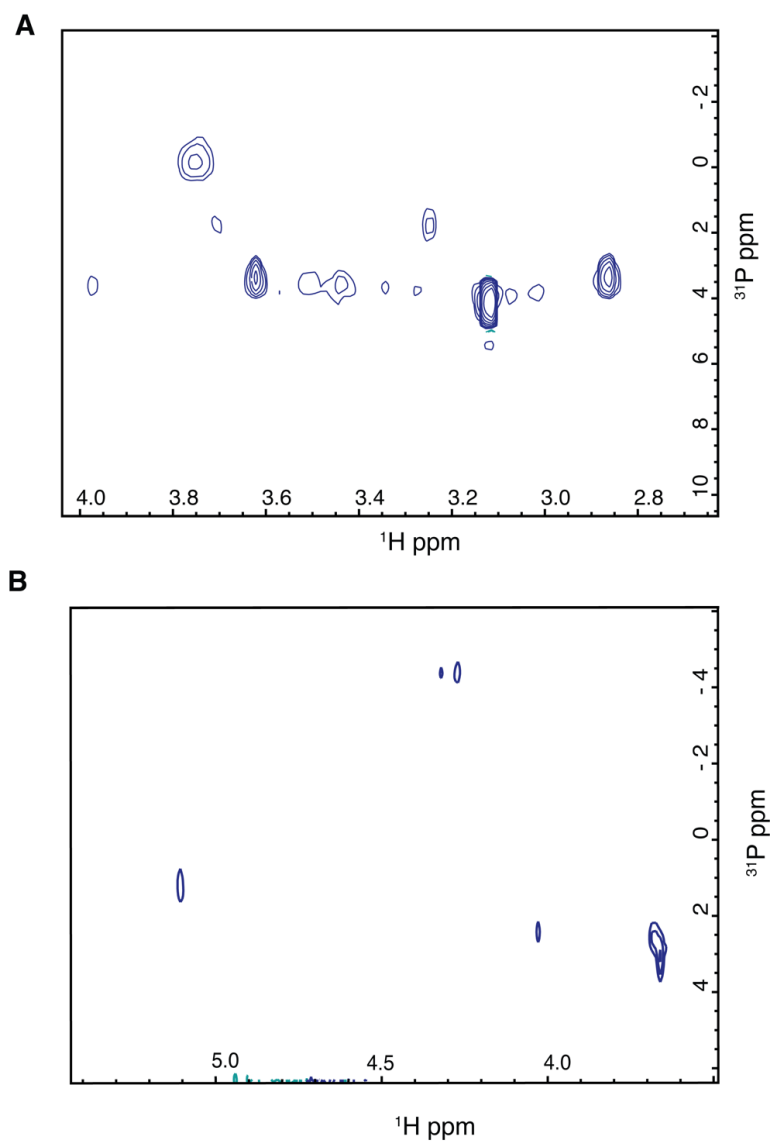
**Figure S1.** Stability of phosphorylated metabolites. Representative 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC TOCSY spectra of the adenosine analog mixture. Overlay of the spectrum before (red) and after freeze drying (blue) and prepared by (A) water bath sonication, (B) wand sonication or (C) in a lysing matrix B using a FastPrep. The peaks are annotated for each of the mixture components as listed in **Table S3**. Metabolite annotation: 1: AMP, 2: ADP, and 3: ATP.



**Figure S2.** Optimization of sample preparation protocols. Overlay of 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC-TOCSY spectra of sugar phosphate mixture after extraction with 100% water or 1:1 water:methanol and with either (A) sonication in a water bath, (B) sonication using a metal wand, or (C) bead beating with a FastPrep. The peaks are annotated to each of the mixture components in **Table S3**. Metabolite annotation: 1: D-Fructose 1,6-bisphosphate, 2: A-D-Glucose 1,6-biphosphate, 3: D-Fructose 6-phosphate, 4: D-Glucose 6-phosphate, 5: D-Glucosamine-6-phosphate, and 6: D-Ribose 5-phosphate.



**Figure S3.** Overlay of 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra highlighting pH-dependent chemical shifts changes. *E. coli* was grown in M9 minimal media supplemented with  $^{15}\text{NH}_3\text{Cl}$  and  $^{13}\text{C}_6$  glucose. Cells were harvested during the log phase. (A) Overlay 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra collected at pH 4 (red) and pH 7 (blue) showing pH-dependent chemical shift variance. (B) 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectrum collected at pH 7 (red) without the pH alterations required for multinuclei metabolomics is overlaid with a 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectrum collected at pH 7 (blue) following the proposed multinuclei metabolomics protocol. The large chemical shift perturbations readily apparent in (A) is no longer visible.



**Figure S4.** 2D  $^{31}\text{P}$  spectra of cell lysates. 2D  $^1\text{H}$ - $^{31}\text{P}$  HSQC TOCSY spectra of (A) stationary phase cell extract of *S. pombe* grown in YES media and (B) A stationary phase cell extract of *M. smegmatis* grown in 7H9 media. Both cells were lysed using Lysing Matrix B in a Fast Prep with a high speed bead beating. None of the observed peaks could be assigned.

**Table S1.** List of phosphorylated-metabolite  $^1\text{H}$ - $^{31}\text{P}$  chemical shifts at pH 4.

No.	Name	1D $^{31}\text{P}$ (ppm) <sup>a,c</sup>	2D HSQC-TOCSY (ppm) <sup>b,c</sup>
1	A-D-Glucose1,6-biphosphatetetra (cyclohexylammonium) salt hydrate	-1.67 (s) 0.48 (s)	-1.70 (3.13, 3.23, 3.42, 3.60, 3.73, 3.80, 5.06, 5.13) 0.46 (3.13, 3.24, 3.43, 3.52 3.60, 3.72, 3.79, 5.15)
2	Adenosine 5'-diphosphate sodium salt	-11.59, -11.07 (d) -0.01, -0.19(d)	-11.71 (3.89, 4.23, 4.08, 4.46, 5.83) -0.06 (3.79, 4.05, 4.16, 4.43, 5.85)
3	Adenosine 5'-diphosphoribose sodium salt	-----	----
4	Adenosine 5'-monophosphate disodium salt	0.01 (s)	0.03 (3.69, 3.80, 4.05, 4.17, 4.43 5.84)
5	Adenosine 5'-triphosphate disodium salt hydrate	-23.36 (s) -10.92, -11.00, -11.06, -11.51, -11.58, -11.61 (m) -0.17 (s)	-11.62 (3.90, 4.06, 4.31, 4.45, 5.84) 0.04 (3.79, 4.06, 4.17, 5.83)
6	Adenosine 5'-triphosphate magnesium salt from bacterial source	-11.63 (m) -0.22 (s)	-11.77 (3.90, 4.07, 4.24)
7	B-Nicotinamide adenine dinucleotide sodium salt	-12.00, -11.93 (d) -11.69, -11.62 (d)	-11.93 (4.05, 4.11, 4.18, 4.22, 5.77) -11.77 (3.60, 3.90, 4.05, 4.18, 5.73,)
8	Coccarboxylase	-0.16 (s) -10.53,-10.46 (d) -11.84, -11.74 (d)	-11.88 (3.00,3.85) -0.16 (3.77) 1.12 (3.23)
9	Creatine phosphate Disodium salt tetrahydrate	-0.19 (s)	---
10	Cyclophosphamide monohydrate	15.36, 15.38, 15.39 (m)	15.48 (3.11, 3.34)
11	Cytidine-5'-monophosphate	-0.16 (s)	-0.26 (3.75, 3.88, 3.95, 3.98)
12	D-Fructose 1,6-bisphosphate trisodium salt hydrate	-0.07 (s) 0.23 (s)	-0.04 (2.59, 3.18, 3.28, 3.37, 3.46, 3.52) 0.28 (3.01, 3.64, 3.77, 3.85)
13	D-Fructose 6-phosphate disodium salt hydrate	0.23 (s)	-2.59 (3.70) 0.22 (4.03, 3.81, 3.76, 3.72, 3.63)
14	D-Glucosamine 6-phosphate	0.39, 0.41 (d)	0.60 (2.66, 2.94, 2.98, 3.17, 3.25, 3.28, 3.33, 3.56, 3.65, 3.73, 3.80, 4.62)
15	D-Glucose 6-phosphate dipotassium salt hydrate	0.48 (s)	0.52 (2.92, 3.16, 3.21, 3.58, 3.69, 3.74, 3.78, 4.32, 4.88)
16	D-Glucose 6-phosphate disodium salt hydrate	0.62, 0.65, 0.66, 0.67, 0.69 (m)	0.67 (2.67, 2.76, 2.91, 3.04, 3.18, 3.22, 3.35, 3.51, 3.60, 3.70, 3.79, 4.33)

17	D-Mannose 6-phosphate disodium salt hydrate	0.49 (s)	0.48 (3.16, 3.31, 3.51, 3.69, 3.72, 3.76, 3.80)
18	D-Mannose 6-phosphate sodium salt	0.45, 0.47 (d)	0.48 (3.15, 3.34, 3.41, 3.52, 3.58, 3.69, 3.72, 3.76, 3.81)
19	D-Ribose 5-phosphate disodium salt dihydrate	0.19 (s)	0.18 (3.61, 3.50, 3.71, 3.85, 3.90, 3.95, 5.06)
20	Dihydroxyacetone phosphate hemimagnesium salt hydrate	-0.19 (s) -0.08, 0.34 (d)	-3.39 (3.49) -0.58 (4.30, 4.08)
21	Glycerol phosphate disodium salt hydrate	-0.11 (s) 0.29 (s) 0.39 (s)	-0.10 (3.39, 3.84) 0.10 (3.95) 0.29 (3.34, 3.56, 3.60, 3.65) 0.39 (3.43, 3.51)
22	Guanosine 3',5'-cyclic monophosphate sodium salt	-1.81 (s)	-1.81 (3.77, 3.91, 3.96, 5.41)
23	Guanosine 5' triphosphate sodium salt	-23.58, -23.65 (m) -19.61 (s) -11.77, -11.69 (d) -11.16, -11.09 (d)	-11.71 (3.70, 3.86, 3.90, 4.01) -11.61 (3.04, 3.46, 3.53, 3.61, 3.66, 3.75, 3.79, 4.20) -11.51 (3.32, 3.63, 3.68) -11.32 (4.36) -9.48 (4.33, 4.27)
24	Inosine 5'-monophosphate	----	----
25	Lithium potassium acetyl phosphate	-11.08, -11.07, -11.03, 11.02, -11.00 (m), -6.17 (s), -0.19 (s)	-6.20 (1.77)
26	NADP	-11.97, -11.90, -11.69, -11.62 (m) -0.57, -0.57 (d)	-11.71 (3.70, 3.74, 3.79, 3.88, 3.89, 3.91, 3.95, 4.04, 4.08, 4.12, 4.19, 4.23, 4.32, 4.36, 4.42, 4.45, 4.76, 5.78, 5.89) -0.5805 (3.86, 3.93, 3.98, 4.03, 4.05, 4.23, 4.75, 4.78, 5.98)
27	NADPH	-11.97, -11.90, -11.69, -11.62 (m) -0.57, -0.57 (d)	-11.71 (3.70, 3.74, 3.79, 3.88, 3.89, 3.91, 3.95, 4.04, 4.08, 4.12, 4.19, 4.23, 4.32, 4.36, 4.42, 4.45, 4.76, 5.78, 5.89) -0.5805 (3.86, 3.93, 3.98, 4.03, 4.05, 4.23, 4.75, 4.78, 5.98)
28	O-Phospho-L-Tyrosine	-4.08 (s)	-4.10 (6.84, 6.93)
29	Phosphocholine chloride calcium salt tetrahydrate	-0.60 (s) 63.69 (s)	-0.71 (3.34)

30	Phosphoserine	0.67(s)	0.67 (3.65, 3.84)
31	Pyridoxal 5'-phosphate	-0.15 (s)	-0.16 (7.83, 4.93, 4.77, 4.69, 4.65, 2.28, 2.23)
32	rac-Glycerol 1-phosphate sodium salt hydrate	0.43 (s)	0.42 (0.35, 3.26, 3.28, 3.35, 3.51, 3.56, 3.59)
33	Riboflavin 5'-phosphate	2.24 (s) 4.33 (s)	4.35 (3.39, 3.67, 3.70)
34	Thiamine pyrophosphate	-11.20, -11.79 (m)	-11.71 (2.99, 3.84) -0.29 (3.76) 1.34 (3.23)
35	Thymidine 5'-monophosphate disodium salt hydrate	-0.15 (s)	-0.17 (2.01, 3.20, 3.50, 3.74, 3.87, 4.27, 6.04)
36	Thymidine 5'-triphosphate sodium salt	-23.78, -23.77, -23.76, -23.75, -23.71, -23.70, -23.68.24, -11.23, -11.22, -11.21, -11.20, -11.19, -11.1, -11.16 (m)	-11.90 (3.85) -11.80 (3.90) -11.76 (4.34) -11.71 (3.74) -9.68 (3.85) -1.42 (5.68)
37	Thymidine 5' monophosphate	0.14(s)	0.14(2.01, 3.75, 3.87, 4.28)
38	$\beta$ -Nicotinamide Adenine dinucleotide	-0.17 (2.01, 3.20, 3.50, 3.74, 3.87, 4.27, 6.04)	-0.17 (2.01, 3.20, 3.50, 3.74, 3.87, 4.27, 6.04)

<sup>a</sup><sup>31</sup>P chemical shifts from 1D <sup>31</sup>P spectrum with peak multiplicity in parenthesis.

<sup>b</sup><sup>31</sup>P chemical shifts from 2D <sup>1</sup>H-<sup>31</sup>P HSQC TOCSY spectrum with correlated <sup>1</sup>H chemical shifts (ppm) in parenthesis.

<sup>c</sup>dash indicates no peaks were detected.



**Table S2:** List of phosphorylated-metabolite  $^1\text{H}$ - $^{31}\text{P}$  chemical shifts at the indicated pH.

No.	Name	pH	1D $^{31}\text{P}$ (ppm) <sup>a,c</sup>	2D HSQC-TOCSY (ppm) <sup>b,c</sup>
1	A-D-Glucose 1,6-bisphosphatetetra (cyclohexylammonium) salt hydrate	7.29	1.82 (s) 4.19 (s)	1.79 (3.41, 3.43, 3.57, 3.68, 3.82) 1.95 (5.35) 4.21 (3.42, 3.56, 3.82, 4.03, 5.35)
2	Adenosine 5'-diphosphate sodium salt	4.0	0.14 (s) -0.04 (s) -10.75, -10.68 (d) -11.39, -11.32 (d)	-11.30 (4.13, 4.31, 4.45, 6.07) 0.09 (4.29) 0.19 (4.41) 0.28 (4.03)
3	Adenosine 5'-diphosphoribose sodium salt	3.82	-11.10, -11.18, -11.25 (t) -11.43, -11.51 (d)	-11.38 (4.32) -11.07 (4.09, 4.12, 4.14) -10.99 (3.94, 3.98, 4.03, 4.00)
4	Adenosine 5'-monophosphate disodium salt	7.32	3.42 (s)	3.43 (3.93) 3.48 (4.43) 3.51 (4.28, 6.04)
5	Adenosine 5'-triphosphate disodium salt hydrate	3.28	-0.03 (s) -10.59, -10.64 (d) -11.31, -11.37 (d) -22.26 (s)	-11.37 (4.32) -11.34 (4.18, 4.48, 6.09)
6	Adenosine 5'-triphosphate magnesium salt from bacterial source		-0.03 (s) -11.33 (s)	-11.38 (4.13, 4.31)
7	B-Nicotinamide adenine dinucleotide sodium salt	6.46	-11.43, -11.69, -11.76 (m)	-11.69 (4.14, 4.45) -11.62 (4.28)
8	Coccarboxylase	2.72	0.01 (s) 0.15 (s) -10.95, -11.02 (d) -11.56, -11.62 (d)	-11.61 (3.24, 4.10) -11.23 (4.09) -0.16 (4.03) 1.56 (3.49)
9	Creatine phosphate Disodium salt tetrahydrate	2.26	-3.20, -3.29 (d)	-----
10	Cyclophosphamide monohydrate	6.38	15.27 (s)	14.73 (3.34) 15.28 (3.23, 3.34, 3.60, 4.27)
11	Cytidine-5'-monophosphate	3.55	-0.02 (s)	-0.41 (3.99) -0.02 (4.02, 4.14, 4.27)
12	D-Fructose 1, 6-bisphosphate trisodium salt hydrate	6.53	1.86 (m)	2.03 (3.82, 4.09) 2.26 (3.72) 3.12 (3.76, 3.82)
13	D-Fructose 6-phosphate disodium salt hydrate	7.16	3.31 (s)	3.20 (3.87, 4.04) 3.59 (3.76)
14	D-Glucosamine 6-phosphate	5.23	0.62 (s)	0.61 (3.58) 0.62 (5.33)

				0.63 (3.52) 0.65 (2.92, 3.50, 3.89, 4.04) 0.66 (3.79, 3.81, 3.98) 0.68 (2.94, 3.22) 0.69 (4.00)
15	D-Glucose 6-phosphate dipotassium salt hydrate		4.15, 4.20 (d)	3.82 (3.93) 4.17 (3.97) 4.19 (5.14) 4.22 (3.52) 4.60 (3.89)
16	D-Glucose 6-phosphate disodium salt hydrate	7.43	4.20, 4.23 (d)	4.13 (3.95) 4.21 (3.16, 3.19, 3.39, 3.47, 3.60, 3.63, 3.76, 3.81, 3.89, 3.93, 5.12)
17	D-Mannose 6-phosphate disodium salt hydrate	7.1	3.95 (s)	3.62 (3.88, 3.95) 3.98 (3.74) 3.99 (3.90) 4.00 (3.32, 3.62) 4.02 (3.55) 4.04 (3.81, 3.84)
18	D-Mannose 6-phosphate sodium salt	5.44	0.79 (s)	0.78 (3.38, 3.67, 3.66, 3.75, 3.80, 3.82, 3.91, 3.95, 3.99, 4.04)
19	D-Ribose 5-phosphate disodium salt dihydrate	7.08	---	----
20	Dihydroxyacetone phosphate hemimagnesium salt hydrate	5.08	-0.04 (s) 0.04 (s) 0.51 (s)	-0.31 (4.50) 0.00 (4.48) 0.39 (4.42, 4.50) 0.47 (3.79) 0.86 (3.75) 1.17 (3.75)
21	Glycerol phosphate disodium salt hydrate	3.46	3.74 (s) 4.10 (s)	3.74 (4.05) 3.82 (3.58) 4.13 (3.52, 3.68, 3.71, 3.76, 3.74)
22	Guanosine 3',5'-cyclic monophosphate sodium salt		-0.04 (s) -1.70 (s)	-1.66 (4.17, 4.22, 4.38, 5.91) -1.65 (4.93)
23	Guanosine 5' triphosphate sodium salt		----	----
24	Inosine 5'-monophosphate		----	----
25	Lithium potassium acetyl phosphate	6.67	0.77 (s) -2.38 (s) -8.74 (s)	-2.14 (2.01)
26	NADP	--	--	--
27	NADPH	--	--	--

28	O-Phospho-L-Tyrosine	-3.94 (s)	-3.97 (4.70) -3.90 (7.10, 7.20)
29	Phosphocholine chloride calcium salt tetrahydrate	7.23 3.15 (s)	-0.47 (4.05) 2.88 (4.05) 2.90 (3.50) 3.20 (4.08) 3.29 (3.38, 3.48)
30	Phosphoserine	6.50 -----	-----
31	Pyridoxal 5'-phosphate	-0.02 (s) 2.87 0.19 (s)	0.00 (5.00, 5.18, 8.08) 0.16 (5.26, 8.29)
32	rac-Glycerol 1-phosphate sodium salt hydrate	7.08 4.11 (s)	4.09 (3.67) 4.10 (3.71) 4.15 (3.57, 3.75) 4.16 (3.52)
33	Riboflavin 5'-phosphate	0.16 (s) 1.13 (s) 6.27 1.69 (s)	1.33 (3.75) 1.48 (3.93) 1.87 (3.98)
34	Thiamine pyrophosphate	-0.06 (s) -0.20 (s) -11.00, -11.07 (d) 2.7 -11.62, -11.69 (d)	-11.69 (3.20, 3.90, 4.08) -11.38 (3.23) -11.30 (4.09) -10.37 (4.09) -9.04 (4.09) -0.15 (4.00)
35	Thymidine 5'-monophosphate disodium salt hydrate	7.68 3.42 (s)	3.41 (2.30) 3.42 (6.26) 3.48 (4.07) 3.53 (3.90)
36	Thymidine 5'-triphosphate sodium salt	0.54(s) -11.37, -11.45 (d)	1.56 (3.93, 4.08) -11.38 (4.08) -11.46 (4.56)
37	Thymidine 5'monophosphate	7.2 0.00 (s)	-0.03 (2.23) 0.00 (3.96, 4.07) 0.03 (2.27) 0.06 (6.25) 0.40 (4.09) 0.41 (3.98)
38	β-Nicotinamide Adenine dinucleotide	-11.41, -11.48 (d) -11.68, -11.75 (d)	-11.67 (4.37, 4.49) -11.59 (4.29) -11.58 (4.15)

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-11.54 (4.11, 6.03)

-11.53 (4.42)

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<sup>a</sup><sup>31</sup>P chemical shifts from 1D <sup>31</sup>P spectrum with peak multiplicity in parenthesis.

<sup>b</sup><sup>31</sup>P chemical shifts from 2D <sup>1</sup>H-<sup>31</sup>P HSQC TOCSY spectrum with correlated <sup>1</sup>H chemical shifts (ppm) in parenthesis

<sup>c</sup>dash indicates no peaks were detected.

**Table S3.** List of phosphorylated-metabolite  $^1\text{H}$ - $^{13}\text{C}$  chemical shifts at pH 4.

<b>Compound Name</b>	<b>2D <math>^1\text{H}</math>-<math>^{13}\text{C}</math> HSQC (ppm)<sup>c</sup></b>
□-D-glucose 1,6-biphosphate tetra(cyclohexylammonium) salt hydrate	23.73 (1.06, 1.22, 1.53, 1.69)30.34 (1.22, 1.86) 50.33 (3.03)63.55 (3.97, 4.01)68.71 (3.47, 3.65, 3.83)94.82 (5.38)
Adenosine 5'-diphosphate sodium salt	6.38 (4.77, 4.65)59.20 (3.60)87.73 (6.05)155.77 (4.80)
Adenosine 5'-diphosphoribose sodium salt	41.79 (3.60)47.11 (3.36)65.00 (3.95, 4.02, 4.12, 4.15, 4.32, 4.35) 69.84 (4.03, 4.10, 4.2, 4.47)75.00 (3.91)80.96 (4.07, 4.12)84.18 (4.319)87.57 (6.09)96.27 (5.24)101.11 (5.10)141.73 (8.56)
Adenosine 5'-monophosphate disodium salt	63.71 (3.92)70.64 (4.38, 4.66)84.51 (4.24, 6.00)139.96 (8.11, 8.44)
Adenosine 5'-triphosphate disodium salt	65.05 (4.19, 4.29)70.33 (4.51, 4.78)84.35 (4.33, 6.09)
Adenosine 5'-triphosphate disodium salt hydrate	65.57 (4.17)70.41 (4.47)74.99 (4.65)84.29 (4.29)87.57 (6.03)141.81 (8.51)
Adenosine 5'-triphosphate magnesium salt from bacterial source	-----
□-Nicotinamide adenine dinucleotide sodium salt	65.00 (4.10, 4.14, 4.26) 70.32 (4.34, 4.40) 74.19 (4.66) 77.57 (4.42)84.02 (4.28)87.09 (4.47, 5.99)128.51 (8.14)99.98 (6.02)139.80 (9.28)140.93 (8.42)142.38 (8.19, 8.76, 9.09, 9.13)
Coccarboxylase	11.16 (2.41)20.99 (2.49)27.60 (3.18)48.88 (3.22, 5.42)64.68 (4.06)
Creatine phosphate Disodium salt tetrahydrate	36.79 (2.91)53.88 (3.79, 3.82)
cyclophosphamide monohydrate	-----
cytidine-5'-monophosphate	63.71 (3.72, 3.74, 3.83, 3.95, 4.05)69.19 (4.06, 4.15, 4.19)74.35 (4.19)83.06 (4.14)89.34 (5.70, 5.84)96.11 (6.04)142.22 (7.92)

D glucose 6-phosphate disodium salt hydrate	63.87 (3.92, 3.98) 69.196 (3.39, 3.45, 3.81)71.45 (3.44, 3.60)74.19 (3.15, 3.37, 3.45)92.24 (4.53, 5.10)
D-fructose 1,6-bisphosphate trisodium salt hydrate	65.65 (3.69, 3.74, 3.79, 3.84, 3.92)74.03 (3.97, 4.07)80.96 (3.84, 3.98, 4.07)3.98)
D-fructose 6-phosphate disodium salt hydrate	48.72 (3.23)62.42 (3.44, 3.46, 3.53, 3.87, 3.96)65.48 (3.84, 3.93)74.19 (4.09, 4.01, 3.96)79.19 (3.81, 4.03)81.77 (4.00)
D-glucosamine 6-phosphate	54.36 (2.90, 3.21)63.55 (3.96, 3.98, 4.01)68.22 (3.20, 3.46, 3.58, 3.78, 3.87)70.48 (3.56, 3.87)75.00 (3.50)88.21 (5.32)
D-Glucosamine 6-phosphate	54.2 (3.24)89.20 (5.34)
D-Glucose 6-phosphate dipotassium salt hydrate	63.87 (3.9, 3.95, 4.92, 4.00)69.19 (3.36, 3.43, 3.58, 3.79)74.19 (3.13, 3.44, 3.36)92.24 (5.10, 4.50)
D-Mannose 6-phosphate disodium salt hydrate	66.2951 (3.70, 3.76, 3.96)
D-Mannose 6-phosphate sodium salt	64.19 (3.93, 3.98, 4.03) 66.13 (3.55, 3.65, 3.74)70.00 (3.33, 3.55, 3.74, 3.82)75.00 (3.39)93.69 (4.80, 5.07)
D-Ribose 5-phosphate disodium salt dihydrate	64.36 (3.840, 3.83, 3.92)70.48 (4.2)75.16 (3.93)81.44 (4.00, 4.14)96.27 (5.30)100.95 (5.15)
Dihydroxyacetone phosphate hemimagnesium salt hydrate	54.36 (2.90, 3.18)63.55 (3.95, 3.98)69.55 (3.20, 3.46, 3.55 3.70, 3.86)74.83 (3.46)89.34 (5.33)
Glycerol phosphate disodium salt hydrate	62.26 (2.90, 3.01, 3.07, 3.13, 3.48, 3.56, 3.65, 3.70)71.29 (3.67, 3.73)74.03 (3.56, 4.02, 4.10)
Guanosine 3',5'-cyclic monophosphate sodium salt	67.26 (4.12, 4.16, 4.34)71.61 (4.11, 4.63)91.92 (5.86)
Guanosine 5' triphosphate sodium salt	----
Inosine 5'-monophosphate	64.36 (3.99)70.48 (4.36)84.19 (4.25)87.42 (6.02)
Lithium potassium acetyl phosphate	20.35 (1.99)

NADP	---
NADPH	---
O-Phospho-L-Tyrosine	35.18 (3.21)55.81 (3.91)121.1 (7.10)
Phosphoserine	---
Phosphocholine chloride calcium salt tetrahydrate	53.88 (2.68, 2.84, 3.08)58.88 (4.14)65.97 (3.52)
Pyridoxal 5'-phosphate	14.22 (2.54)15.35 (2.38, 2.42)60.81 (5.14, 5.23, 5.27)
rac-glycerol 1-phosphate sodium salt hydrate	62.0673 (3.49)38, 3.56)65.677 (3.71, 3.76)70.99 (3.76)
Riboflavin 5'-phosphate	20.67 (2.26, 2.38)66.29 (3.95, 4.25)71.29 (3.95, 3.86)116.91 (7.67)130.29 (7.55)
Thiamine pyrophosphate	11.00 (2.42)20.99 (2.49)27.60 (3.20)49.04 (3.22, 5.42)64.68 (4.06)144.31 (7.74)
Thymidine 5'-monophosphate disodium salt hydrate	11.48 (1.81) 38.73 (2.26)64.68 (3.97, 4.07)71.13 (4.47)85.47 (4.07, 6.26)137.38 (7.67)
thymidine 5'-triphosphate sodium salt	----
Thymidine 5' monophosphate	----
β-Nicotinamide Adenine dinucleotide	62.5875 (3.44, 3.53, 4.25, 4.12, 4.09)70.80 (4.33, 4.39)72.09 (3.65)74.19 (4.39, 4.69, 5.95)90.98 (4.64, 5.99)

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<sup>13</sup>C chemical shifts from 2D <sup>1</sup>H-<sup>13</sup>C HSQC spectrum with correlated <sup>1</sup>H chemical shifts (ppm) in parenthesis.

**Table S4.** Composition of the mixtures

<b>No.</b>	<b>Adenosine analogs</b>	<b>No.</b>	<b>Sugar phosphates</b>
1	Adenosine 5'-diphosphate sodium salt	1	D-Fructose 1,6-bisphosphate trisodium salt hydrate
2	Adenosine 5'-monophosphate disodium salt	2	A-D-Glucose 1,6-biphosphate tetra (cyclohexylammonium) salt hydrate
3	Adenosine 5'-triphosphate disodium salt hydrate	3	D-Fructose 6-phosphate disodium salt hydrate
3	Adenosine 5'-triphosphate magnesium salt from bacterial source	4	D-Glucose 6-phosphate disodium salt hydrate
		4	D-Glucose 6-phosphate dipotassium salt hydrate
		5	D-Glucosamine-6-phosphate
		6	D-Ribose 5-phosphate disodium salt dihydrate



## References

1. Worley, B.; Powers, R., Deterministic multidimensional nonuniform gap sampling. **2015**.
2. Bhinderwala, F.; Lonergan, S.; Woods, J.; Zhou, C.; Fey, P. D.; Powers, R., Expanding the Coverage of the Metabolome with Nitrogen-Based NMR. *Analytical Chemistry* **2018**, *90* (7), 4521-4528.
3. Wishart, D. S.; Jewison, T.; Guo, A. C.; Wilson, M.; Knox, C.; Liu, Y.; Djoumbou, Y.; Mandal, R.; Aziat, F.; Dong, E.; Bouatra, S.; Sinelnikov, I.; Arndt, D.; Xia, J.; Liu, P.; Yallou, F.; Bjorn Dahl, T.; Perez-Pineiro, R.; Eisner, R.; Allen, F.; Neveu, V.; Greiner, R.; Scalbert, A., HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acids Res* **2013**, *41* (Database issue), D801-7.
4. Markley, J. L.; Ulrich, E. L.; Berman, H. M.; Henrick, K.; Nakamura, H.; Akutsu, H., BioMagResBank (BMRB) as a partner in the Worldwide Protein Data Bank (wwPDB): New policies affecting biomolecular NMR depositions. *Journal of Biomolecular NMR* **2008**, *40*, 153-155.
5. Akiyama, K.; Chikayama, E.; Yuasa, H.; Shimada, Y.; Tohge, T.; Shinozaki, K.; Hirai, M. Y.; Sakurai, T.; Kikuchi, J.; Saito, K., PRIME: a Web site that assembles tools for metabolomics and transcriptomics. *In Silico Biol* **2008**, *8* (3-4), 339-45.