Supplementary information

Colorimetric Discrimination of Nucleoside Phosphates Based on Catalytic Signal Amplification Strategy and its Application to Related Enzyme assays

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S1. Materials

2,6-Bis(hydroxymethyl)-p-cresol, manganese acetate tetrahydrate (Mn(OAc)$_2 \cdot$4H$_2$O) and sodium perchlorate (NaClO$_4$) were purchased from Sigma-Aldrich. 2,2'-Dipicolylamine and hydrogen peroxide 35% in water (H$_2$O$_2$) were purchased from Tokyo Chemical Industry (TCI). Thionyl chloride (SOCl$_2$), anhydrous sodium sulfate (Na$_2$SO$_4$) and triethylamine (Et$_3$N) were purchased from Daejung Chemical Industry. Sodium acetate trihydrate (NaOAc $\cdot$3H$_2$O) was purchased from Junsei Chemical Industry. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 3,3',5,5'-tetramethylbenzidine (TMB) was purchased from Alfa-Aesar.

Adenosine 5'-monophosphate monohydrate from yeast (AMP), adenosine 5'-triphosphate disodium salt hydrate (ATP), adenosine 3',5'-cyclic monophosphate (cAMP), guanosine 5'- diphosphate sodium salt (GDP), guanosine 5'-triphosphate sodium salt hydrate (GTP), cytidine 5'-monophosphate disodium salt (CMP), cytidine-5'-triphosphate disodium salt (CTP), thymidine, sodium phosphate dibasic (Pi), and sodium pyrophosphate decahydrate (PPi) were purchased from Sigma-Aldrich. Adenosine 5'-diphosphate disodium salt hydrate (ADP) and cytidine 5'-diphosphate trisodium salt hydrate (CDP) was purchased from TCI. Guanosine 5'- monophosphate disodium salt hydrate (GMP) was purchased from Acros. Adenosine was purchased from Alfa-Aesar.

For enzyme assay, phosphodiesterase I (EC 3.1.4.1, from *Crotalus adamanteus venom*), hexokinase (EC 2.7.1.1, type F-300, from *Saccharomyces Cerevisiae*), acetate kinase (EC 2.7.2.1, from *Escherichia coli*), 3-isobutyl-1-methylxanthine (IBMX), glucose-6-phosphate sodium salt (G6P), lithium potassium acetyl phosphate (AcP), and magnesium perchlorate (Mg(ClO$_4$)$_2$) were purchased from Sigma-Aldrich. β-D-glucose, N-acetyl-D-glucosamine (NAG), and N-benzoyl-D-glucosamine (NBG) were purchased from TCI. Allyl isothiocyanate (AITC) was purchased from Alfa-Aesar.

S2. Synthesis of H-bpmp Ligand [S1]
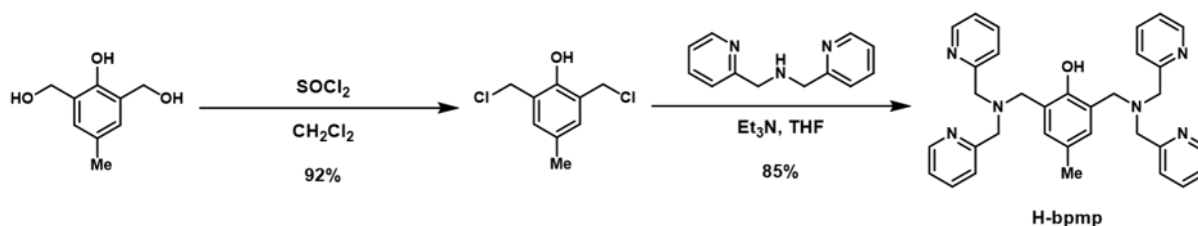
S2.1. Synthesis of 2,6-bis(chloromethyl)-4-methylphenol

2,6-Bis(hydroxymethyl)-*p*-cresol (2.5 g, 15 mmol) in CH_2Cl_2 (30 mL) was added to $SOCl_2$ (10 mL, 140 mmol). After stirring for 12 h, the yellow mixture was washed with water and brine. The organic layer was dried with Na_2SO_4 and concentrated under the reduced pressure to give a white solid. Yield 2.8 g (92%). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.09 (s, 2H), 5.52 (s, 1H), 4.66 (s, 4H), 2.28 (s, 3H).

S2.2. Synthesis of 2,6-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-methylphenol (H-bpmp)

2,6-Bis(chloromethyl)-4-methylphenol (2.8 g, 14 mmol) was dissolved in 30 mL of THF in an ice bath. Then, a solution of 2,2'-dipicolylamine (4.9 mL, 27 mmol) and Et$_3$N (11.6 mL, 55 mmol) in 30 mL of THF was added dropwise. After stirring for 5 days at room temperature, the mixture was filtered and concentrated under reduced pressure. The mixture was dissolved in CH_2Cl_2 then washed three times with brine. The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The mixture was hot filtered with diethyl ether to give a pale-yellow solid. Yield 6.2 g (85%). mp 105-107 °C; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.80 (s, 1H), 8.49 (dq, J = 4.9, 0.8 Hz, 4H), 7.72 (td, J = 7.7, 1.8 Hz, 4H), 7.47 (d, J = 7.8 Hz, 4H), 7.24 (ddd, J = 7.4, 4.9, 1.1 Hz, 4H), 6.97 (s, 2H), 3.75 (s, 8H), 3.66 (s, 4H), 2.17 (s, 3H); ^{13}C-NMR (101 MHz, DMSO-d_6) δ 158.69, 153.24, 148.76, 136.86, 129.48, 126.44, 123.49, 122.62, 122.21, 58.88, 53.87, 20.31.

S3. Screening colorimetric response of Mn$_2$(bpmp)/ABTS/H$_2$O$_2$ system in the presence of various phosphate anions
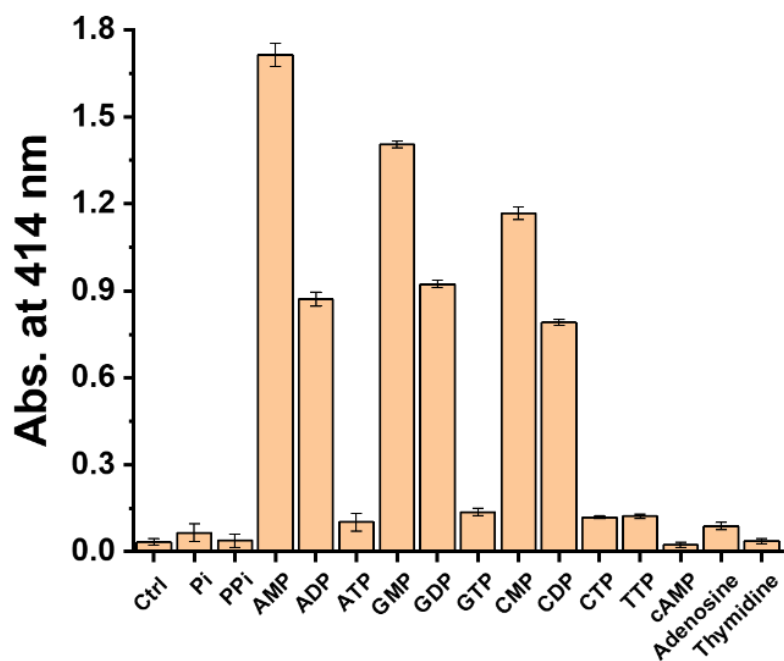
Fig. S1 Screening of colorimetric response of Mn$_2$(bpmp)/ABTS/H$_2$O$_2$ system in the presence of various phosphate anions (0.5 mM) after 300 s in aqueous solution (20 mM pH 7.0 Tris buffer, 5% DMSO).

S4. Optimization of colorimetric response of the Mn₂(bpmp)/ABTS/H₂O₂ system

S4.1. Effect of pH, temperature, and concentration of Mn₂(bpmp) on the Mn₂(bpmp)/ABTS/H₂O₂ system
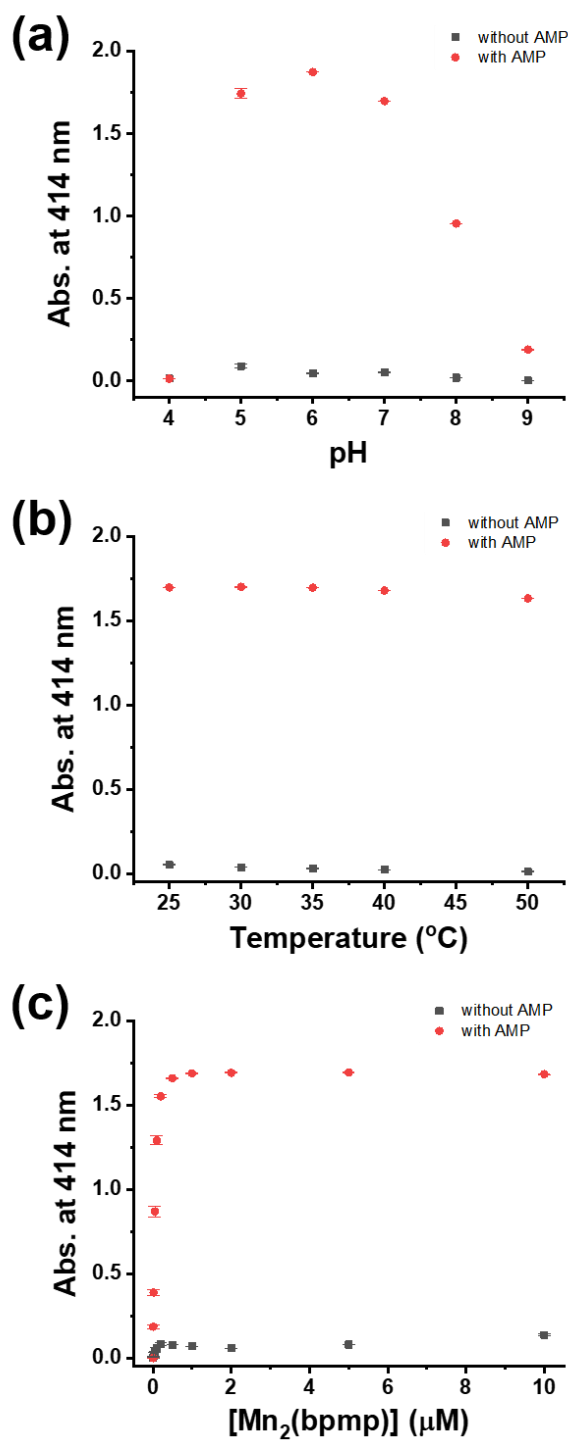

Fig. S2 The effects of (a) pH 4-5 (acetate, 20 mM), pH 6 (MES, 20 mM), pH 7-9 (Tris, 20 mM), (b) temperature (25-50 °C), and (c) concentration of Mn₂(bpmp) (0-10 μM) in the presence and absence of AMP. [Mn₂(bpmp)] = 2 μM, [ABTS] = 0.1 mM, [AMP] = 0.5 mM, [H₂O₂] = 10 mM.

S4.2. Oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by Mn_2(bpmp)/H_2O_2 system
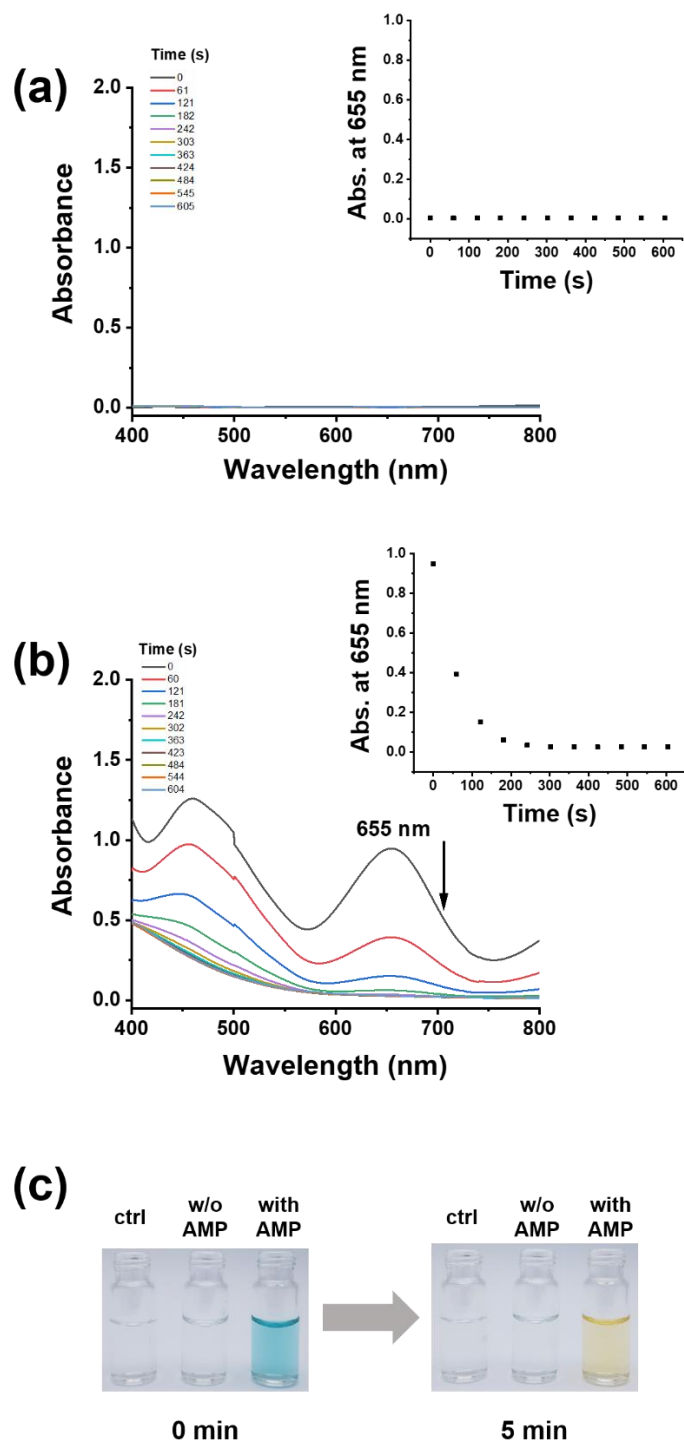

Fig. S3 UV-Vis spectral changes of the Mn_2(bpmp)/TMB/H_2O_2 system in the (a) absence and (b) presence of AMP in buffered solution (pH 7.0 Tris, 20 mM, DMSO 5%), Inset: Plot of the absorbance at 655 nm versus time. (c) Photograph of Mn_2(bpmp)/TMB/H_2O_2 system without and with AMP. [Mn_2(bpmp)] = 2 μM, [AMP] = 0.5 mM, [TMB] = 0.1 mM, [H_2O_2] = 10 mM.

S5. AMP titration and determination of limit of detection (LOD)
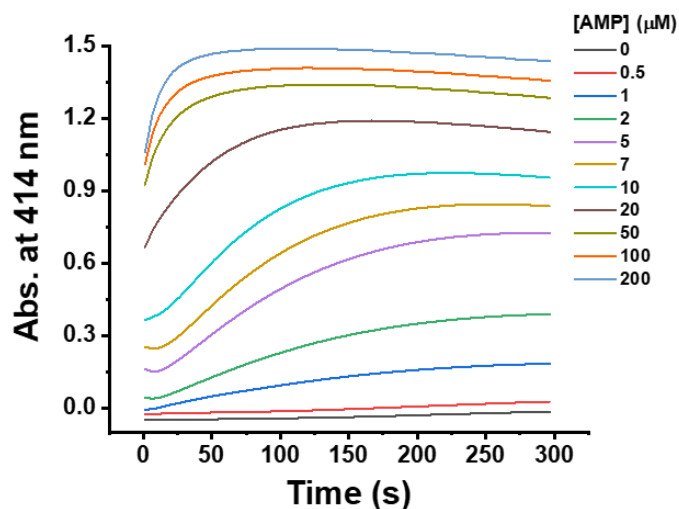
Fig. S4 Change in the absorbance of the $Mn_2(bpmp)/ABTS/H_2O_2$ system in the presence of various AMP concentrations (0–200 μM).
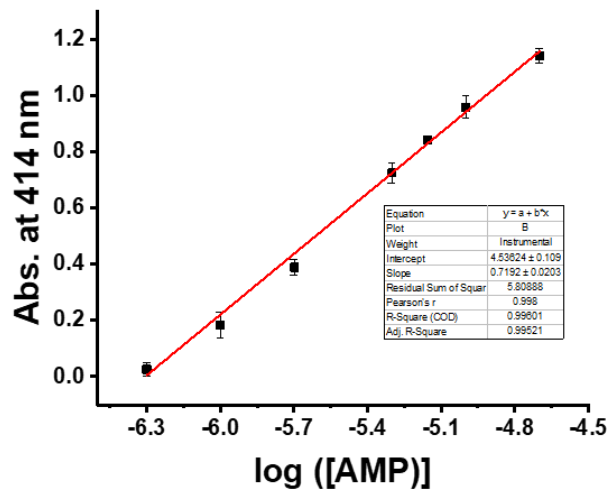
Fig. S5 Limit of detection of AMP.[3]

The limit of detection (LOD) for AMP was obtained from the low concentration range of AMP (0.5~20 μM) in the AMP titration (see Fig. 1(b)). As shown in Fig. S5, a linear function was obtained when the x-axis is log[AMP] and the y-axis is Abs. at 414 nm. The LOD was estimated from the x-intercept of this function.
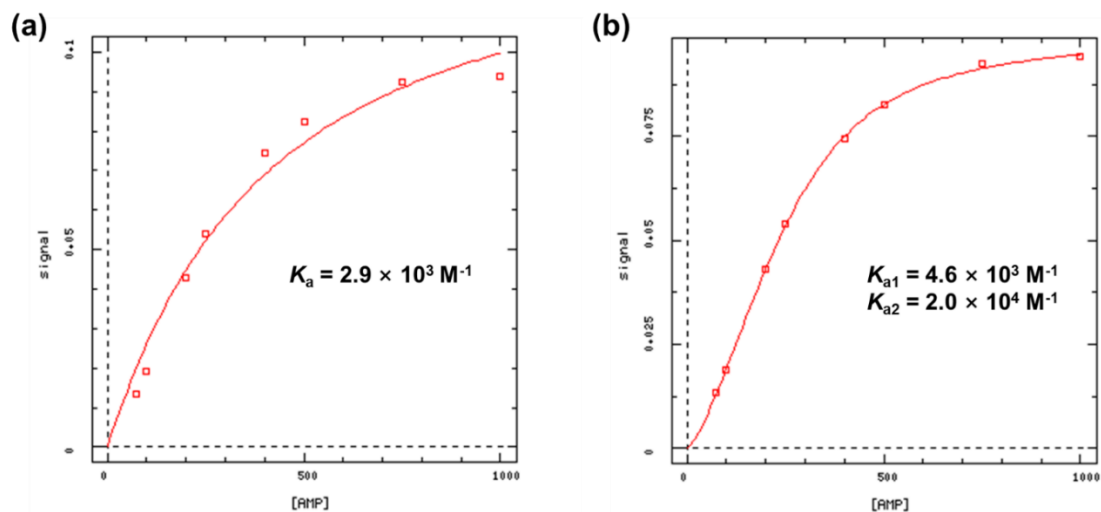
intercept = 4.5362

Slope = 0.7192

R^2 = 0.99

LOD = 0.5 μM

S6. Binding study between Mn₂(bpmp) and AMP

Fig. S6 The absorption changes at 505 nm observed from AMP titration and the fitting to two binding model; (a) 1:1 binding model, (b) 1:1 and 1:2 binding model.

S6.1. Mass based binding study

To identify AMP-Mn₂(bpmp) adduct mass analysis was conducted. To a buffered solution (200 µM pH 7 Tris, 0.5% DMSO) of Mn₂(bpmp), AMP (100 µM) was added then allowed to equilibrate for 30 min before measurements. The final concentration following as: [Mn₂(bpmp)] = 10 µM, [AMP] = 100 µM. As shown in Fig. S7, both 1:1 and 2:1 adduct was founded.
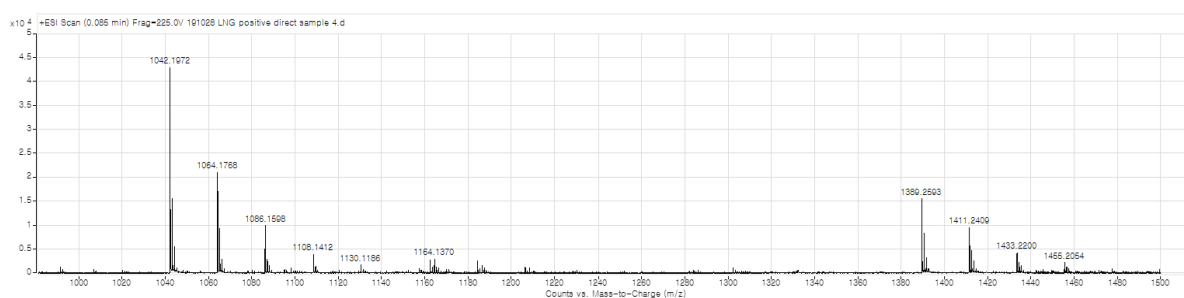

Fig. S7 Mass spectrum of AMP-Mn₂(bpmp) adduct.

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Table S1. Assignments of mass spectrometry peaks.

found *m/z*	Compound (cald)	Manganese oxidation state
1042.1972	$[Mn_2(bpmp)(\mu\text{-}OAc)(AMP)]^+$ (1042.2000)	III, III
1389.2593	$[Mn_2(bpmp)(\mu\text{-}OAc)(AMP)_2]^+$ (1389.2631)	III, III

The peak of 1164.1370 was found to be impurity signal in blank sample.

S6.2. ITC based binding study

Aqueous Mn$_2$(bpmp) (0.2 M pH 7 HEPES-NaOH, 5 % DMSO, 0.25 and 0.5 mM) and phosphate derivatives (0.2 M pH 7 HEPES-NaOH, 5 % DMSO, 5 mM) were prepared, respectively. The samples were extensively degassed prior to titration to prevent air bubble formation during the procedure. The Mn$_2$(bpmp) solution (1 mL) was placed in the sample cell of the calorimeter, and phosphate derivatives (250 μL) were loaded in the syringe. Then, phosphate derivative solutions (10.0 μL per injection) were titrated with 300 s intervals into the Mn$_2$(bpmp) solution over 7,500 s at 25 °C. Through the ITC measurement, the thermodynamic information including heat rate curve depending on time, entropy change (ΔS, cal/mol·K), enthalpy change (ΔH, kcal/mol), association constant (K_a, M^{-1}), and molar ratio of the reaction (n) was obtained.
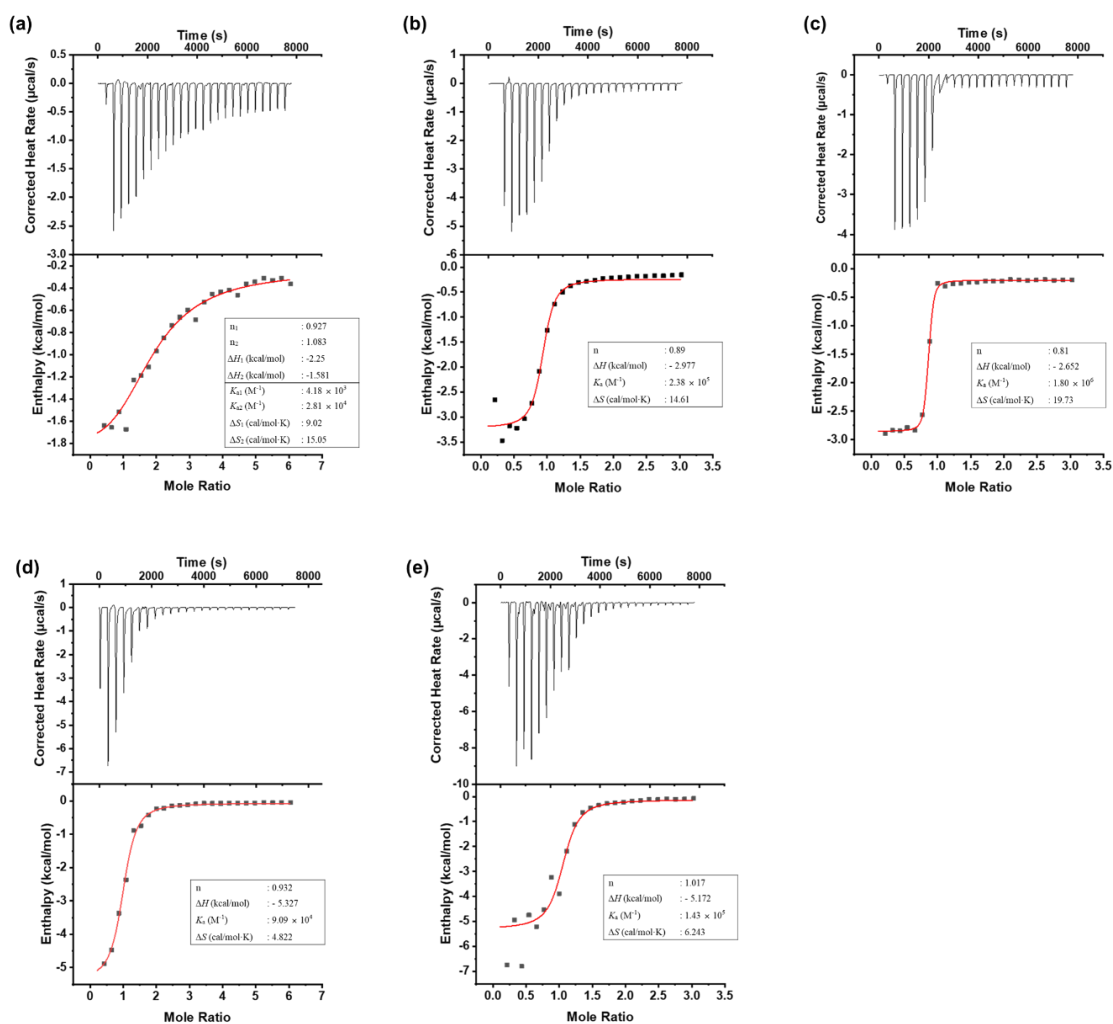

Fig. S8 ITC data; Top: heat rate along with reaction time, Bottom: the normalized fit curve of ΔH depending on mole ratio of the phosphate derivatives/Mn$_2$(bpmp). (a) AMP, (b) ADP, (c) ATP, (d) Pi, and (e) PPi.

Table S2. Thermodynamic parameter of binding event of Mn_2(bpmp) and phosphate derivatives from ITC.

Thermodynamic parameter	AMP	ADP	ATP	Pi	PPi
n	0.927 (n_1) 1.083 (n_2)	0.89	0.81	0.932	1.017
K_a (M^{-1})	4.18×10^3 (K_{a1}) 2.81×10^4 (K_{a2})	2.38×10^5	1.80×10^6	9.09×10^4	1.43×10^5
ΔH (kcal/mol)	-2.25 (ΔH_1) -1.581 (ΔH_2)	-2.977	-2.652	-5.327	-5.172
ΔS (cal/mol K)	9.02 (ΔS_1) 15.05 (ΔS_2)	14.61	19.73	4.822	6.243

S7. Effect of AMP in the reaction between Mn_2(bpmp) and H_2O_2
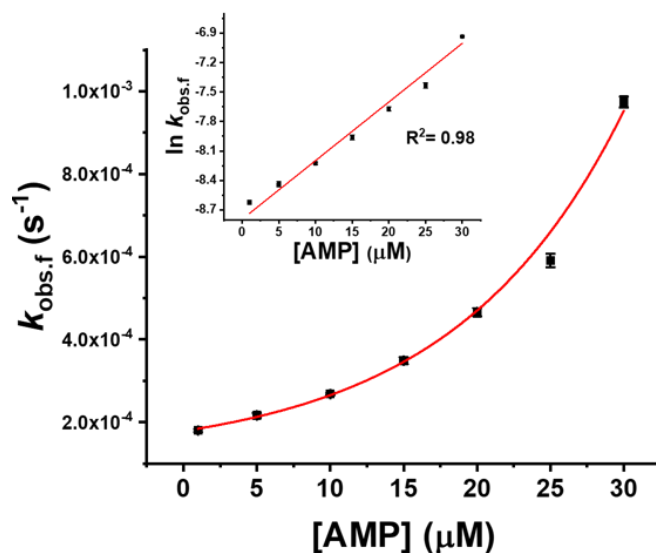
Fig. S9 Change of $k_{obs.}$ during the reaction of Mn_2(bpmp) (100 µM) with H_2O_2 (1 mM) in a buffered solution (pH 7.0) in the absence and presence of AMP; Inset: $\ln k_{obs.}$ vs. AMP concentration.

S8. PDE assay

S8.1. Inhibitory assay

To buffered solution (20 mM, pH 7 Tris) containing PDE (5 mU/mL) and different concentration of IBMX (10, 40, 100, 150, 200, 400 μM), cAMP (1 mM) was added. After 10 min at 25 °C, $Mn_2(bpmp)$ (2 μM), ABTS (0.5 mM), H_2O_2 (4 mM) was added. After 10 s, UV-vis spectra were recorded at 414 nm at 2 s intervals for 300 s. Using the absorbance at 414 nm (100 s), plotting was performed for concentration of IBMX.
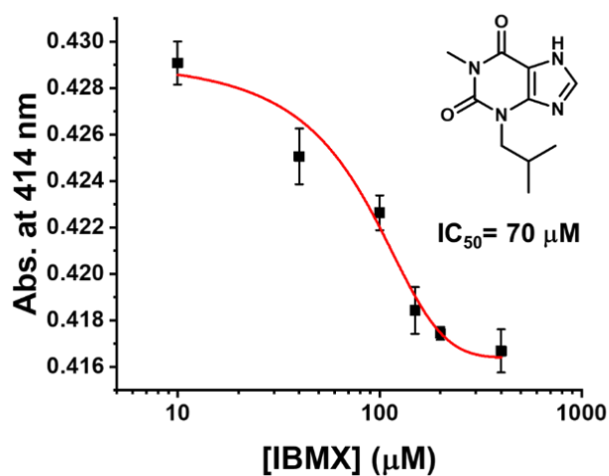

Fig. S10 Inhibitory assay for PDE using IBMX

S9. Small molecule kinase assay

### S9.1. Discrimination of ADP and ATP using Mn$_2$(bpmp)/ABTS/H$_2$O$_2$ system

Various ratios of ATP to ADP (total concentration set to 2.0 μM) were added to a buffer solution (HEPES-NaOH, 20 mM, pH 7.0) containing ABTS (100 μM), Mn$_2$(bpmp) (2 μM, 5% DMSO), and Mg(ClO$_4$)$_2$ (100 μM). One minute after adding H$_2$O$_2$ (10 mM) to the above solution, the UV-vis spectra were recorded for 300 s.
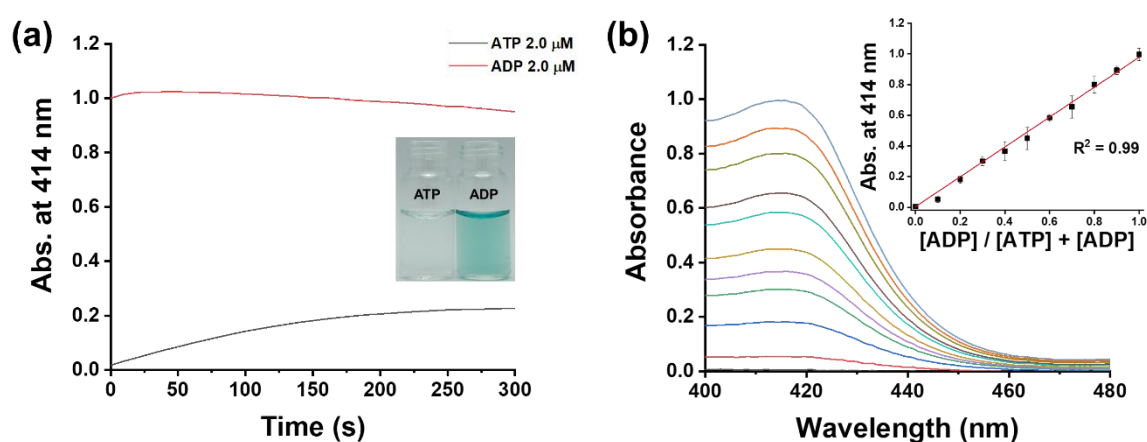
Fig. S11 (a) Time dependent increase in absorbance of the Mn$_2$(bpmp)/ABTS/H$_2$O$_2$ system at 414 nm in the presence of 2.0 μM of ATP or ADP. Inset: corresponding color 1 min after H$_2$O$_2$ addition. (b) Change in the absorption spectra with varying ratios of ADP/ATP, [ADP] + [ATP] = 2 μM. Inset: Absorbance at 414 nm as a function of ADP ratio.

S9.2. Test of kinase substrate/product/inhibitor effect on the assay system

To buffered solution (HEPES-NaOH, 20 mM, pH 7.0) containing ABTS (100 μM), Mn$_2$(bpmp) (2 μM, 5% DMSO), Mg(ClO$_4$)$_2$ (100 μM), and ATP (2 μM), kinase substrate, product, inhibitor or enzyme was added. One minute after adding H$_2$O$_2$ (10 mM) to the above solution, the UV-vis spectra were recorded at 414 nm at 10 s intervals for 10 min. The final concentration: [ADP] = 2 μM, [Glucose] = 1 mM, [Sodium acetete] = 5 mM, glucose-6-phosphate sodium salt (G6P) [G6P] = 2 μM, lithium potassium acetyl phosphate (AcP) [AcP] = 2 μM, [NAG] = 10 mM, [NBG] = 5 mM, [AITC] = 200 μM, [HK] = 1 U/mL, and [AcK] = 1 U/mL.
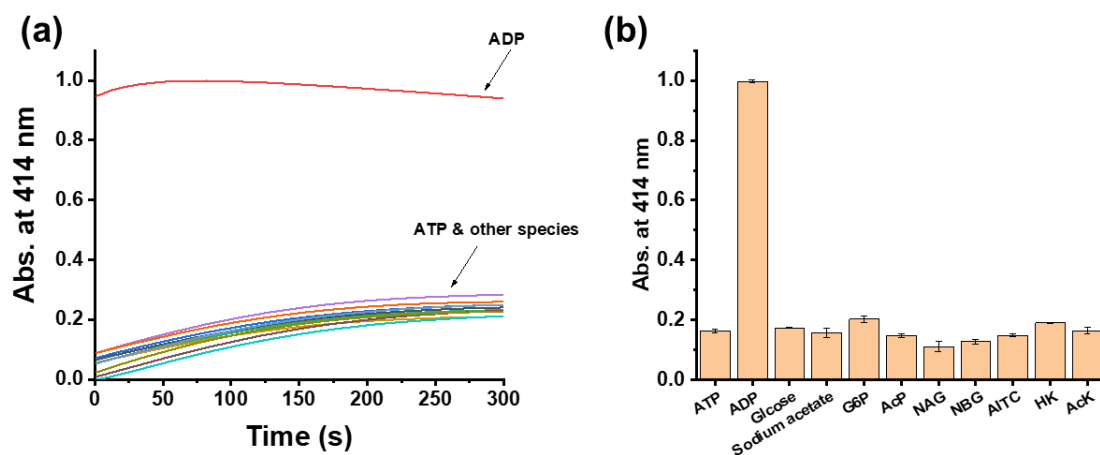
Fig. S12 (a) The time-resolved absorbance of $Mn_2(bpmp)/ABTS/H_2O_2$ system in the presence of kinase substrate, product, inhibitor or enzyme (b) Plot of absorbance of 414 nm at 100 s.

S9.3. General method for kinase activity assay

To a buffered solution (HEPES-NaOH, 20 mM, pH 7.0) containing [ABTS (100 μM), $Mn_2(bpmp)$ (2 μM, 5% DMSO), $Mg(ClO_4)_2$ (100 μM), substrate (glucose (1 mM) or acetate (5 mM)), and ATP (2 μM)], H_2O_2 (10 mM) was added. After 1 min, various concentrations of hexokinase (from 0.01 to 0.3 U/mL) or acetate kinase (from 0.2 to 1.0 U/mL) were added. With no further incubation, UV-Vis spectra were recorded at 414 nm at 10 s intervals for 10 min. The observed rate (k_{obs}) of the reaction was determined based on the change in absorbance at 414 nm for 2 min.
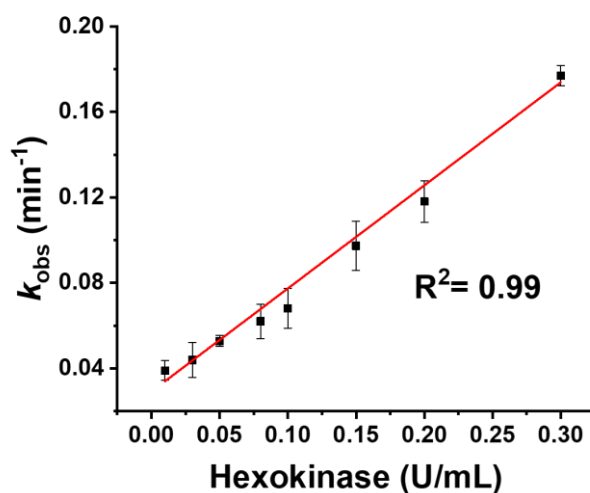
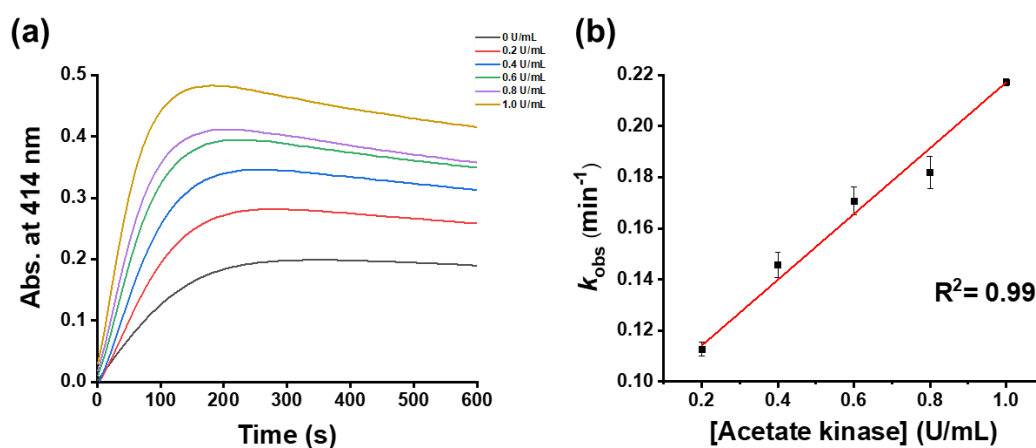**Fig. S13** Change in k_{obs} as a function of hexokinase concentrations.

Fig. S14 (a) Real-time monitoring of different concenration of acetate kinase using the time-resolved absorbance of $Mn_2(bpmp)/ABTS/H_2O_2$ system. (b) Change of k_{obs} *versus* concentrations of acetate kinase.

S9.4. Inhibitory screening for HK in a well-plate

To a buffer solution (HEPES-NaOH, 20 mM, pH 7.0) containing [ABTS (1 mM), $Mn_2(bpmp)$ (2 μM, 5% DMSO), $Mg(ClO_4)_2$ (100 μM), glucose (100 μM), ATP (2 μM) and NAG, NBG, or AITC (5 mM)], H_2O_2 (10 mM) was added. After 1 min, hexokinase (1.0 U/mL) was added. Photographs was captured on a SAMSUNG GALAXY Note 8.

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S9.5. Inhibitory assay for HK using NAG and NBG

To a buffer solution (HEPES-NaOH, 20 mM, pH 7.0) containing [ABTS (100 µM), Mn_2(bpmp) (2 µM, 5% DMSO), $Mg(ClO_4)_2$ (100 µM), glucose (100 µM), ATP (2 µM), and various concentrations of NAG or NBG], H_2O_2 (10 mM) was added. After 1 min, hexokinase (0.1 U/mL) was added. With no further incubation, UV-Vis spectra were recorded at 414 nm at 10 s intervals for 10 min. Inhibition efficiency was defined by the following equation:

Inhibition efficiency (%) = [($A_{con.}$ - A_{sample})/($A_{con.}$ - A_{blank})] × 100 (%)

$A_{con.}$: Absorbace of 414 nm at 10 min without inhibitor.

A_{sample}: Absorbace of 414 nm at 10 min with various concentration of inhibitor.

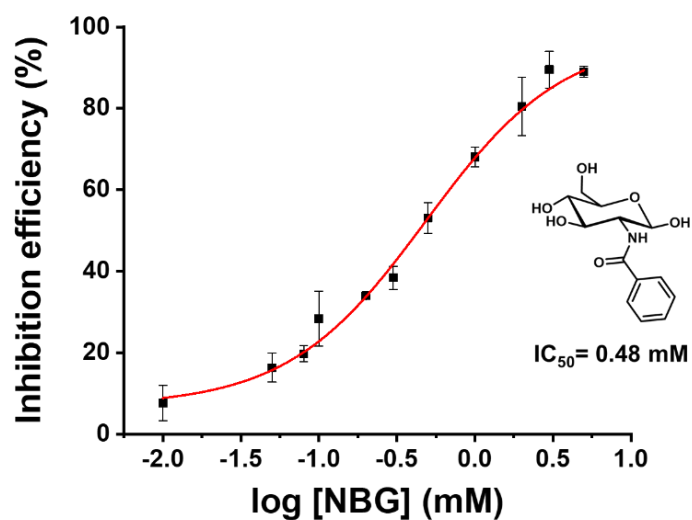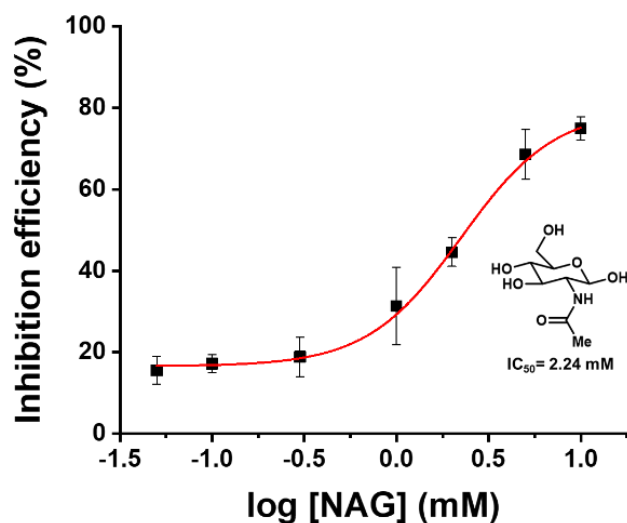A_{blank} : Absorbance of 414 at 10 min without HK.

Fig. S15 Inhibition efficiency of NBG for HK.

Fig. S16 Inhibition efficiency of NAG for HK.

To compare with parameter of previous report, the IC_{50} value was converted to the inhibition constant (K_i) using Cheng-Prusoff equation.[s2]

Cheng-Prusoff equation: $K_i = IC_{50} / 1 + ([S] / K_m)$

K_m: Michaelis constant of D-glucose for HK. (0.12 mM from Sigma-Aldrich)

[S]: Concentration of D-glucose.

Table S3. Inhibition constant of NAG and NBG for HK.

Inhibitor	IC_{50} (mM)	K_i (mM)	K_i (mM) *from ref.*
NAG	2.24	1.22	5.1[s3]
NBG	0.48	0.26	0.33[s4]

S9.6. Inhibitory assay for AcK using AITC

Acetate kinase (0.4 U/mL) was incubated in a solution containing various concentrations of AITC (0 to 200 µM) and H_2O_2 (10 mM). After 5 min of incubation, it was added to a solution containing ABTS (100 µM), Mn_2(bpmp) (2 µM, 5% DMSO), $Mg(ClO_4)_2$ (100 µM), sodium acetate (5 mM), and ATP (2 µM). With no further incubation, UV-vis spectra were recorded at 414 nm at 10 s intervals for 10 min.
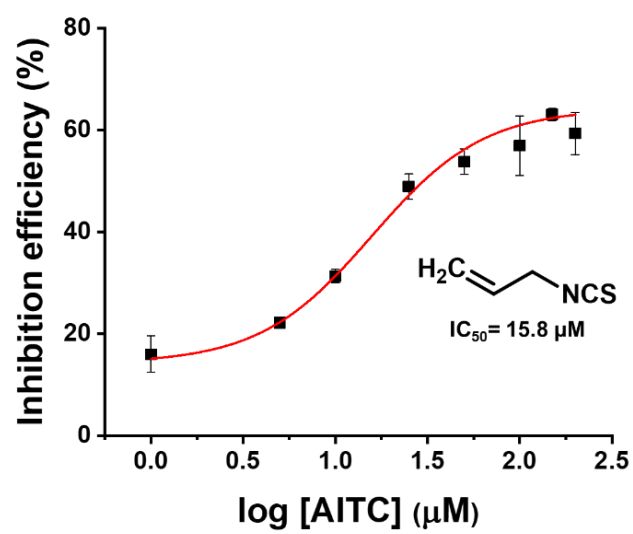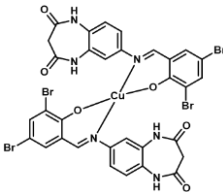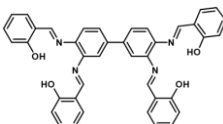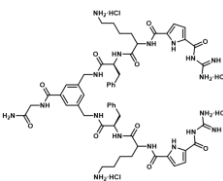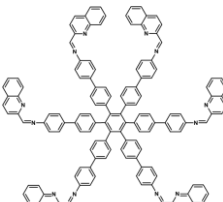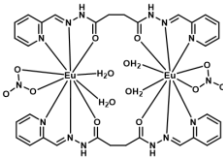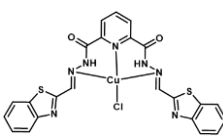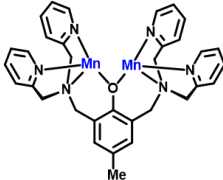
Fig. S17 Inhibition efficiency of AITC for AcK.

Table S4. Representative AMP sensors.

Structure	Detection method	Sensing Selectivity & Association constant	Media	LOD (μM)	Ref.
	UV/Vis	AMP > ADP > ATP K_a (AMP) = 4.0×10^4 M^{-1} K_a (ADP) = 4.1×10^3 M^{-1} K_a (ATP) = 1.2×10^3 M^{-1}	DMSO/H$_2$O (9/1)	No data	[S5]
	Fluorescence	AMP >> ADP > ATP K_a (AMP) = 2.0×10^4 M^{-1}	MeCN/H$_2$O (95/5)	1.1	[S6]
	UV/Vis	AMP > ADP > ATP K_a (AMP) = 7.6×10^4 M^{-1} K_a (ADP) = 3.2×10^4 M^{-1} K_a (ATP) = 3.8×10^3 M^{-1}	pH 7.0 Tris	No data	[S7]
	Fluorescence	AMP > ADP,ATP	EtOH/THF (3/1)	0.9	[S8]
	Luminescence	AMP >> ADP, ATP K_a (AMP) = 1.0×10^4 M^{-1}	pH 7.4 HEPES	2.0	[S9]
	UV/Vis & Colorimetric	AMP >> ADP, ATP K_a (AMP) = 1.47×10^5 M^{-1}	DMSO/H$_2$O (8/2)	0.08	[S10]
	UV/Vis & Colorimetric	AMP > ADP >> ATP K_{a1} (AMP) = 4.6×10^3 M^{-1} K_{a1} (AMP) = 2.0×10^4 M^{-1} K_a (ADP) = 2.4×10^5 M^{-1} K_a (ATP) = 1.8×10^6 M^{-1}	pH 7.0 Tris, 5% DMSO	0.5	This work

S10. References

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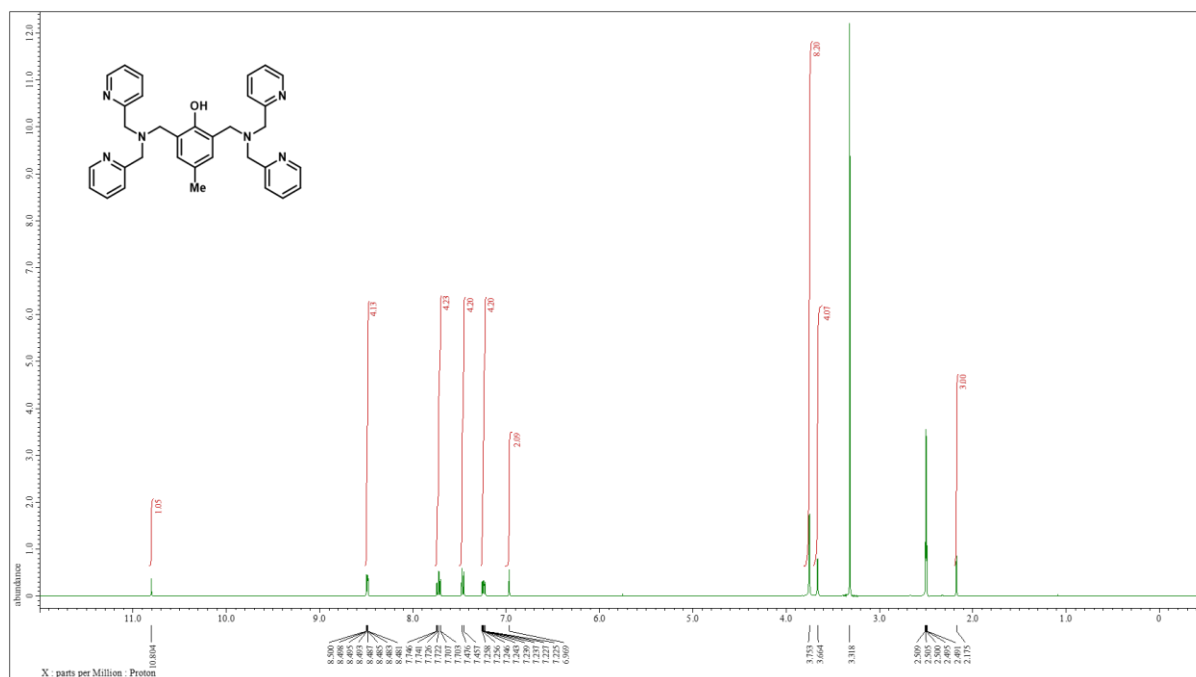
S11. Spectra

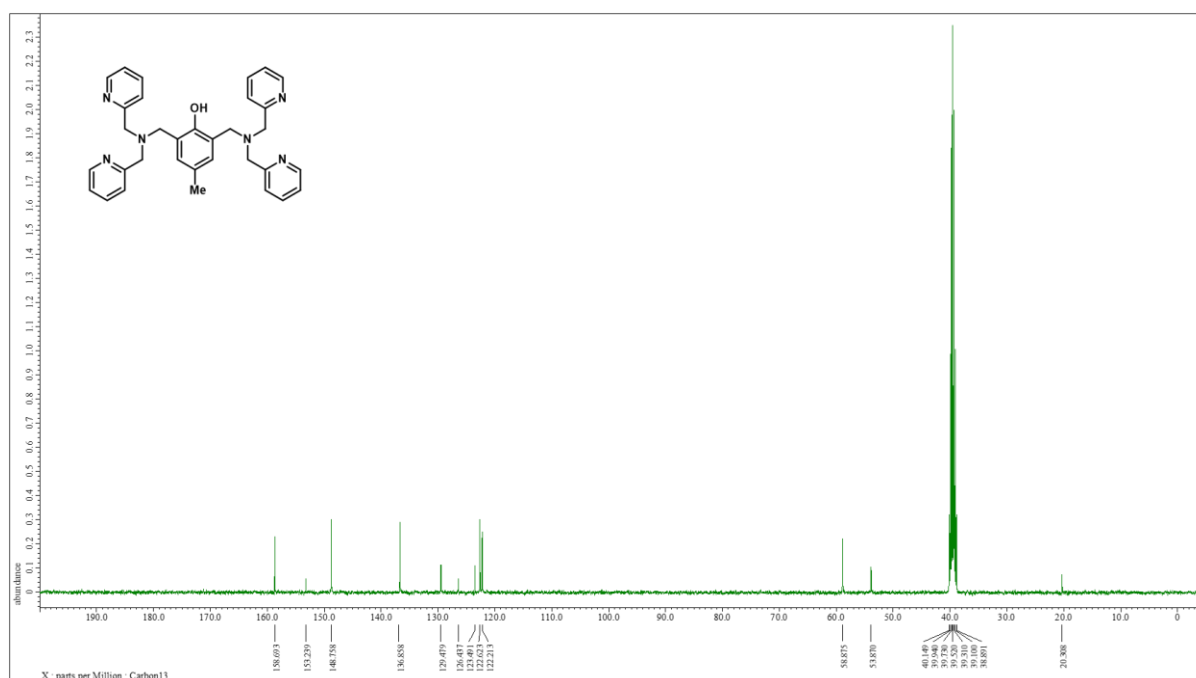**Fig. S18** ¹H NMR spectrum of H-bpmp in DMSO-*d*₆.

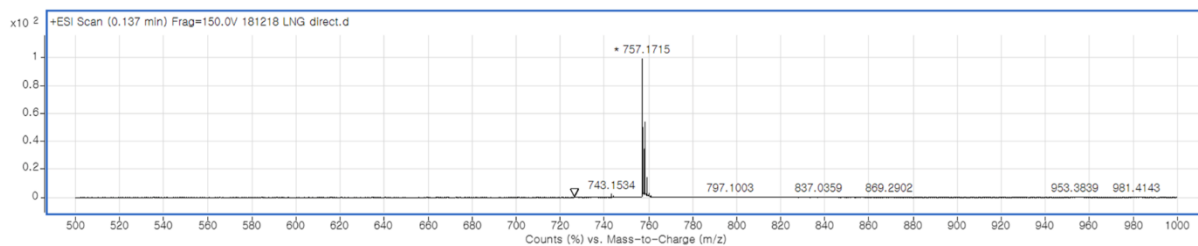**Fig. S19** ¹³C NMR spectrum of H-bpmp in DMSO-*d*₆.

Fig. S20 HRMS spectrum of $Mn_2(bpmp)(OAc)_2(ClO_4)$.
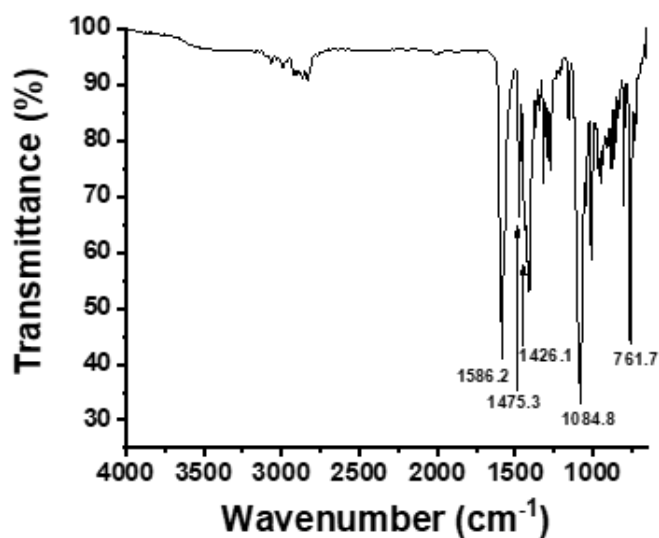
Fig. S21 FT-IR spectrum of $Mn_2(bpmp)(OAc)_2(ClO_4)$.
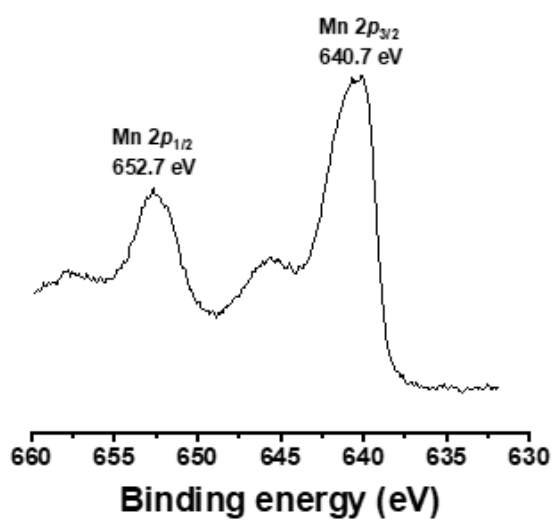
Fig. S22 XPS spectrum of Mn 2p of $Mn_2(bpmp)(OAc)_2(ClO_4)$.