Electronic Supplementary Information for:

Phenanthroimidazole-based zinc(II) complex as a fluorescent probe for pyrophosphate ion as generated in polymerase chain reactions and pyrosequencing

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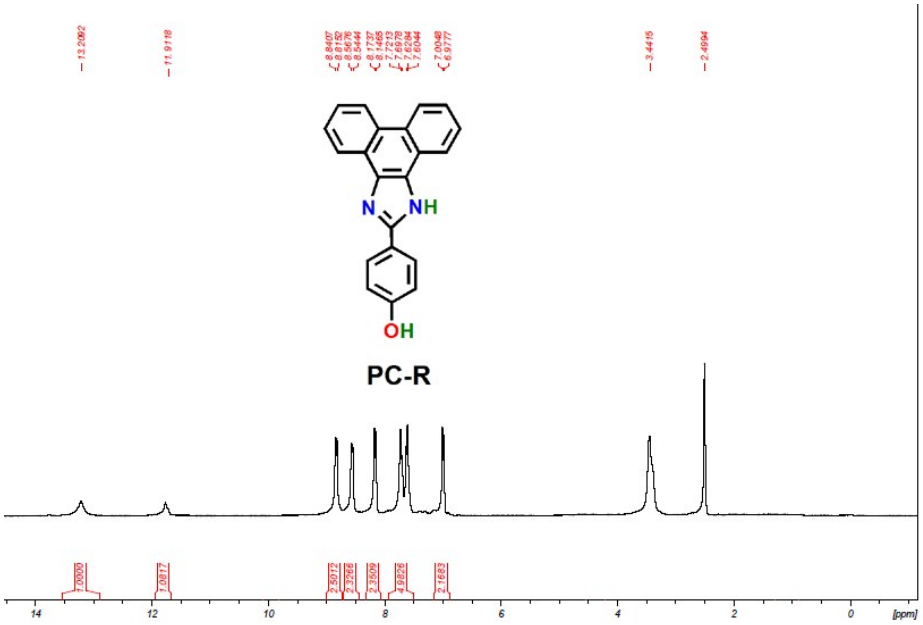

Fig. S1 ^{1}H NMR spectrum of **PC**–**R** recorded in DMSO–d^{6}.

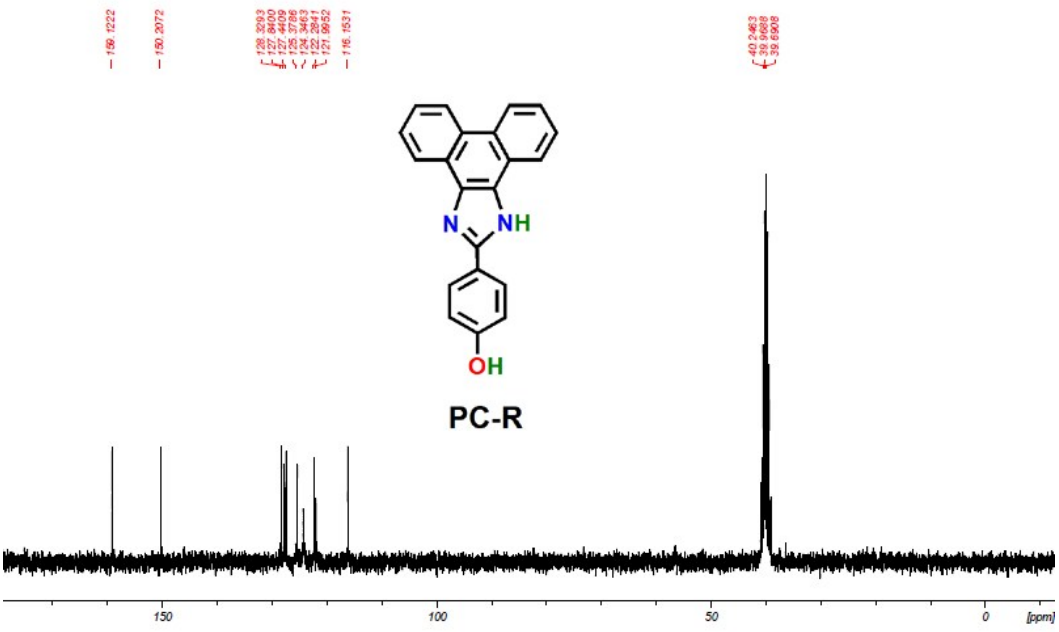

Fig. S2 ^{13}C NMR spectrum of **PC**–**R** recorded in DMSO–d^{6}.

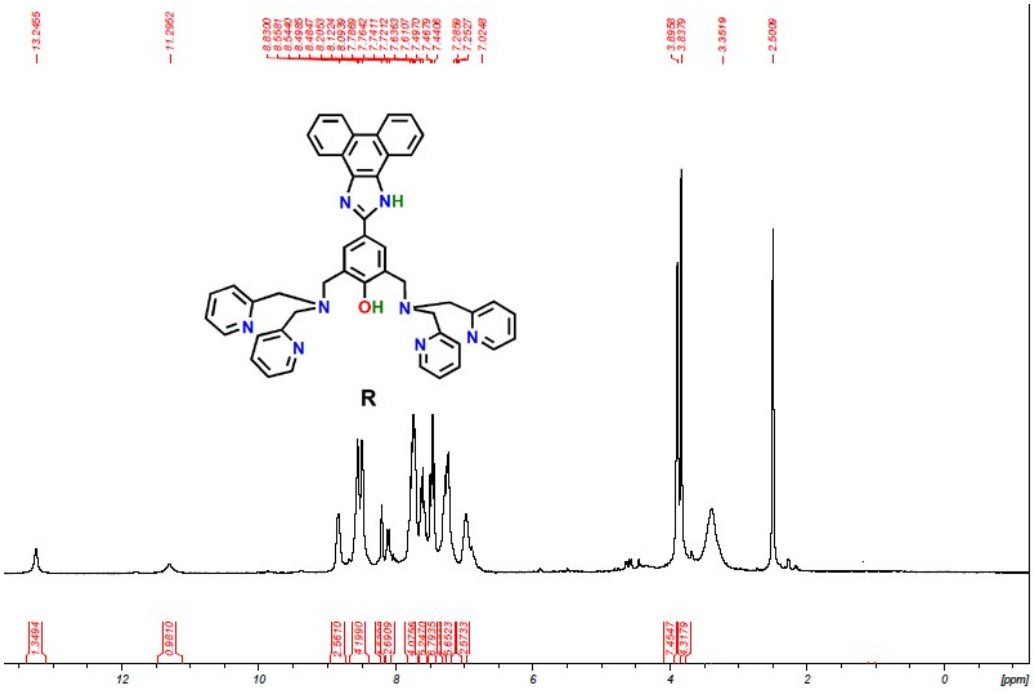

Fig. S3 ^{1}H NMR spectrum of **R** recorded in DMSO–d^{6}.

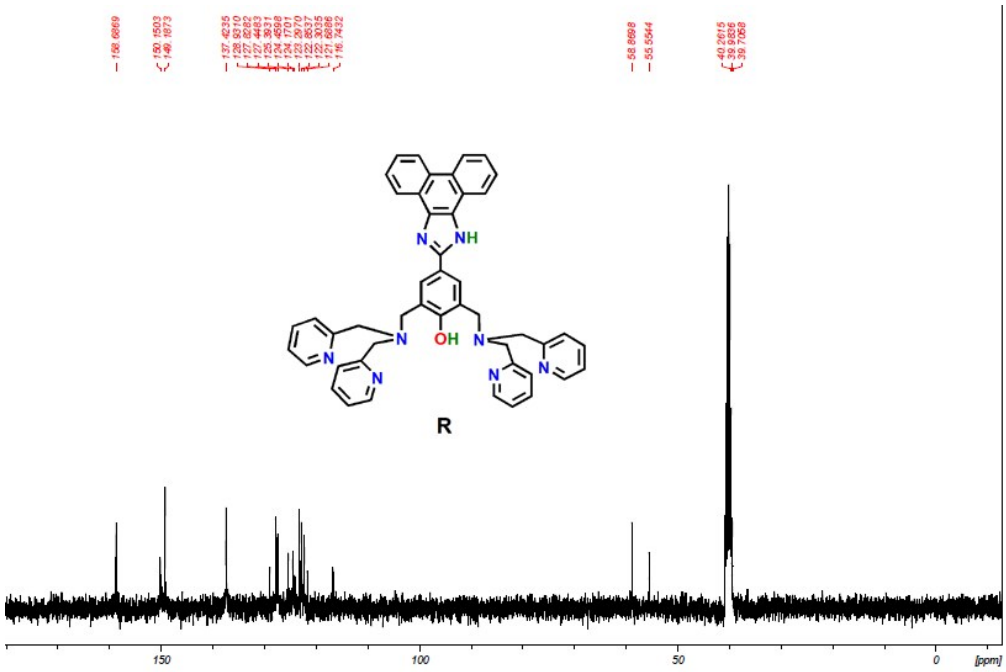

Fig. S4 ^{13}C NMR spectrum of **R** recorded in DMSO–d^{6}.

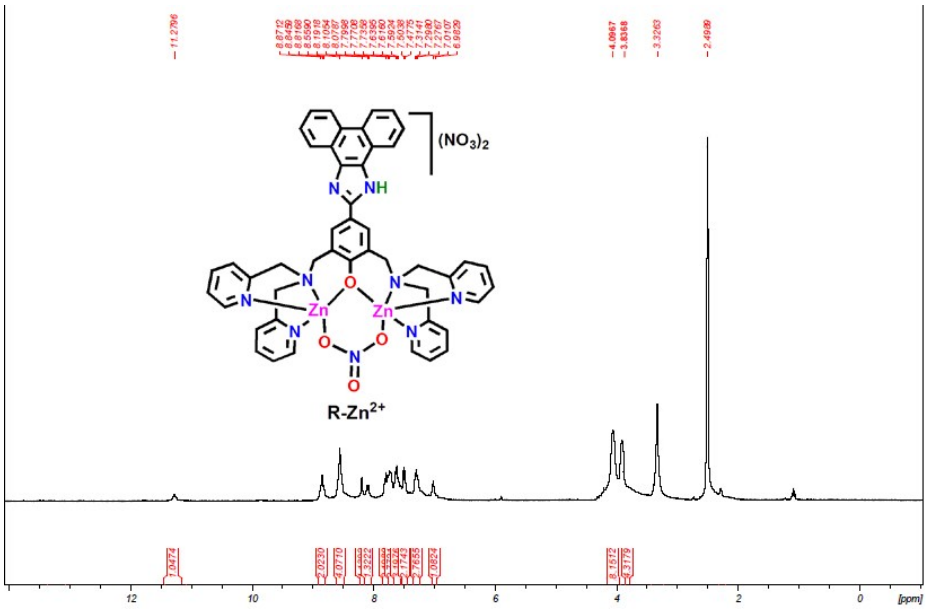

Fig. S5 ^{1}H NMR spectrum of **R-Zn^{2+}** recorded in DMSO–d^{6}.

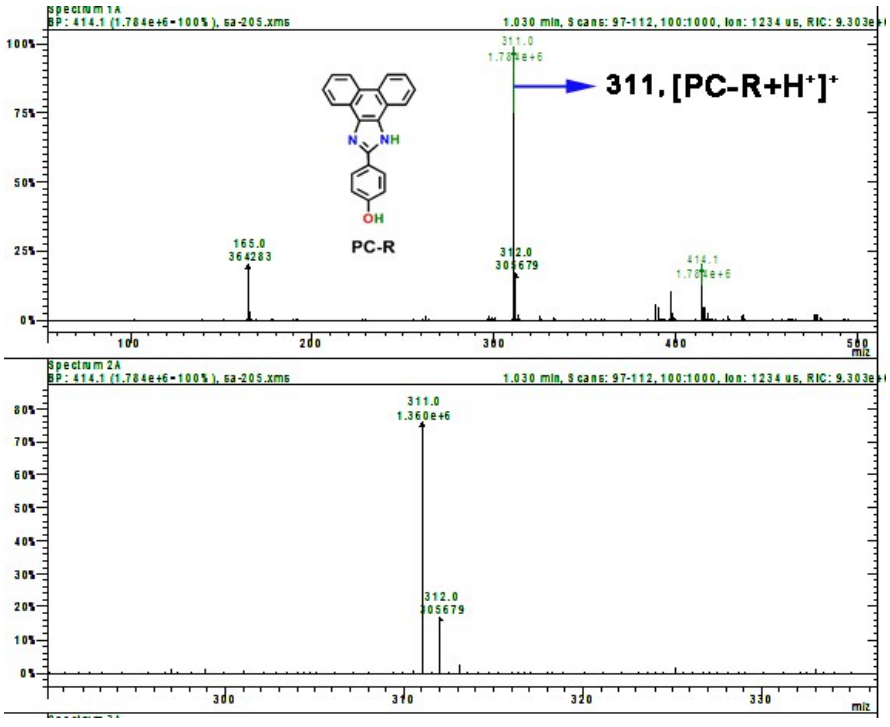

Fig. S6 ES-MS spectrum of PC–**R** recorded in CH_3CN.

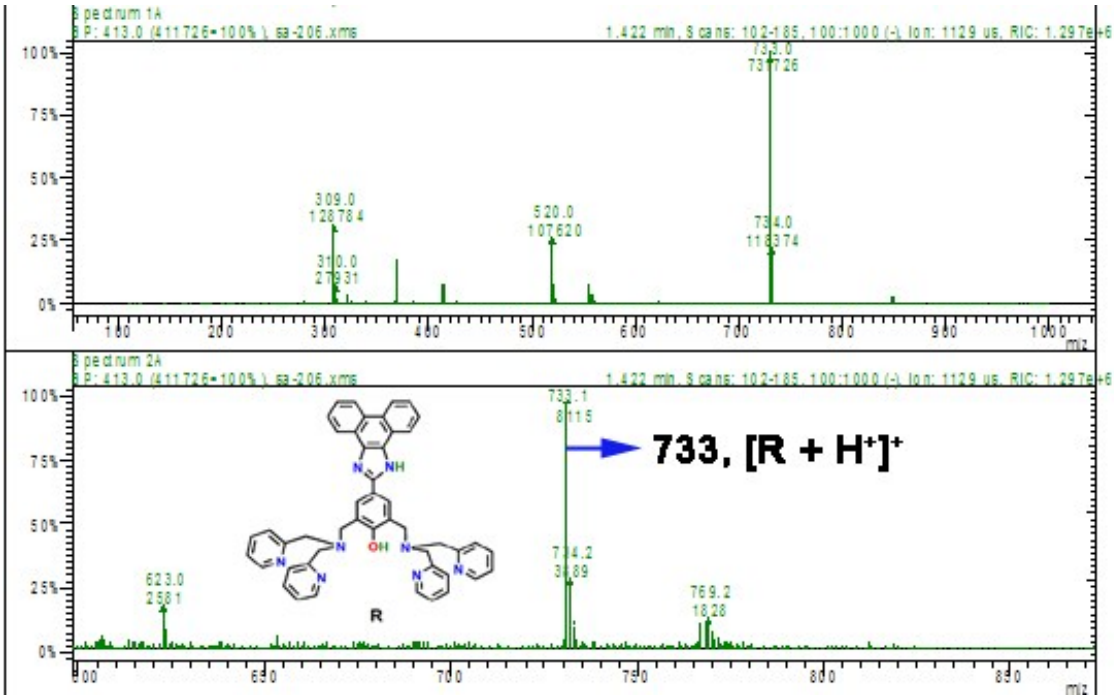

Fig. S7 ES-MS spectrum of **R** recorded in CH_3CN.

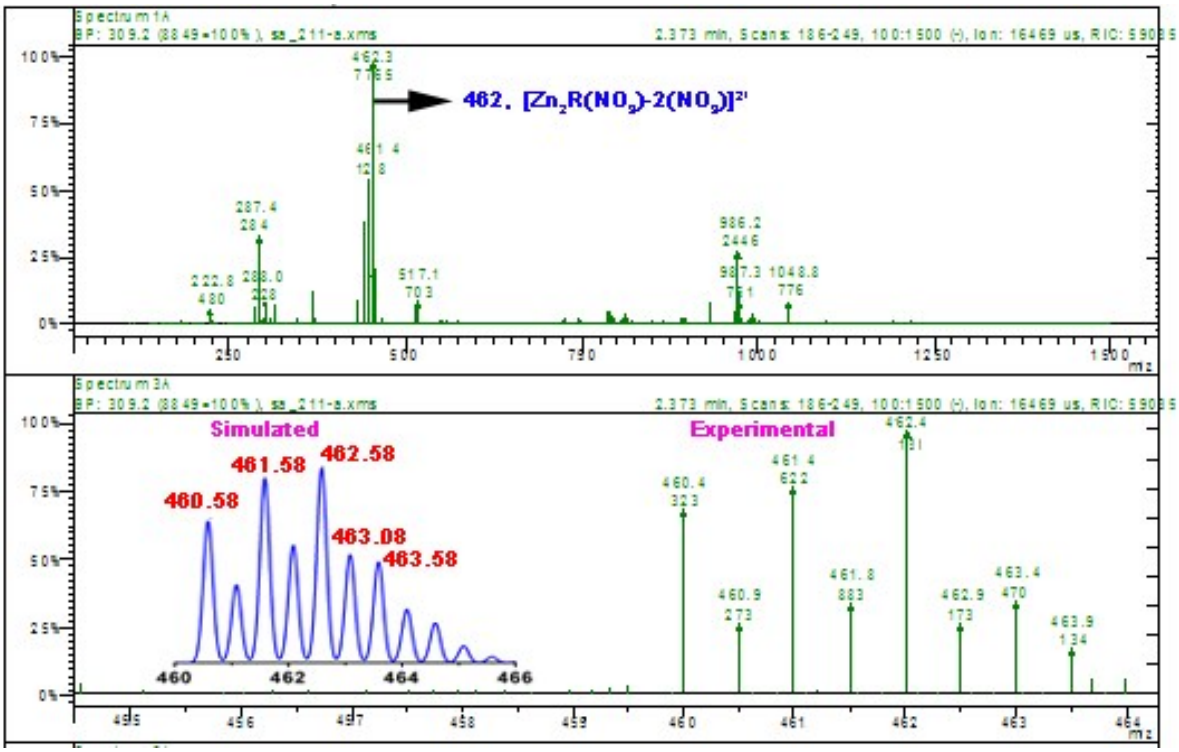

Fig. S8 ESI–MS spectrum of the probe **R–Zn^{2+}** recorded in CH_3CN.

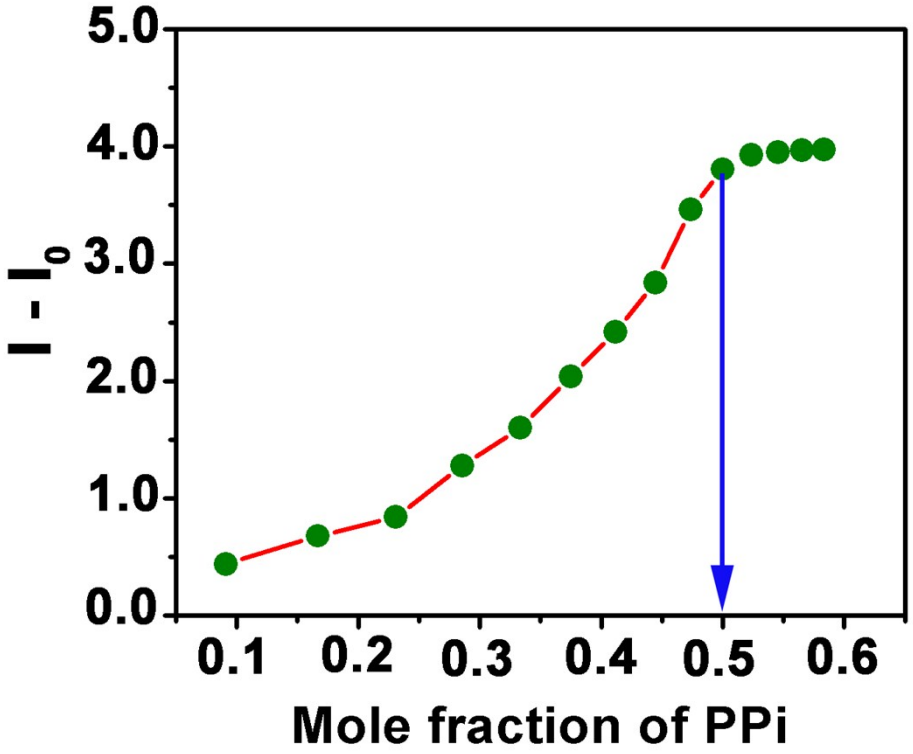

Fig. S9 Stoichiometry plot of ($F-F_0$) against mole fraction of PPi (F and F_0 are the fluorescent intensities of **R-Zn^{2+}** at a particular concentration of PPi and in the absence of PPi, respectively).

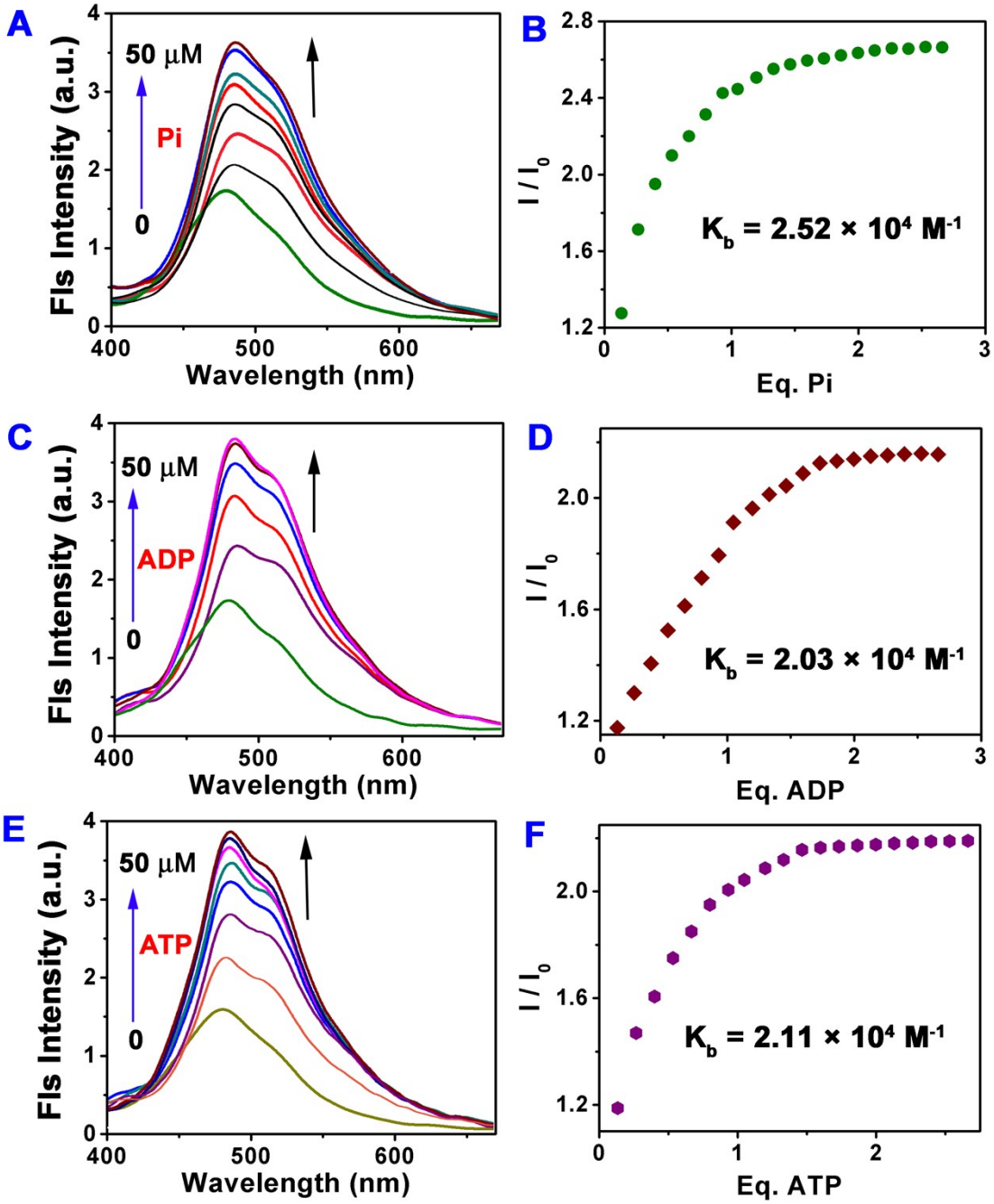

Fig. S10 Change in the initial fluorescence intensities of probe **R–Zn^{2+}** (5 μM) upon gradual increase in concentration of Pi (A), ADP (C) and ATP (E) (0−50 μM) in 0.02 M HEPES at pH = 7.8, and corresponding binding isotherms (B, D and F).

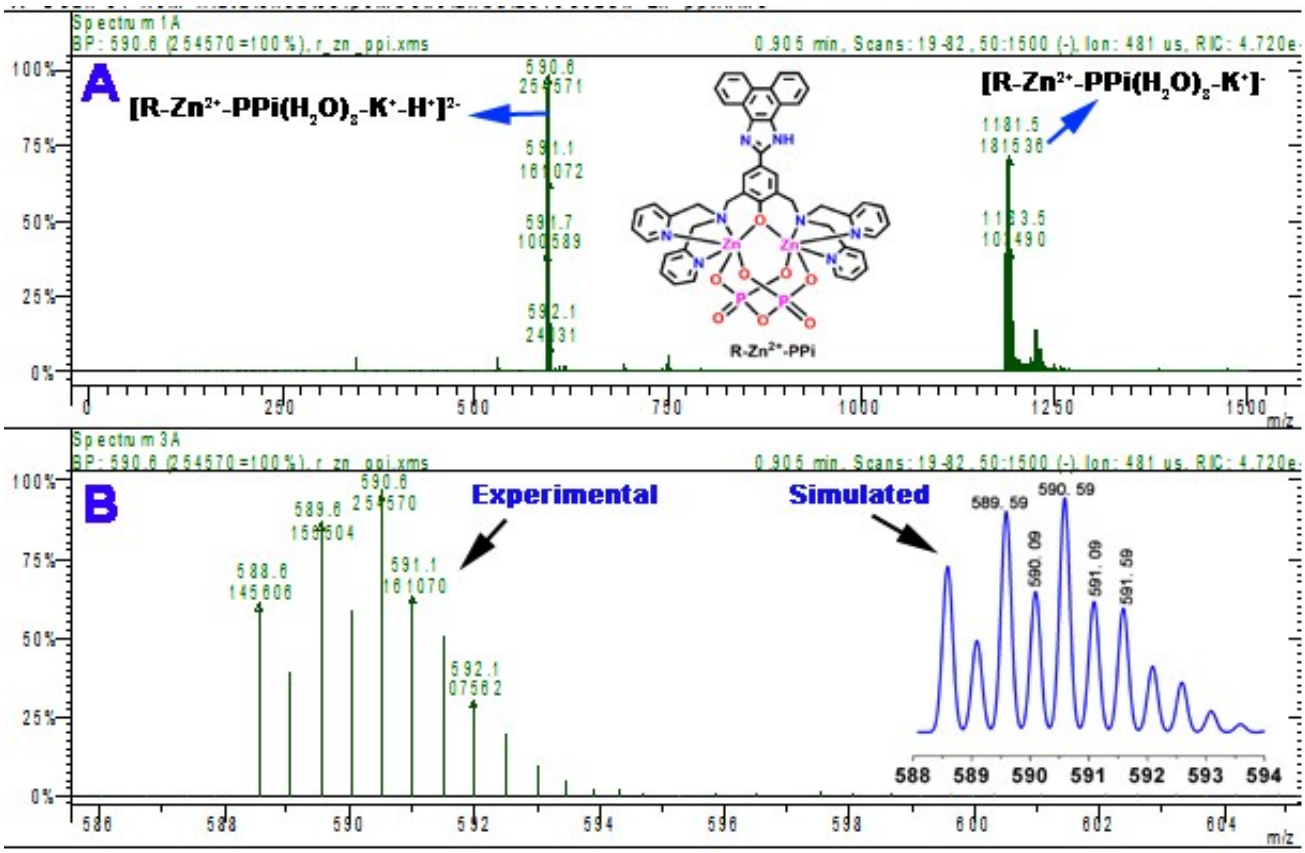

Fig. S11 (A) Mass spectrum of **R–Zn^{2+}-PPi** obtained in negative mode. Peaks at m/z = 590 and 1181, correspond to $[C_{47}H_{55}N_8O_{16}P_2Zn_2]^{2-}$ (= [**R–Zn^{2+}-PPi** $(H_2O)_8$ –K^+–H^+]$^{2-}$) and $[C_{47}H_{56}N_8O_{16}P_2Zn_2]^-$ (= [**R–Zn^{2+}-PPi** $(H_2O)_8$ – K^+]$^-$), respectively. (B) Measured and simulated isotopic distributions of peak at m/z = 590.

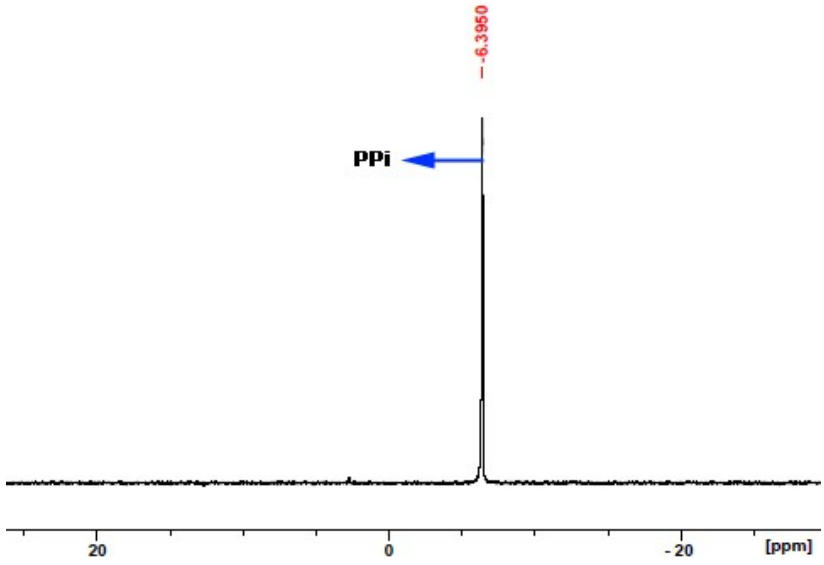

Fig. S12 ^{31}P NMR spectrum of inorganic pyrophosphate (PPi) (5 mM) in D_2O.

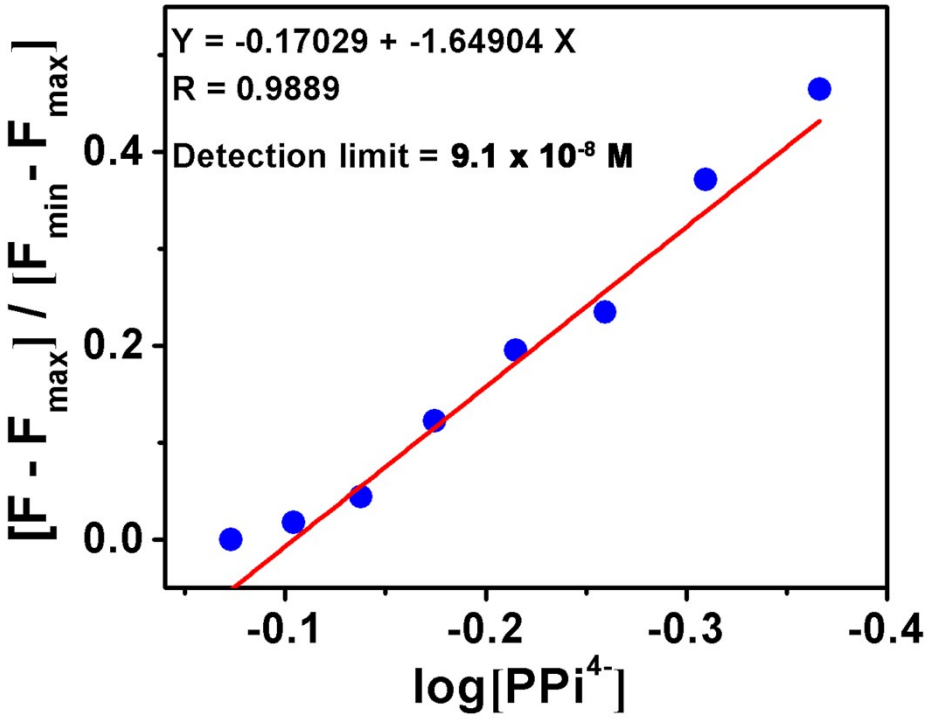

Fig. S13 Detection limit plot of $[(F-F_{max})/(F_{min}-F_{max})]$ against $\log[PPi^{4-}]$ (concentration in nM) (D), where F_{min}, F and F_{max} indicate the emission intensity in absence of, at intermediate and at infinite concentration of PPi, respectively.

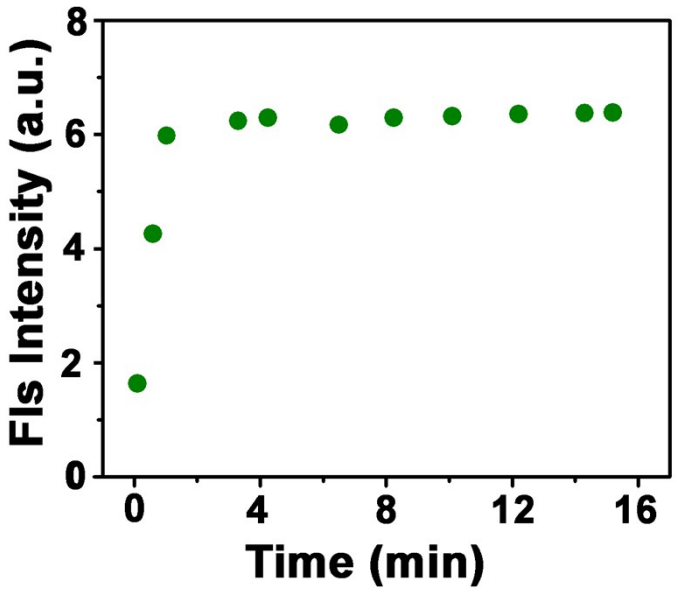

Fig. S14 Time dependence of the fluorescence intensity of probe **R–Zn**$^{2+}$ (5 μM) in the presence of PPi (5 μM) in 0.02 M HEPES at pH = 7.8.

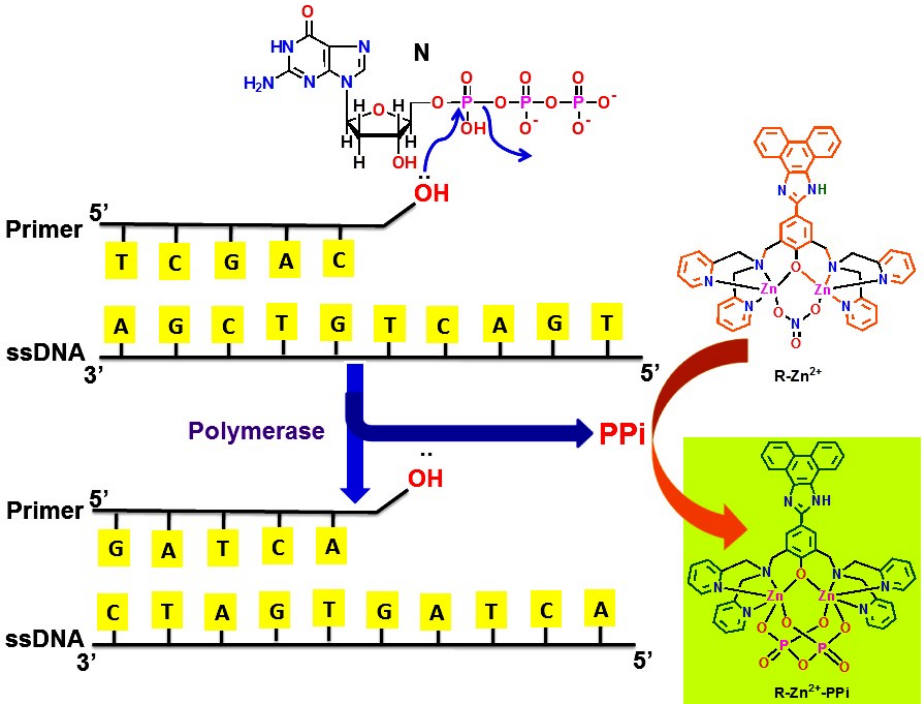

Fig. S15 Mechanism of sensing PPi released from PCR by **R–Zn**$^{2+}$.

Table S1. Selective probes for PPi detection

Probe	Analyte	Exptl. conditions; method	Comments	Ref
Eu(III)-tetracycline	PPi	pH = 7.4 (MOPS) - Fluorescense	LOD = 10^{-6} M	1
Pyrene-dpa-Zn(II)	PPi	pH = 7.4 (HEPES) - Fluorescense	LOD = 10^{-5} M	2
Spiropyran-Zn(II)	PPi	pH = 7.4 (HEPES) - Fluorescense	LOD = 10^{-7} M	3
Terpyridine-Zn(II)	PPi	pH = 7.4 (HEPES) - Fluorescense	LOD = 10^{-9} M	4
Quinoline-Tm(III)	PPi	Water - Fluorescense	LOD = 10^{-8} M	5
Phos-AuNPs	PPi	pH = 7.0 (HEPES) – naked eye	LOD = 10^{-9} M	6
Acedan-Zn(II)	PPi	pH = 7.4 (HEPES) - Fluorescense	LOD = 10^{-6} M	7
Naphthyl-dpa-Zn(II)	PPi	pH = 7.4 (HEPES) - Fluorescense	LOD = 10^{-9} M	8

dpa = 2,2'-dipycolylamine; LOD = Limits of Detection

Materials and methods

All the materials for synthesis of **R** were purchased from various commercial sources and used without further purification. Spectroscopic grade CH_3CN solvent was used for all titration experiments. ^{1}H, ^{13}C and ^{31}P NMR spectra were run on a Bruker 400 MHz spectrometer. The chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (Me_4Si) as internal standard (0.0 ppm) or (for ^{1}H NMR spectra) proton resonance resulting from incomplete deuteration of the NMR solvent: DMSO-d_6. Infra-red spectra were recorded on a Bruker ALPHA FT-IR spectrometer. Electronic absorption spectral measurements were done using a Perkin-Elmer LAMBDA 750 UV-visible spectrophotometer and the fluorescence emission studies were carried out on a HORIBA JOBIN YVON Fluoromax-4 spectrometer. ESI-MS spectra of the compounds were recorded on a VARIAN 500-MS spectrometer. Fluorescence quantum yield

was determined by using an optically matching solution of quinine sulfate (Φ_{fr} = 0.577 in 0.1M H_2SO_4) as standard at an excitation wavelength of 350 nm and it was calculated using the equation:

$$\Phi_{fs} = \Phi_{fr} \times \frac{1\text{-}10^{-ArLr}}{1\text{-}10^{-AsLs}} \times \frac{N_s^2}{N_r^2} \times \frac{D_s}{D_r}$$

Φ_{fs} and Φ_{fr} are the radiative quantum yields of sample and the reference, respectively, A_s and A_r are the absorbance of the sample and the reference, respectively, D_s and D_r the respective areas of emission. L_s and L_r are the lengths of the absorption cells of sample and reference, respectively. N_s and N_r are the refractive indices of the sample and reference solutions (pure solvents were assumed), respectively.

Synthesis of compound PC–R

4-hydroxybenzaldehyde (1.22 g, 10.0 mmol), 9,10-phenanthrenedione (2.30 g, 11.0 mmol) and ammonium acetate (15.4 g, 200 mmol) were stirred in acetic acid (50 mL), and the mixture was refluxed for 2 h with continuous stirring. On cooling to room temperature a colorless solid precipitated. It was collected by filtration and washed several times with water and then air-dried. The finally obtained dirty white solid was purified by silica gel column chromatography using 2:4 ratio of dichloromethane and hexane mixture as an eluent. Isolated yield = 2.7 g (76%). Anal. Calcd for $C_{21}H_{14}N_2O$: C, 81.27; H, 4.55; N, 9.03; Found: C, 81.54; H, 4.59; N, 9.14. IR (KBr, cm^{-1}): ν 3422s (-OH), 3287br (-NH), 1628s (-C=N). ^{1}H NMR (DMSO-d^6, 400 MHz) δ (ppm): 13.21 (s, H, phenolic-OH), 11.91 (s, H, NH), 8.83 (d, 2H, *J* = 10.2 Hz, H-phenanthrene), 8.55 (d, 2H, *J* = 9.28 Hz, H-phenanthrene), 8.16 (d, 2H, *J* = 10.88 Hz, ArH-phenol), 7.65 (dd, 4H, *J* = 9.5 Hz, H- phenanthrene), 6.99 (d, 2H, *J* = 10.84 Hz, ArH-phenol). ^{13}C NMR (DMSO-

d^6, 100 MHz) δ (ppm): 118.15, 121.99, 122.28, 124.34, 125.37, 127.44, 127.84, 128.33, 150.21 and 159.12. ESI-MS in CH_3CN: *m/z* calcd. for [**PC-R** + H^+]$^+$ 310.35, found = 311.01.

Synthesis of compound R

Paraformaldehyde (60 mg, 1.0 mmol) and di(2-picolyl)amine (399 mg, 2.00 mmol) were added to ethanol (30 mL) and acetic acid (4 mL) mixture at room temperature. This mixture was stirred for 24 h to obtain a clear solution, then the precursor compound **PC-R** (310 mg, 1.0 mmol) was added and the resulting mixture was refluxed. After 5 days brown oil was isolated by removal of solvent at reduced pressure. Water (100 mL) and dichloromethane (80 mL) were added to the residue, and the pH of the aqueous phase was neutralized with sodium bicarbonate. The aqueous phase was extracted with dichloromethane and dried over sodium sulfate. Evaporation of the solvent gave a dirty white solid. Chromatography on silica gel (acetone) afforded the receptor **R** as a white solid. Isolated yield = 300 mg (46%). Anal. Calcd. for $C_{47}H_{40}N_8O$: C, 77.03; H, 5.50; N, 15.29; Found: C, 77.11; H, 5.61; N, 15.36. IR (KBr, cm^{-1}): ν 3433s (-OH), 3281br (-NH), 2937s (-CH), 1221 (-CO), 1634s (-C=N). ^{1}H NMR (DMSO-d^6, 400 MHz) δ (ppm): 13.25 (s, H, phenolic-OH), 11.29 (s, H, NH), 8.69 (s, 2H, *J* = 8.64 Hz, H-phenanthrene), 8.53 (d, 4H, *J* = 5.64 Hz, H-pyridine), 8.49 (d, 2H, *J* = 5.52 Hz, H-phenanthrene), 8.10 (d, 4H, *J* = 11.4 Hz, H-pyridine), 7.75 (dd, 4H, *J* = 8.52 Hz, H- phenanthrene), 7.62 (d, 4H, *J* = 10.24 Hz, H-pyridine), 7.49 (d, 4H, *J* = 9.2 Hz, H-pyridine), 7.45 (d, 4H, *J* = 10.92 Hz, H-pyridine), 7.27 (d, 4H, *J* = 13.28 Hz, H-pyridine), 7.03 (s, 2H, H-phenyl), 4.09 (s, 8H, CH_2) and 3.84 (s, 4H, CH_2). ^{13}C NMR (DMSO-d^6, 100 MHz) δ (ppm): 55.55, 58.87, 116.74, 121.69, 122.30, 122.85, 123.30, 124.17, 124.46, 125.39, 127.45, 127.83, 128.93, 137.42, 149.19, 150.15 and 158.69. ESI-MS in CH_3CN: *m/z* calcd. for [**R** + H^+]$^+$ 732.87, found = 733.07.

Synthesis of the probe R–Zn^{2+}

To a vigorously stirred solution of **R** (100 mg, 0.136 mmol) in methanol (5 mL) was added dropwise $Zn(NO_3)_2.6H_2O$ (86 mg, 0.272 mmol) in methanol (10 mL) and the resulting mixture was refluxed. After 3 h, the resulting brown solution was filtered under hot conditions and kept for slow evaporation at room temperature. A yellow solid was isolated and washed with cold MeOH and dried in vacuum. Isolated yield (114 mg, 61%). Anal. Calcd for $C_{47}H_{39}N_{11}O_{10}Zn_2$: C, 53.83; H, 3.75; N, 14.69; Found: C, 53.97; H, 3.84; N, 14.75. IR (KBr, cm^{-1}): ν 3283br (-NH), 2922s (-CH), 1447vs (N-O), 1252s (N-O), 1221 (-CO), 1634s (-C=N). λ_{max}, nm (ε, M^{-1} cm^{-1}) in in 0.02 M HEPES buffer: 272 (32,000), 293 (54,000) and 374 (64,000). ^{1}H NMR (DMSO-d^6, 400 MHz) δ (ppm): 11.28 (s, H, NH), 8.86 (s, 2H, *J* = 10.12 Hz, H-phenanthrene), 8.80 (d, 4H, *J* = 8.48 Hz, H-pyridine), 8.51 (d, 2H, *J* = 10.8 Hz, H-phenanthrene), 8.11 (d, 4H, *J* = 10.7 Hz, H-pyridine), 7.77 (dd, 4H, *J* = 10.4 Hz, H-phenanthrene), 7.59 (d, 4H, *J* = 9.6 Hz, H-pyridine), 7.62 (d, 4H, *J* = 9.4 Hz, H-pyridine), 7.49 (d, 4H, *J* = 10.5 Hz, H-pyridine), 7.29 (d, 4H, *J* = 6.4 Hz, H-pyridine), 6.98 (s, 2H, H-phenyl), 3.90 (s, 8H, CH_2) and 3.83 (s, 4H, CH_2). ESI-MS in CH_3CN: *m/z* calcd. for $[Zn_2R(NO_3)\text{-}2(NO_3)]^{2+}$ 462.58, found = 462.38.

Procedures of anion sensing

Stock solutions (1×10^{-3} M) of the potassium salts of F^-, Cl^-, Br^-, I^-, CH_3COO^-, HCO_3^-, CO_3^{2-}, ClO_4^-, SO_4^{2-}, PO_4^{3-}, AMP, ADP and ATP in 0.02 M HEPES (pH 7.8) were prepared. Stock solutions of the probe **R–Zn^{2+}** (1.0×10^{-3} M) were also prepared in 0.02 M HEPES (pH 7.8). A 3 mL stock solution of **R–Zn^{2+}** was placed in a quartz cell of 1 cm width and the tested anion solution was added in an incremental fashion. Their corresponding fluorescence spectra were recorded at 298 K. Each titration was repeated at least two times to get a consistent value. For all measurements, λ_{ex} = 365 nm and the emission wavelength was monitored from 400–660 nm.

There was no considerable change in shape of the emission spectra except a significant enhancement of the initial fluorescence intensity of **R–Zn^{2+}** upon the gradual addition of anions solution. The binding constant K_b was calculated by fitting the change of absorbance and fluorescence intensity from the non-linear regression analysis of the following equations (1) and (2), respectively:

$$A = [A_{ini} + A_{fin} K_b [A^-]/[1 + K_b [A^-]] \text{---------- (1)}$$

$$I/I_0 = ([A^-]_0 + [R]_0 + 1/K_b - (([A^-]_0 + [R]_0 + 1/K_b)^2 - 4[R]_0 [A^-]_0)^{1/2}) \Delta I_{max} / 2[R]_0 \text{ ----(2)}$$

(where A= Absorbance, R = **R–Zn^{2+}** and A^- = Anion)

Using sensor R–Zn^{2+} to detect PPi released from PCR

Total RNA was extracted from 2–week–old fungal mycelium of *Pestalotiopsis microspora* (Taxol (Anticancer drugs) producing strain) using the trizol (Chomczynski and Sacchi 1987) methods.[9] Isolated RNA was used as a template for first strand cDNA synthesis in a 20 µl reaction with M–MuLVRT Reverse Transcriptase (RT) and oligo $(dT)_{18}$ primer (5'–TTTTTTTTTTTTTTTT– 3') as the primer according to the manufacture's protocol. The cDNA (complementary DNA) was amplified by PCR using the fungal gene specific primers of geranylgeranyl pyrophosphate synthase (GGPPS) Forward and Reverse primer, designed and screened. The PCR reaction, including the following components to a PCR microcentrifuge tube, was prepared on ice: 10X PCR buffer, plus Mg^{2+} (10 mM Tris–HCl, pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$) to final concentration of 1X, 10 mM dNTP mixture to final concentration of 0.2 mM each, primer mix to final concentration of 0.2 µM each, template cDNA to final concentration of 100 ng and Taq DNA polymerase to final concentration of 2.5 unites. The PCR program started at 96 °C for 3 min, then 96 °C for 30 s denaturation, 56 °C for 50 s annealing, 72 °C for 2 min extension and a final extension at 72 °C for another 10 min. The cycles were increased from 29

to 32. After PCR, the reaction mixture was allowed to equilibrate to room temperature. 10 μL PCR products of different cycles were added to 400 μL, 0.4 – 0.6 mM of probe **R–Zn^{2+}** with 0.02 M HEPES buffer (pH 7.4). The fluorescence change was measured at 500 nm by spectroscopy and the gel electrophoresis of PCR products was carried out on 1% agrose gel to compare the existence of amplified DNA bands and corresponding fluorescence change.

References

1. M. Schferling and O. S. Wolfbeis, *Chem. Eur. J*., 2007, **13**, 4342.

2. H. K. Cho, D. H. Lee and J.-I. Hong, *Chem. Commun*., 2005, 1690.

3. N. Shao, H. Wang, X. D. Gao, R. H. Yang and W. H. Chan, *Anal. Chem.*, 2010, **82***,* 4628.

4. S. Bhowmik, B. N. Ghosh, V. Marjomäki and K. Rissa, *J. Am. Chem. Soc*., 2014, **136**, 5543.

5. M. R. Ganjali, M. Hosseini, F. Aboufazeli, F. Faridbod, H. Goldooz and A. R. Badiei, *Luminescence*, 2012, **27**, 20.

6. A. S. Rao, S. Singha, W. Choib and K. H. Ahn, *Org. Biomol. Chem*., 2012, **10**, 8410.

7. S. Kim, M. S. Eom, S. K. Kim, S. H. Seo and M. S. Han, *Chem. Commun*., 2013, **49**, 152.

8. D. H. Lee, S. Y. Kim and J.–I. Hong, *Angew. Chem., Int. Ed.*, 2004, **43**, 4777.

9. P. Chomczynski and N. Sacchi, *Anal. Biochem.*, 1987, **162**, 156.