Electronic Supplementary Information for:

A Colorimetric Sensor Array for the Classification of Biologically Relevant Tri-, Di- and Mono-Phosphates

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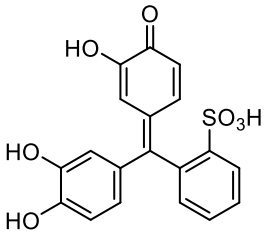
General Experimental

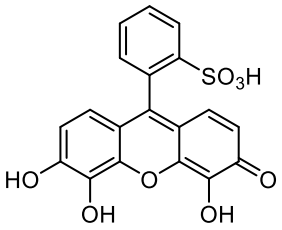
All chemicals and solvents were of reagent grade (≥ 95%) and used as received unless otherwise noted. Solid-phase peptide synthesis was performed using Torviq polypropylene syringes (10 mL) equipped with a porous polypropylene disc at the bottom. Melting points were manually observed using a Stanford Research Systems Optimelt melting point apparatus. Optical rotations were obtained using a Perkin Elmer Model 341 polarimeter at 589 nm and 20 °C, using the indicated spectroscopic grade solvents. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker Advance III 500 and a Bruker Avance DPX 400, and are reported as parts per million (ppm), referenced to residual undeuterated solvent. The data are reported as chemical shift (d), multiplicity (br = broad, s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (J Hz) and relative integral. UV-vis data were recorded on a Varian Cary 4000 UV-vis spectrophotometer at 25 °C. Temperature control was provided by a Varian Cary PCB 150 Water Peltier System. Low resolution mass spectra were recorded on a Bruker amaZon SL mass spectrometer (ESI) operating in positive mode as indicated. High resolution ESI spectra were recorded on a Bruker BioApex Fourier Transform Ion Cyclotron. pH values were obtained using an Activon Model 209 pH/mV meter. Reversed-phase column chromatography was performed on a Biotage Isolera Prime automated flash purification system equipped with a dual wavelength UV-Vis detector. The detection wavelengths were 254 and 280 nm, respectively.

Experimental Methods and Characterisation Data

Indicators



PV



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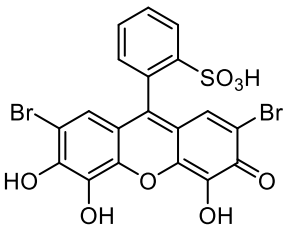

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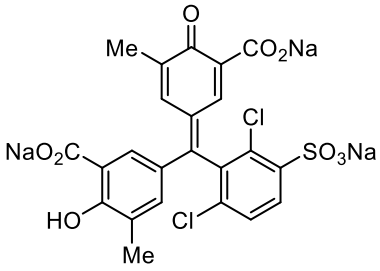

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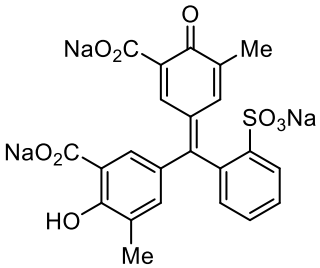

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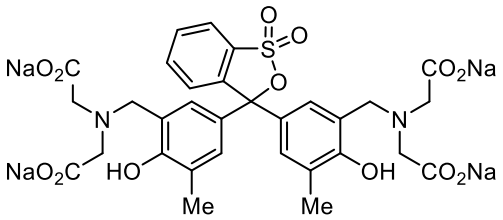
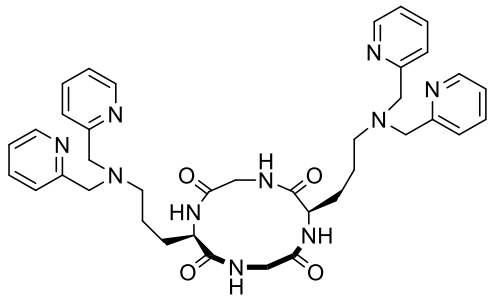


XO

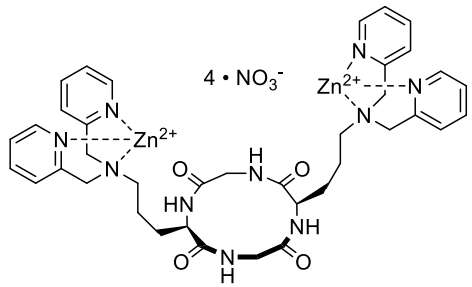
Cyclic DPA appended peptide 5



5

The peptide backbone was synthesised on 2-chlorotrityl chloride resin (0.25 g, 0.2 mmol/g) using standard Fmoc-SPPS procedures as described previously.[1] Following cleavage off the resin, the crude peptide (28 mg, 0.038 mmol) was dissolved in a solution of DMTMM.BF_4 (17.0 mg, 0.050 mmol) and DIPEA (20 µL, 0.12 mmol) in DMF (0.8 mL) and stirred for 72 h. The reaction mixture was subjected to reversed-phase column chromatography using a gradient of 0 to 100% MeCN in H_2O (0.1% aq. 25% NH_4OH) to afford **2** as a colourless fluffy solid upon lyophilisation (7 mg, 18%). m.p. 32-33°C; $[\alpha]_D^{20}$=+6*.*7 (*c* 0.20 MeOH); ^{1}H NMR (400 MHz, MeOD) δ 8.49-8.38 (m, 4H), 7.83-7.72 (m, 4H), 7.66-7.54 (m, 4H), 7.34-7.20 (m, 4H), 4.33-4.23 (m, 2H), 4.07-3.81 (m, 4H), 3.81 3.67 (s, 8H), 2.62-2.44 (m, 4H), 1.86-1.45 (m, 4H), 1.37-1.28 (m, 4H); ^{13}C NMR (125 MHz, MeOD) δ 175.4 , 173.3, 160.7, 149.4, 138.7, 124.9, 123.8, 61.1, 55.1, 54.9, 45.0, 28.0, 24.3; IR (ATR) ν_{max}= 3358, 2972, 1681, 1109, 836 cm^{-1}; HRMS (+ESI) calc. for $C_{38}H_{47}N_{10}O_4$: 707.3776 $[M+H]^+$, found: 707.3764.

Cyclic Receptor 1



1

A solution of the DPA-functionalised peptide **5** in MeOH (2 mg, 2.83 µmol, 10 mg mL^{-1}) was added to an aqueous solution of $Zn(NO_3)_2{\cdot}4H_2O$ (2 equiv. relative to peptide, 10 mg mL^{-1}) and the mixture was stirred at rt for 2 h. Lyophilisation afforded **1** as a colourless fluffy solid (3 mg, quant.); HRMS (+ESI) calc. for $C_{38}H_{44}N_{10}O_4Zn_2$: 416.1060 $[M\text{-}4NO_3\text{-}2H]^{2+}$, found: 416.1060.

NMR and Mass Spectra

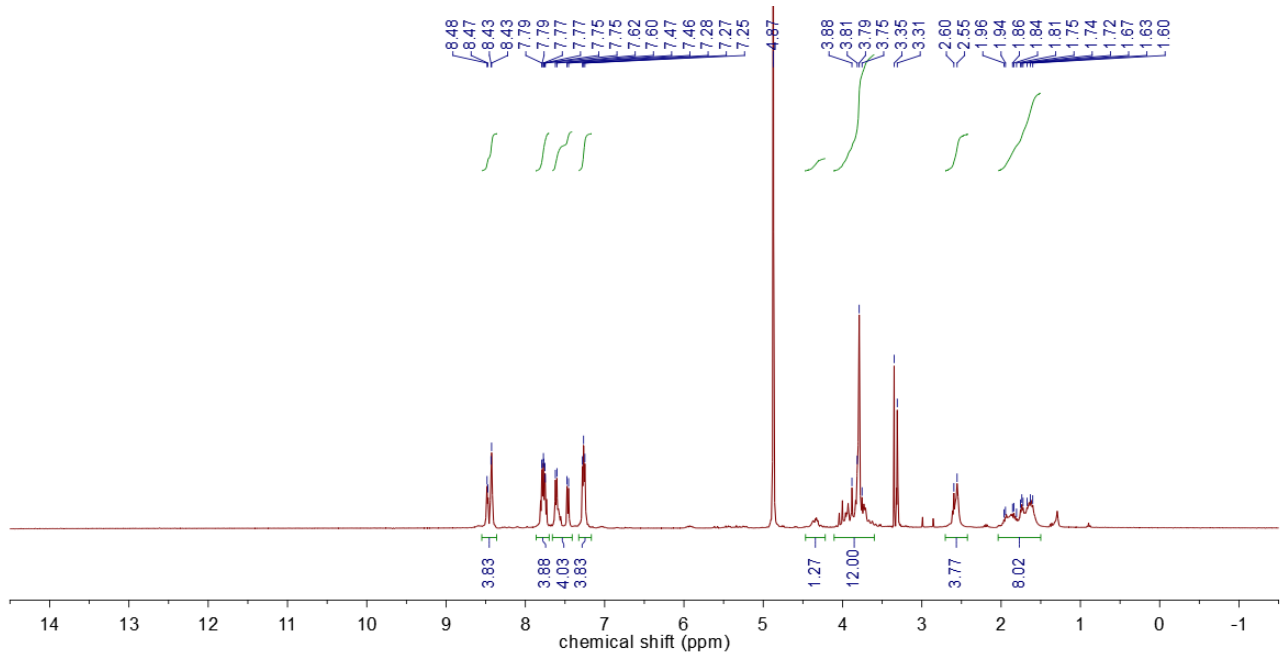


Figure S1: ^{1}H NMR (400 MHz, MeOD) of **5**

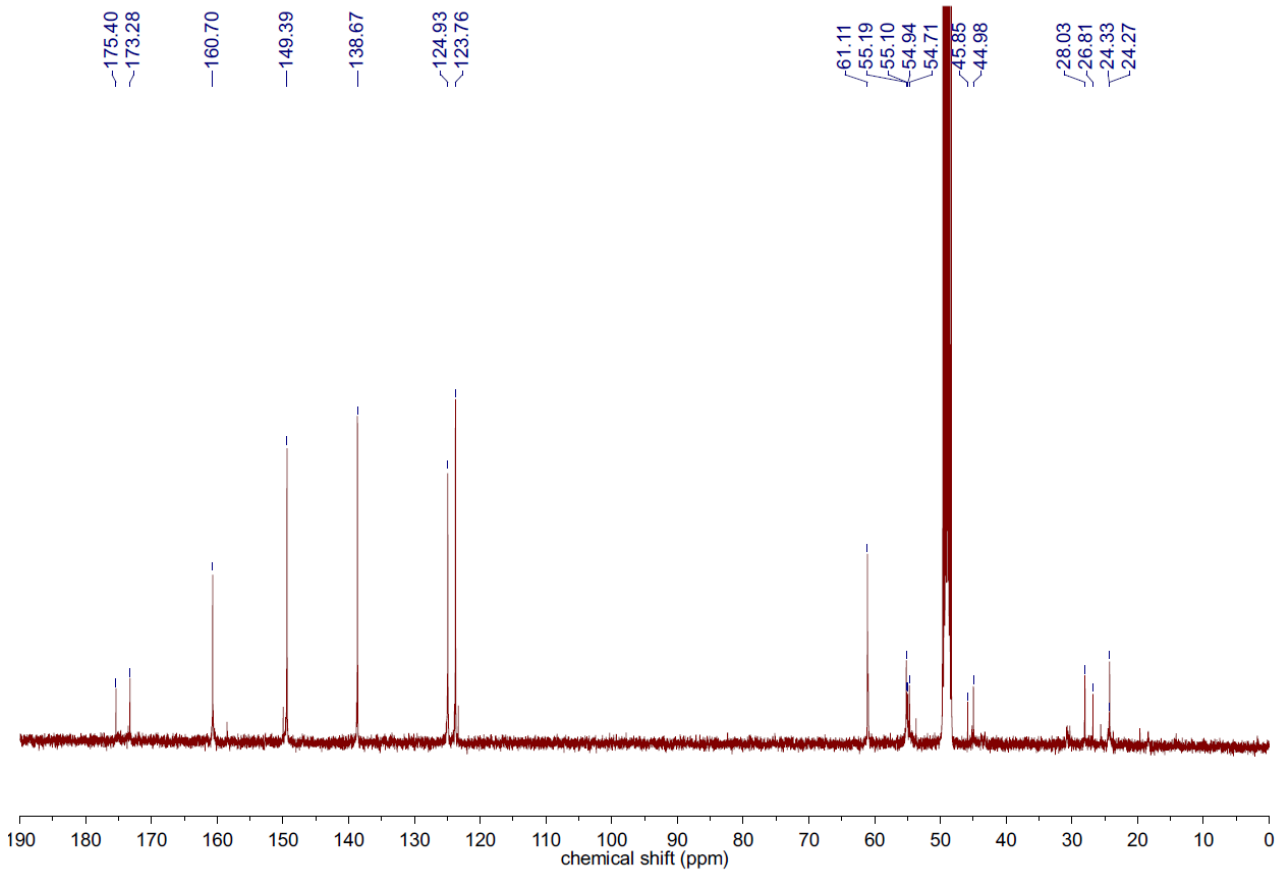


Figure S2: ^{13}C NMR (125 MHz, MeOD) of **5**

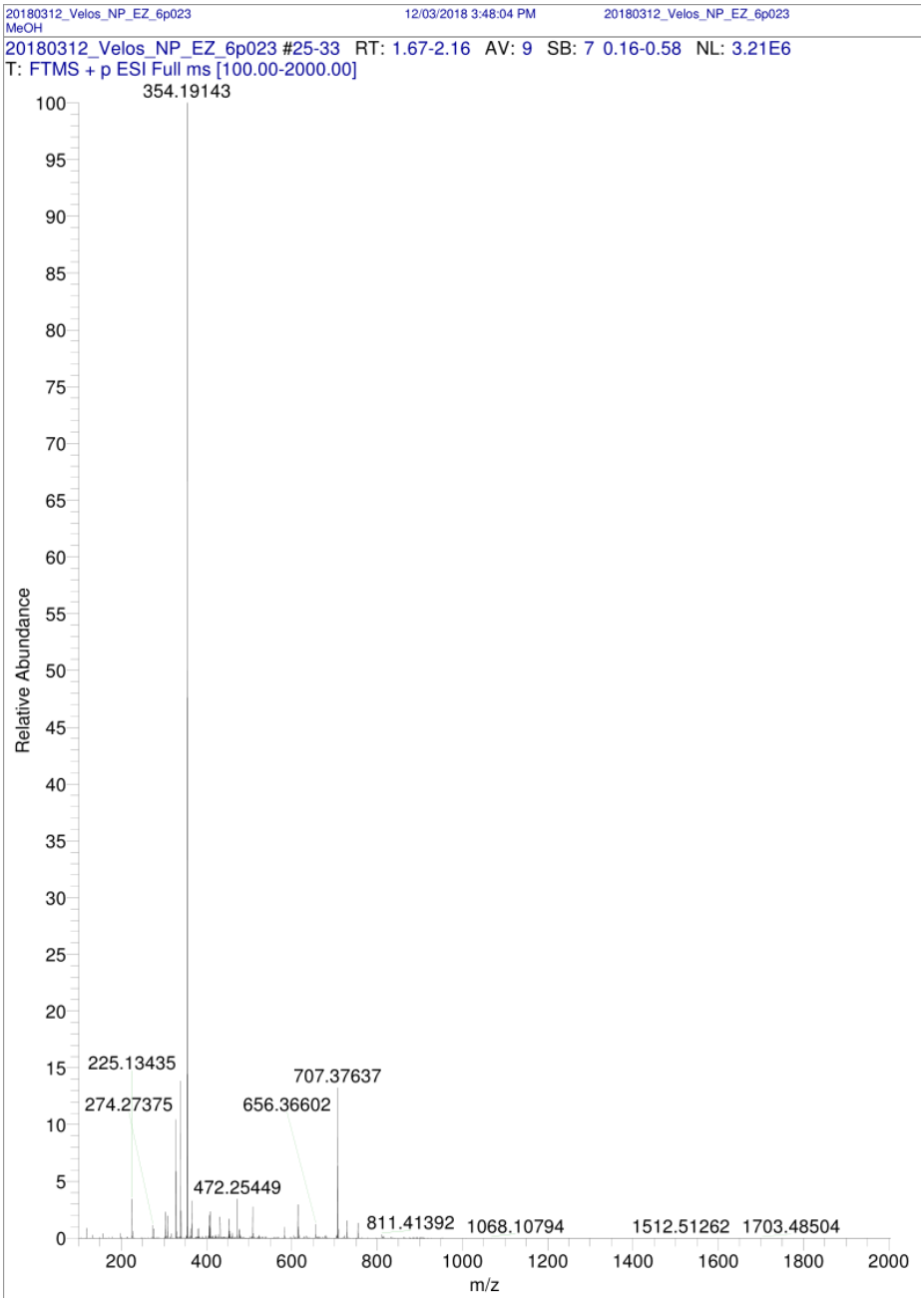


Figure S3: HRMS (+ESI) calc. for $C_{38}H_{47}N_{10}O_4$: 707.3776 $[M+H]^+$, found: 707.3764. for **5**

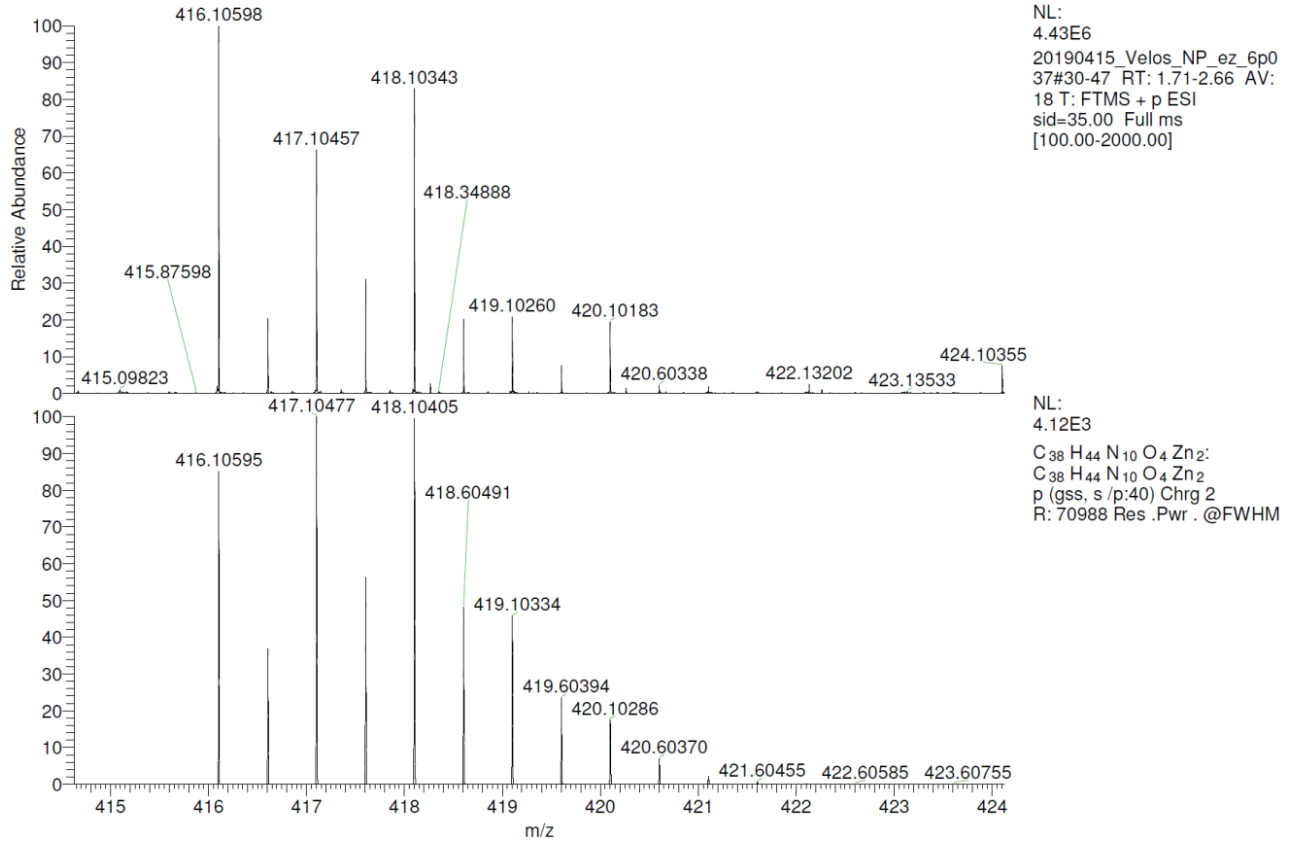


Figure S4: HRMS (+ESI) of complex **1**. Isotope pattern of found (top) and predicted for $C_{38}H_{44}N_{10}O_4Zn_2$ (bottom).

Titrations of pyrocatechol violet PV with receptor 1

To a 1 cm quartz glass cuvette was added a solution of the indicator **PV** (2.5 mL, 20 µM) and to another matched quartz glass cuvette was added the buffer solution as the blank (2.5 mL). The absorbance spectrum was recorded from 250-750 nm. Aliquots of a solution containing **1** (1000 µM) were then added to both the sample and the blank cuvettes. After each addition, the resulting solution was stirred for at least 30 seconds before the absorbance spectrum was recorded. Typically, up to 10 equivalents of the receptor were added to the solution. To determine association constants for the receptor-indicator complexes, global analysis of the absorbance data over the range of 300-720 nm was carried out using a nonlinear least-squares curve fitting procedure, based on the equilibria described for 1 : 1 binding, using the commercially available software program HypSpec (Hyperquad package).[2]

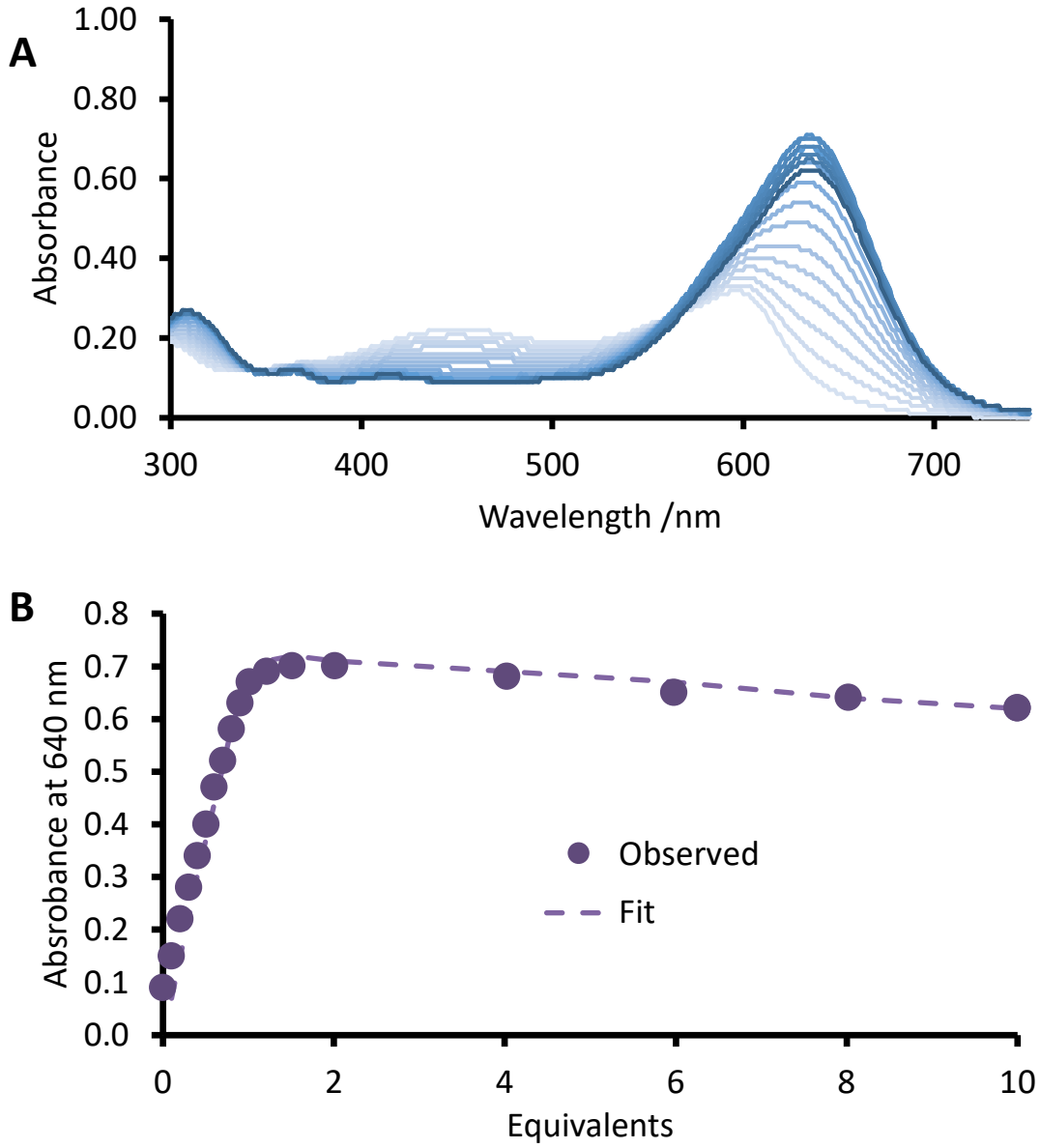


Figure S5: A) Representative UV-Vis spectra from the titration of **1** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of pyrocatechol violet (PV) (light to dark blue) **B)** Fitting of the data to a 1:1 binding model

Binding studies: 1.Indicator with Analytes

Stock solutions of the respective 1:1 – receptor **1**:**PV** ensemble (20 µM both) and of the tested phosphoanions as sodium salts (2000 µM) were prepared. To a 1 cm quartz glass cuvette was added a solution of the receptor-indicator ensemble (2.5 mL) and to another matched quartz glass cuvette was added a solution of the same concentration of the receptor as the blank (2.5 mL). The absorbance spectrum was recorded from 250-750 nm. Aliquots of the respective anion solution were then added to both the sample and the blank cuvettes. After each addition, the resulting solution was stirred for at least 30 seconds before the absorbance spectrum was recorded. Typically, up to 10 equivalents of the anion were added to the solution. All titrations were repeated three times. To determine the association constants for the receptor-anion complexes, global analysis of the absorbance data over the range of 300-720 nm was carried out using a nonlinear least-squares curve fitting procedure, based on the equilibria described for indicator displacement,[3] using the commercially available software program HypSpec (Hyperquad package).[2]

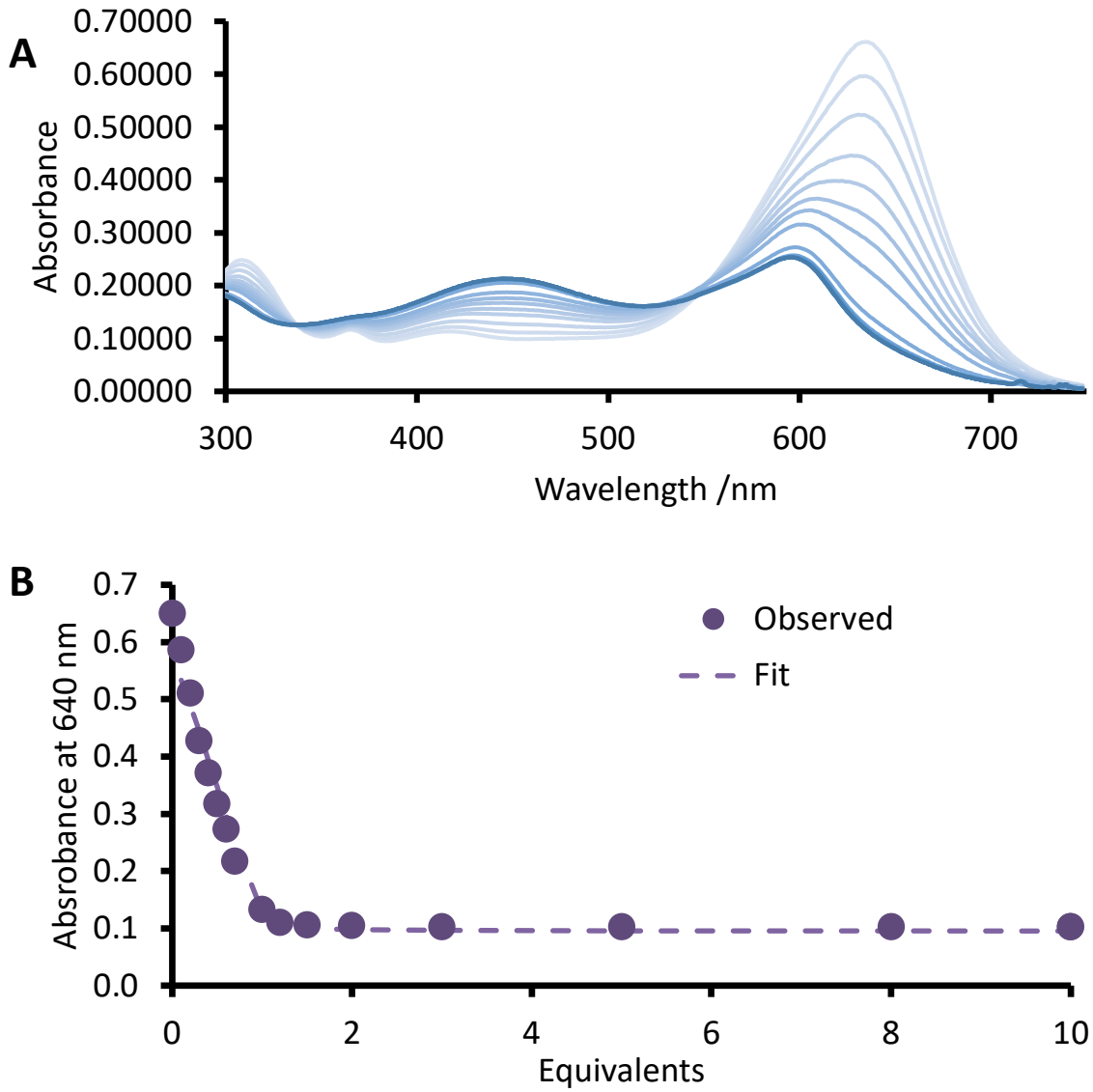


Figure S6: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of PPi (light to dark blue) **B)** 1:1 fitting curve at 640 nm.

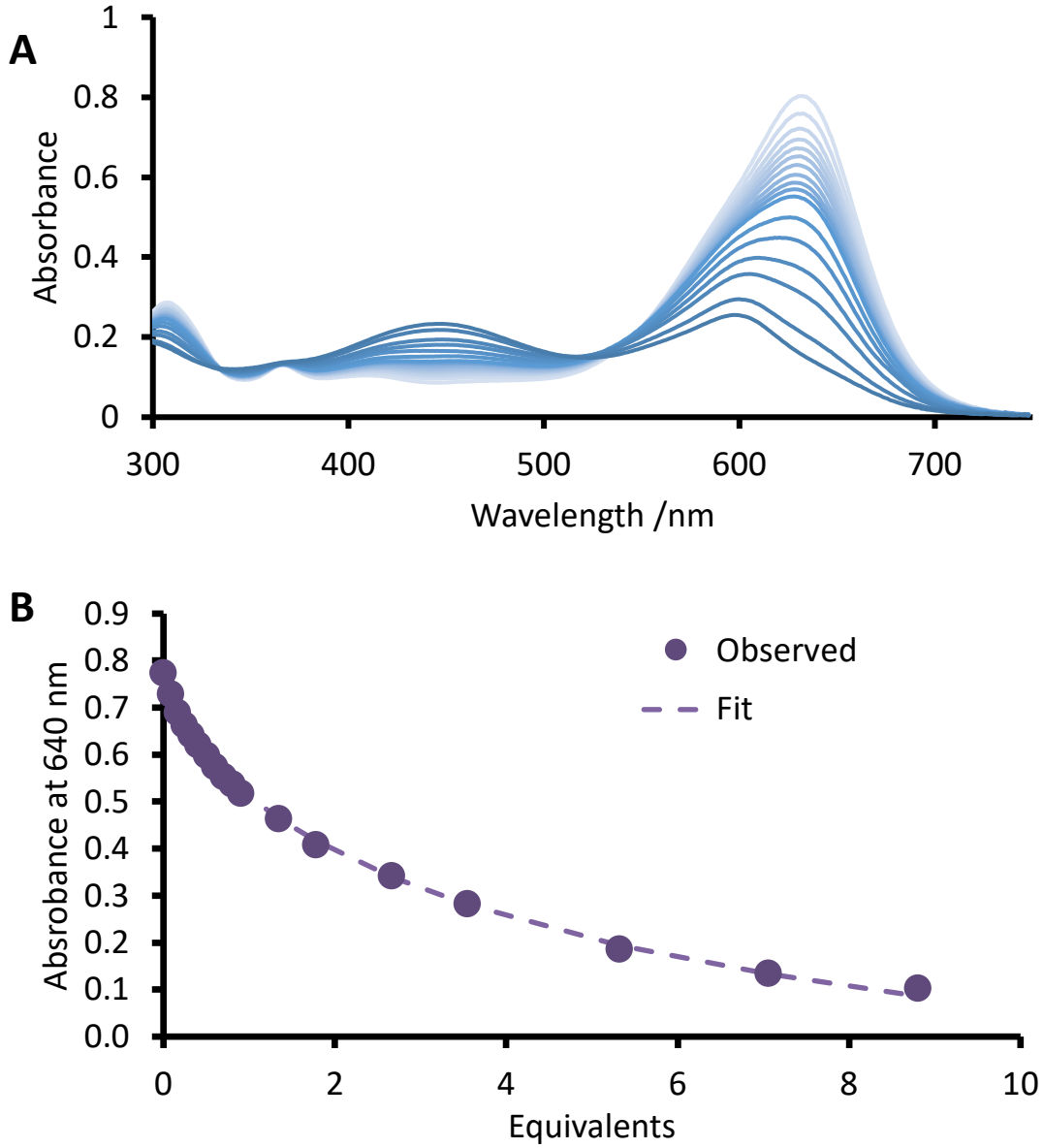


Figure S7: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of ADP (light to dark blue) **B)** 1:1 fitting curve at 640 nm.

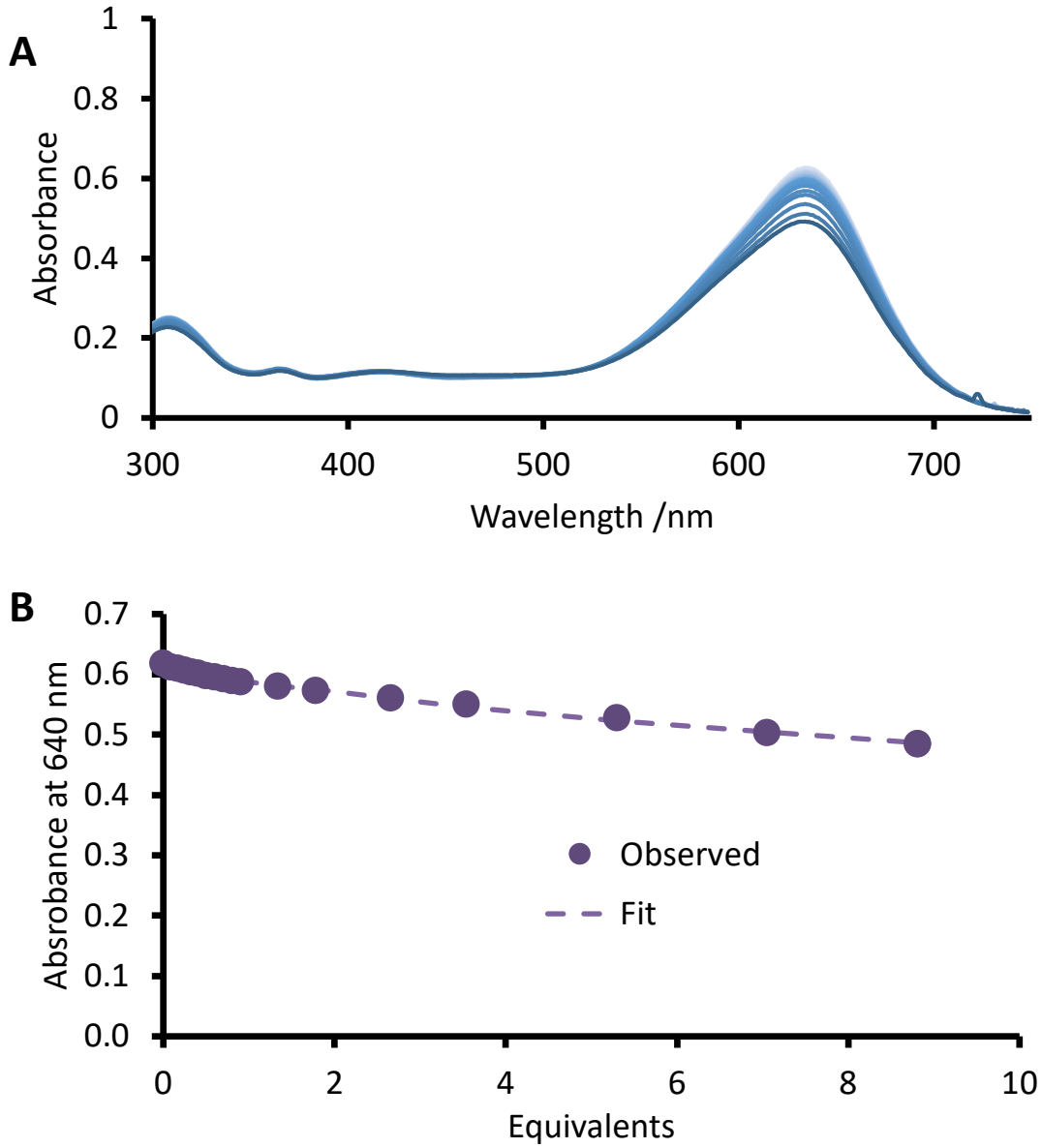


Figure S8: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of CDP (light to dark blue) **B)** 1:1 fitting curve at 640 nm.

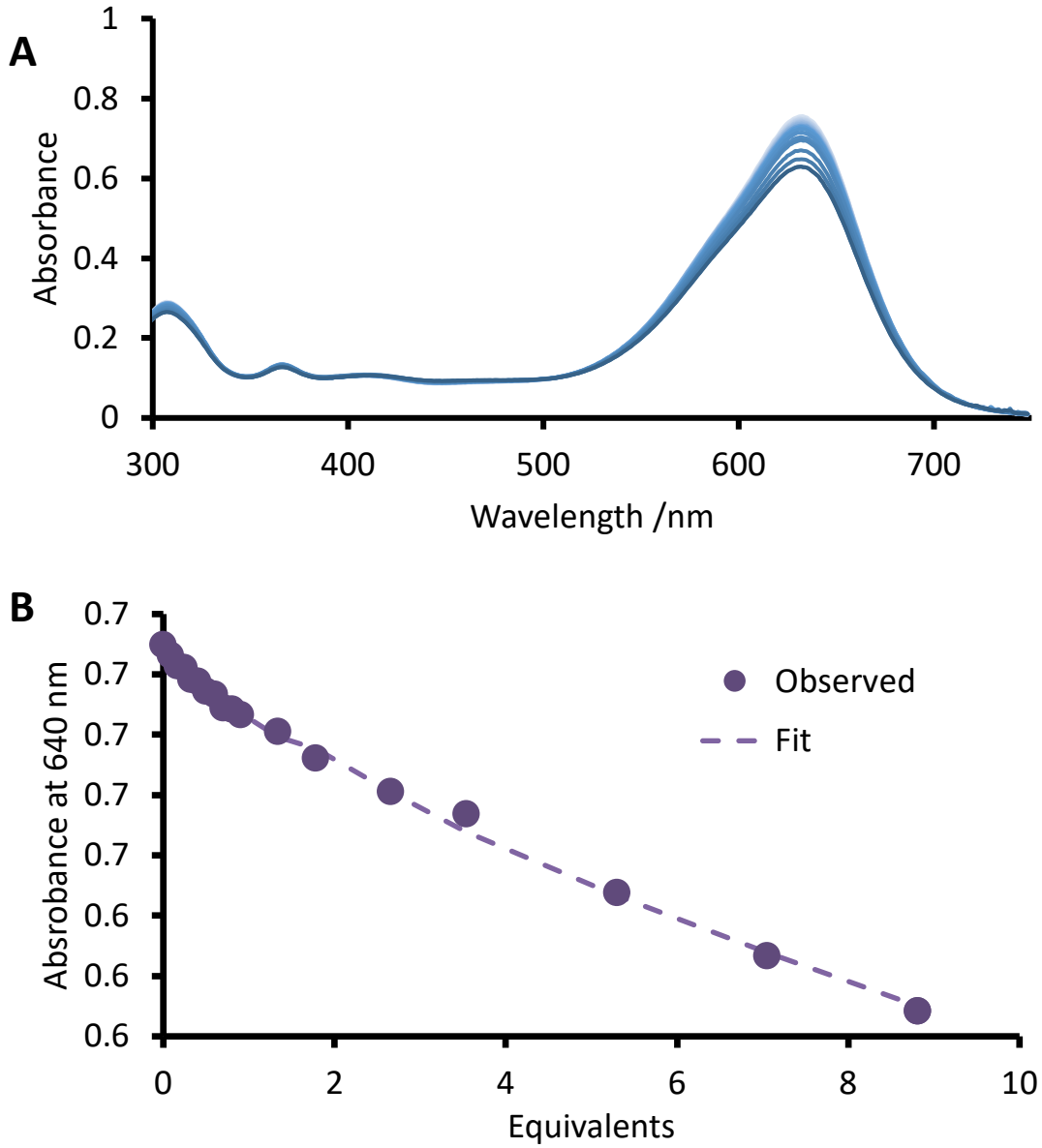


Figure S9: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of GDP (light to dark blue) **B)** 1:1 fitting curve at 640 nm.

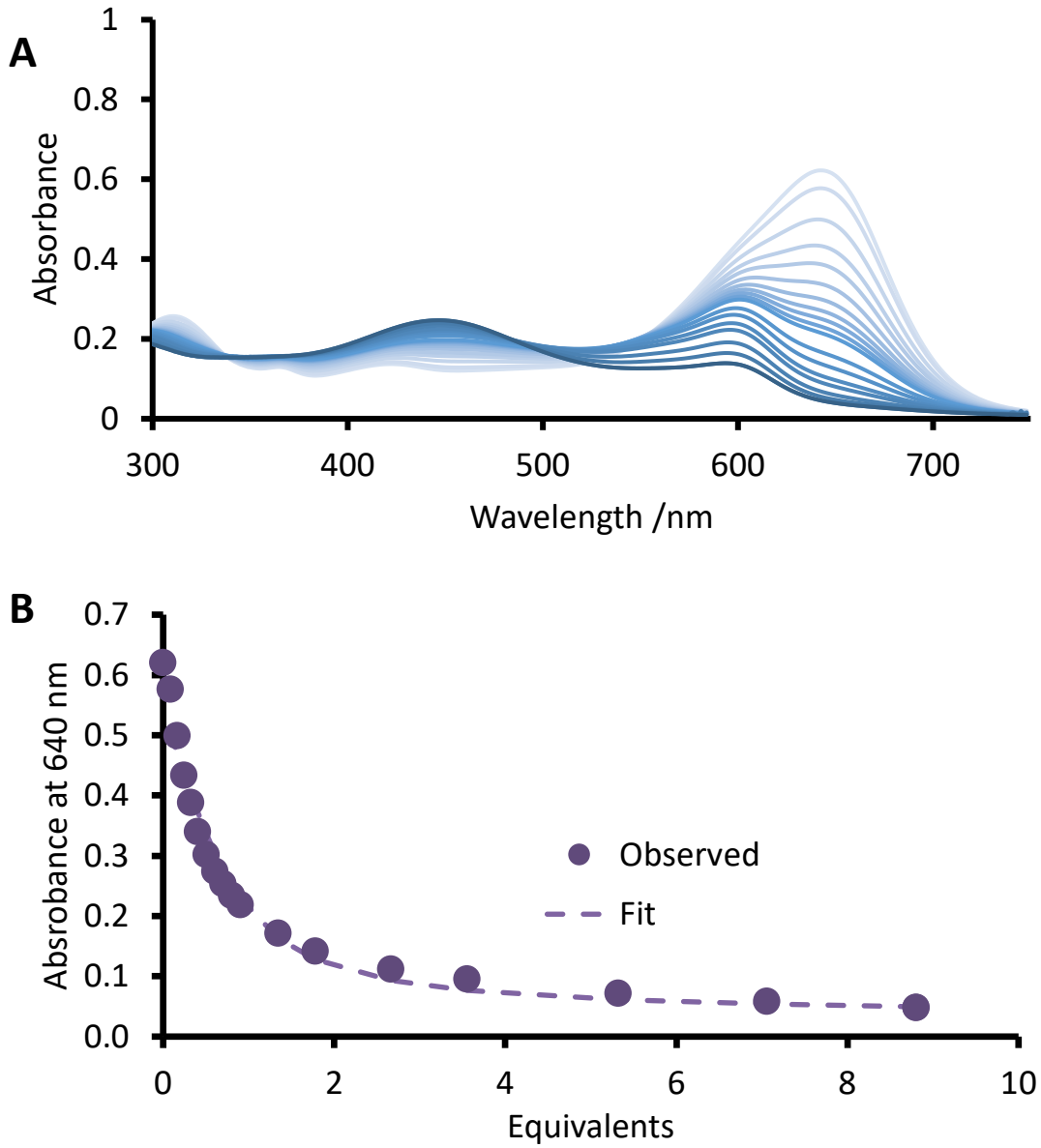


Figure S10: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of ATP (light to dark blue) **B)** 1:1 fitting curve at 640 nm.

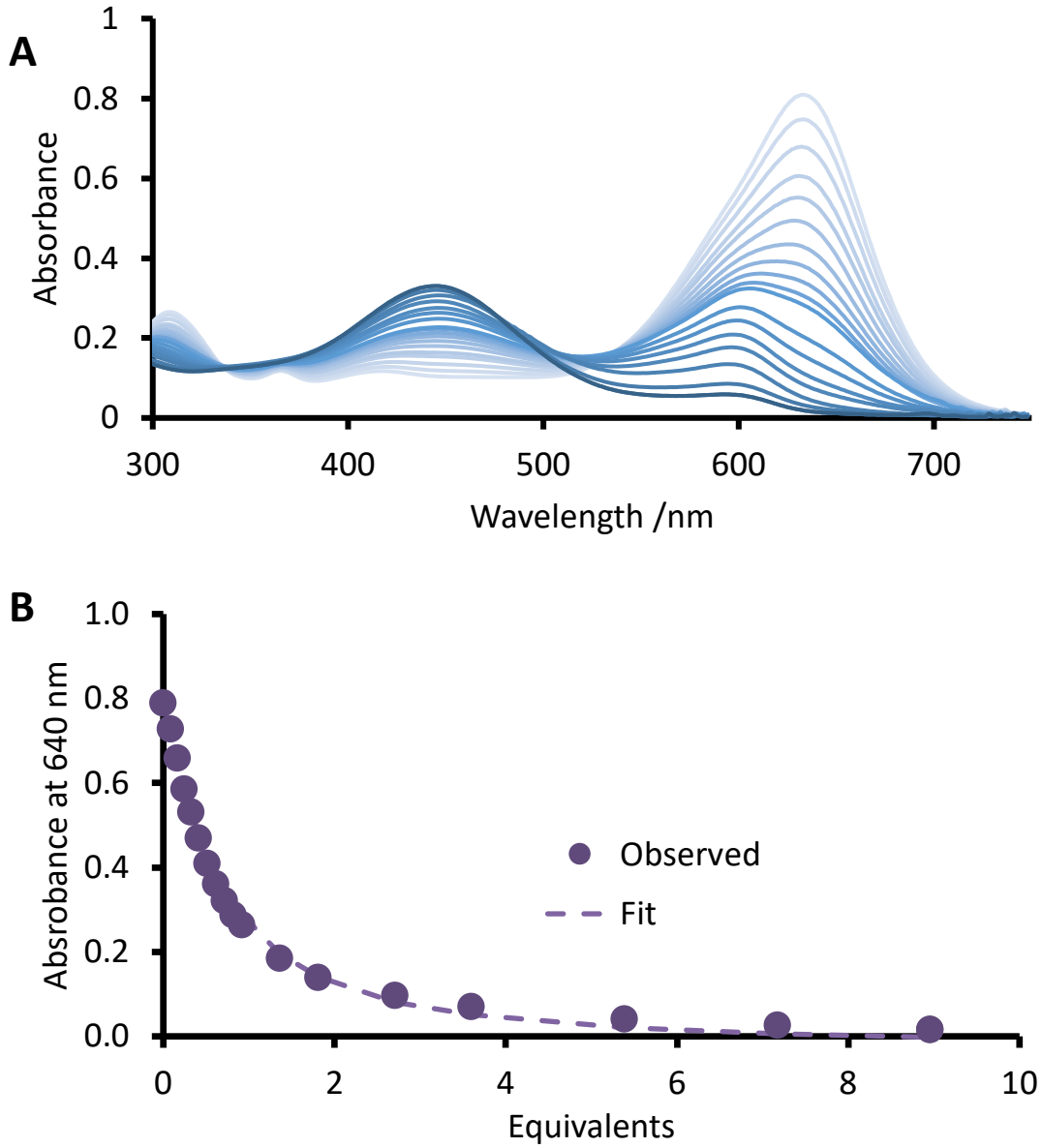


Figure S11: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of CTP (light to dark blue) **B)** 1:1 fitting curve at 640 nm.

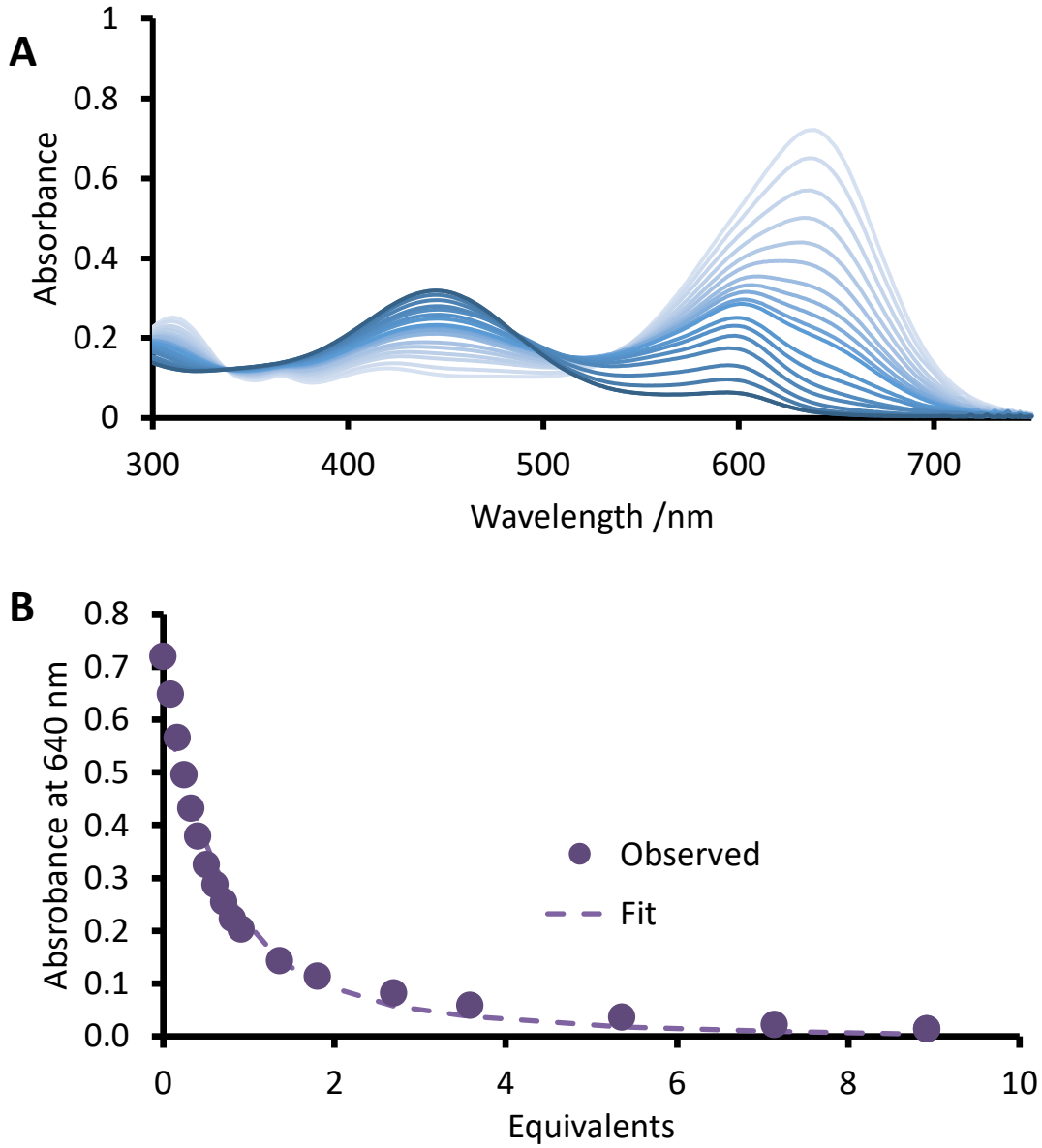


Figure S12: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of GTP (light to dark blue) **B)** 1:1 fitting curve at 640 nm.

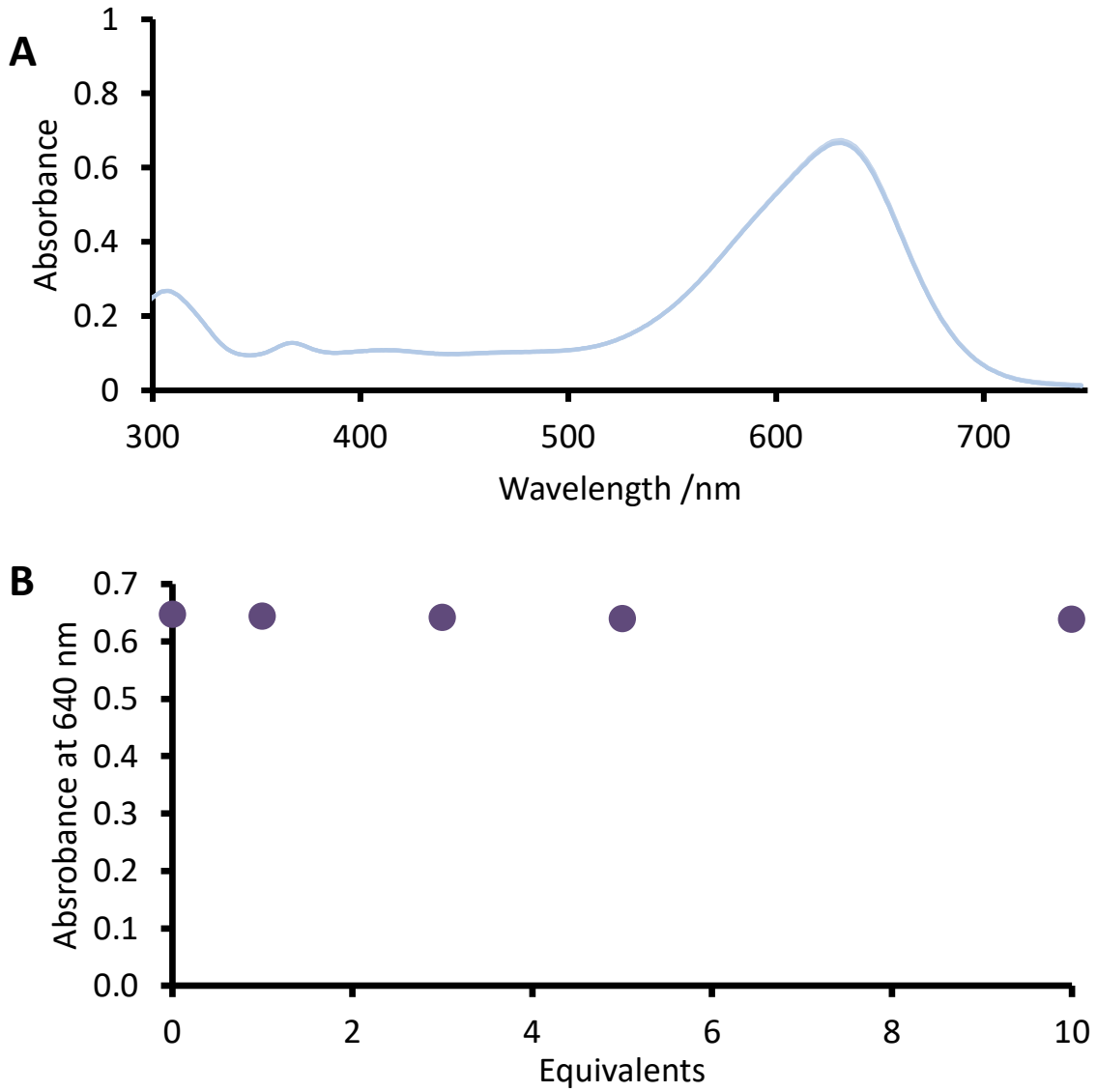


Figure S13: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of AMP (light to dark blue) **B)** Isotherm at 640 nm.

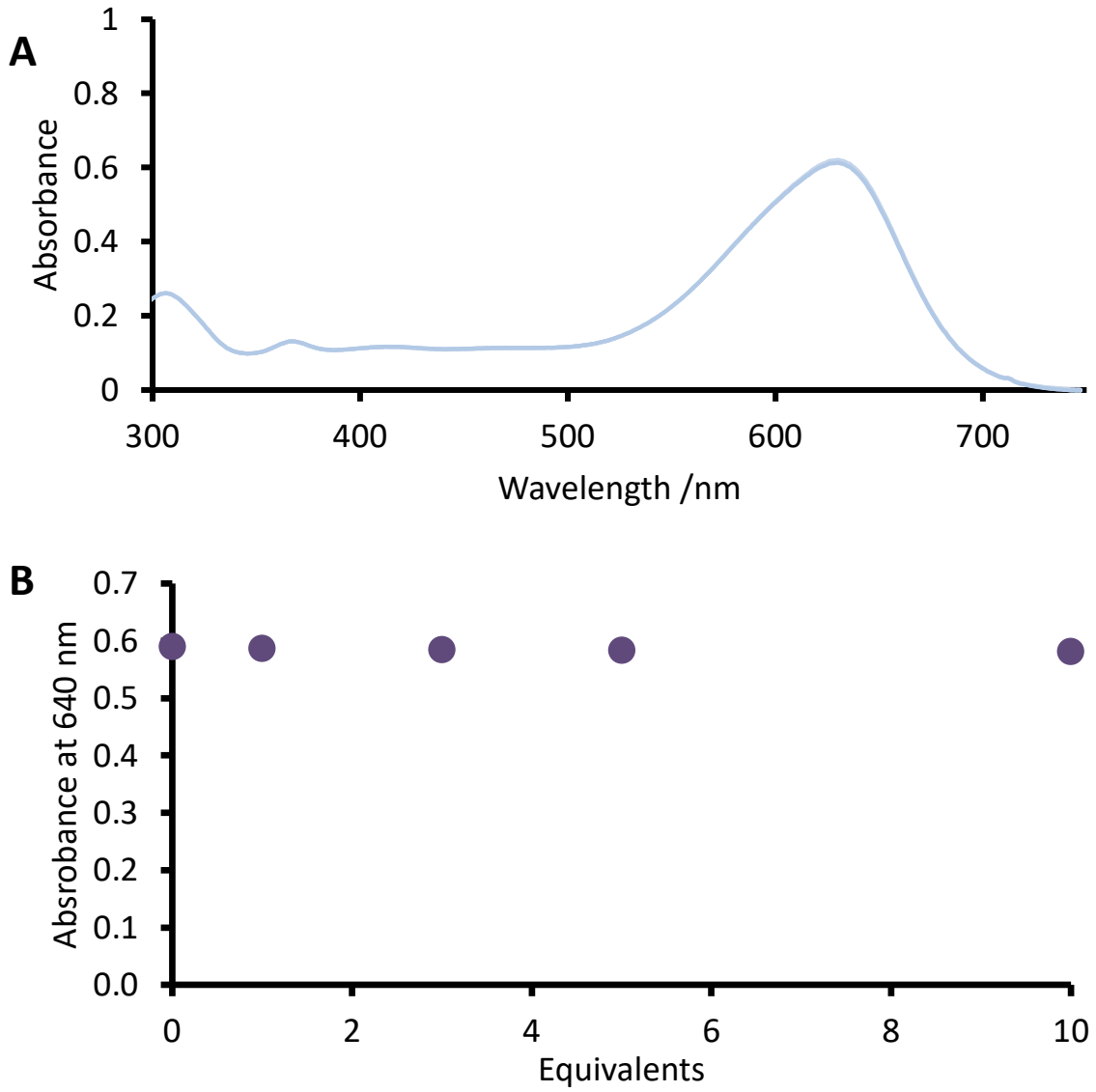


Figure S14: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of CMP (light to dark blue) **B)** Isotherm at 640 nm.

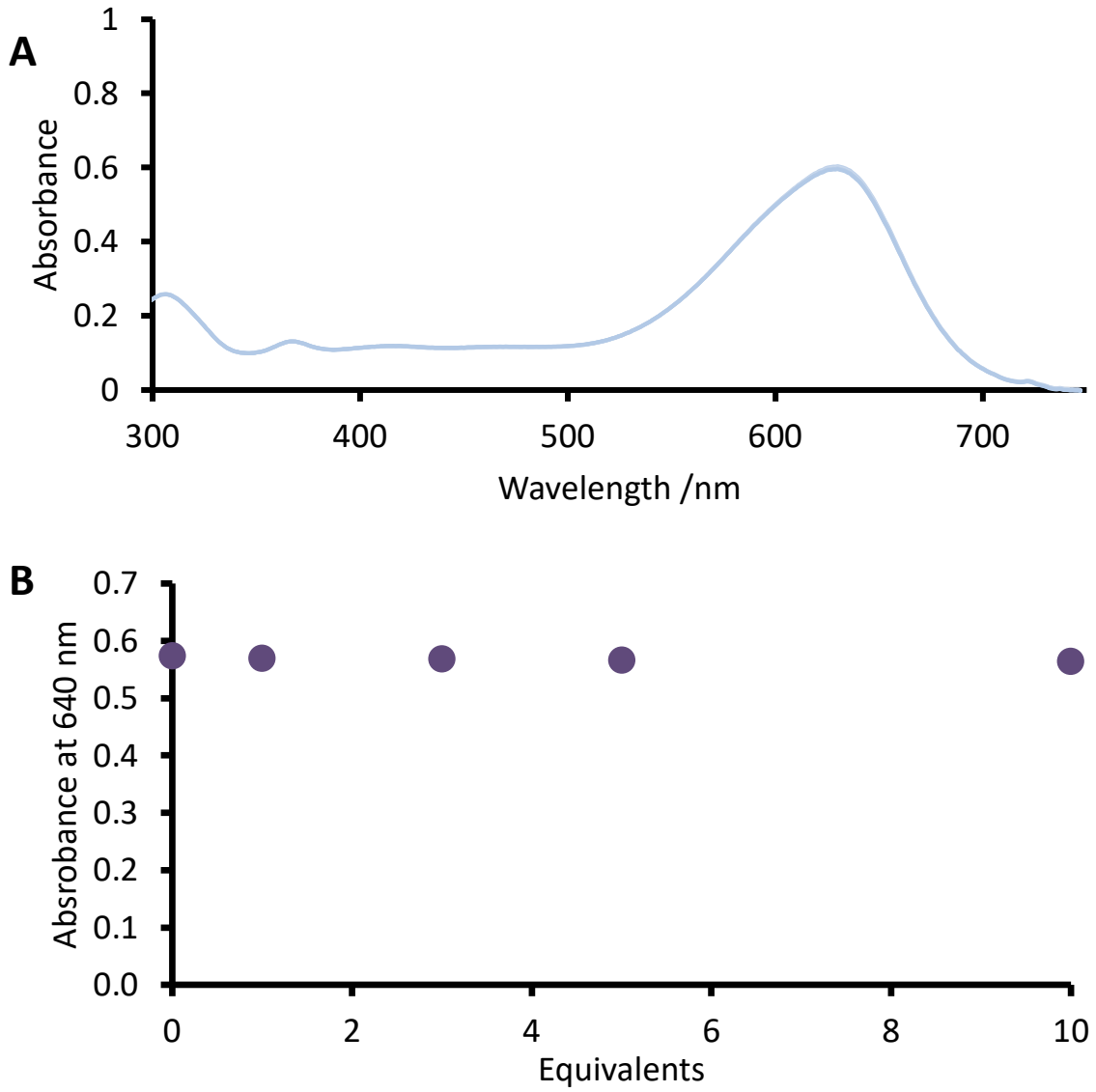


Figure S15: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of GMP (light to dark blue) **B)** Isotherm at 640 nm.

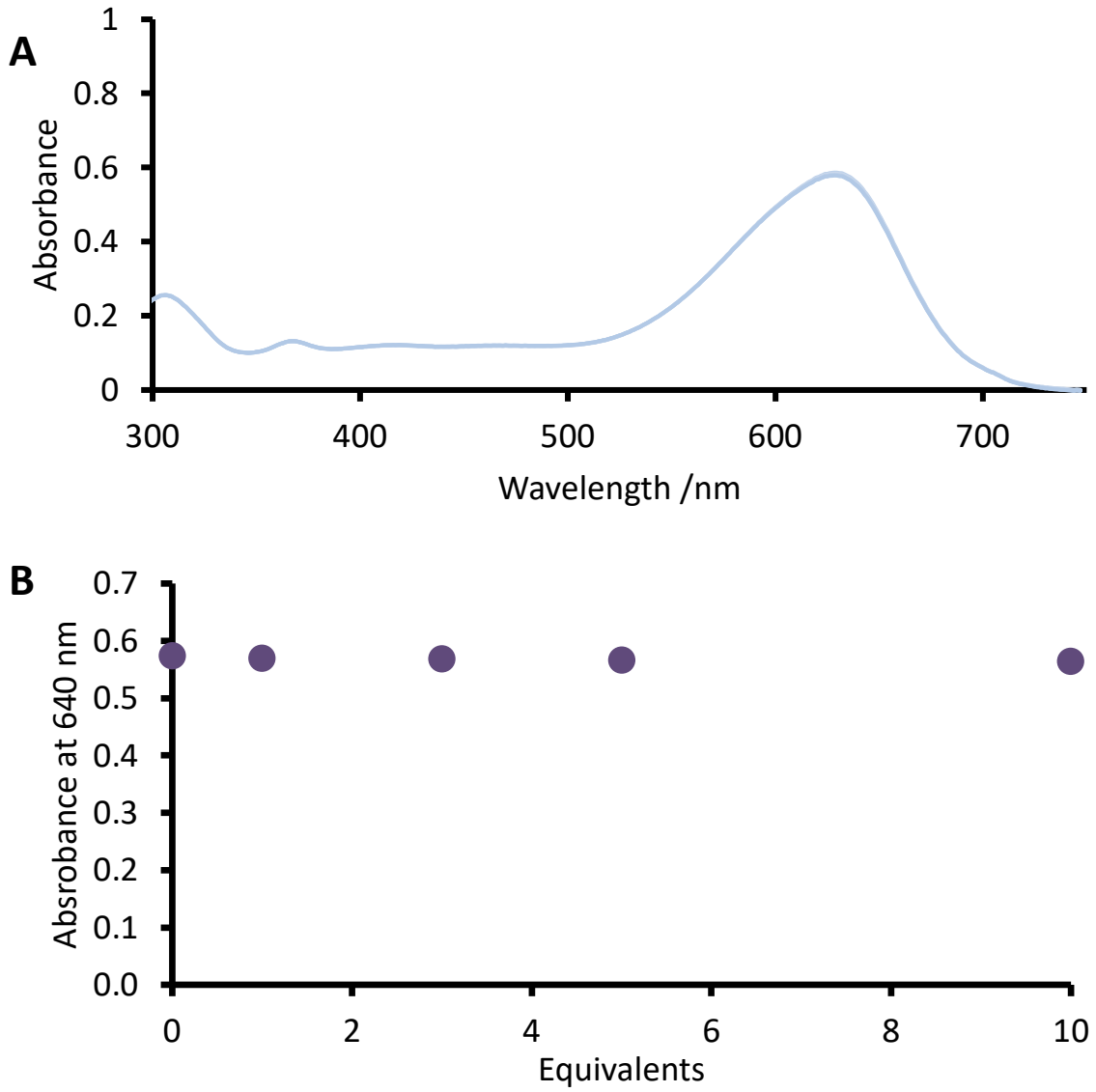


Figure S16: Representative UV-Vis spectra from the titration of **1·PV** (20 µM) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) with increasing amounts of Pi (light to dark blue) **B)** Isotherm at 640 nm.

Array Procedure

Sample preparation and data collection

Stock solutions of the sensor elements were typically prepared at 1.2 mM in HEPES buffer (5 mM, 145 mM NaCl, pH 7.4). Stock solutions of the phosphates were typically prepared at 3 mM concentration in the same buffer. The array experiments were performed in 96-well black polypropylene plates. Spectra were collected before and after the addition of the respective phosphates. For the preparation of each replicate in the colorimetric array, 20 µL of the phosphate stock solution were added to a well containing 280 µL of the receptor-indicator ensemble in HEPES buffer (the final concentration of the receptor-indicator ensemble was 20 µM). Measurements were

recorded on a Perkin-Elmer Enspire plate reader. For the colorimetric array, the absorbance was collected from 300-750 nm.

Data processing and statistical analysis

The array data was processed by calculation of the relative integrated absorbance in the regions indicated in Table S1. The obtained data was used directly in SPSS Statistics version 24 for principal component analysis and linear discriminant analysis.

Table S1: Regions of the absorbance spectra used as input data for array statistical analysis.

Indicator	Region 1 /nm	Region 2 /nm
PV	425-500	560-685
PGR	475-550	580-630
BPG	510-630	
CA	390-460	530-630
ECR	390-460	530-600
XO	490-560	

Colorimetric Response

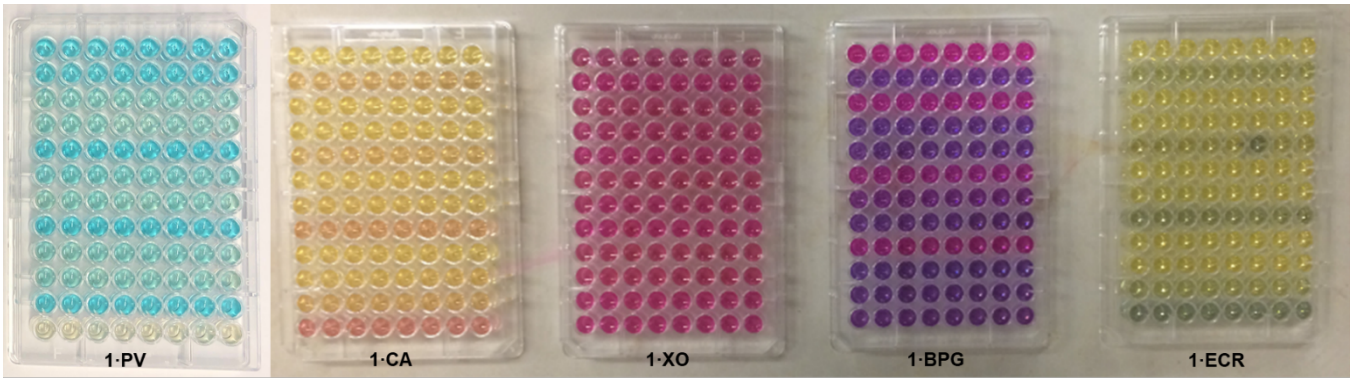


Figure S17: Colour changes of each **1·Indicator** solution (20 μM of each) in solution (5 mM HEPES, pH 7.4, 145 mM NaCl) upon addition of different phosphate containing analytes. Anions were added as the sodium salts and 10 equivalents (200 μM) were added.

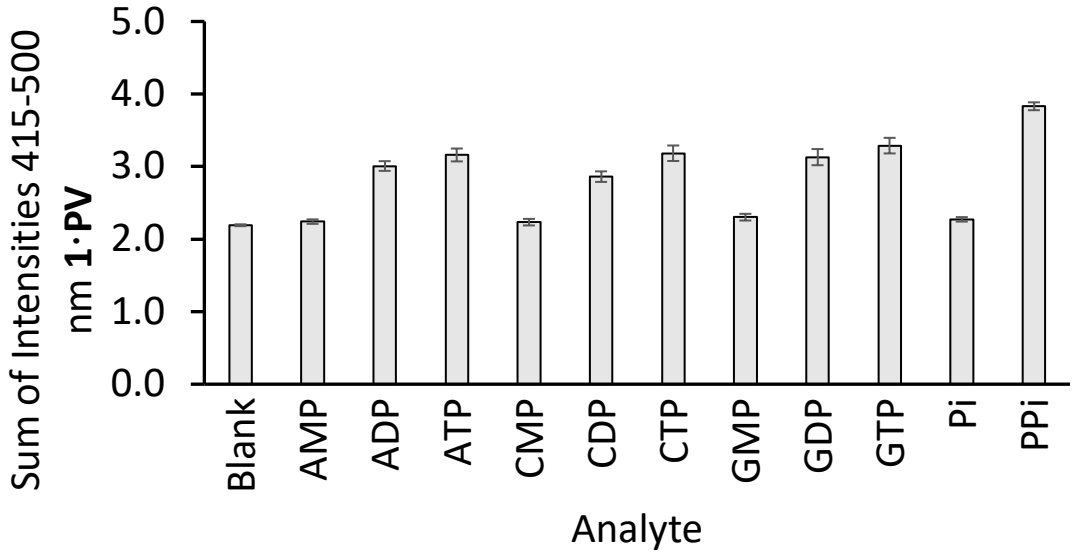


Figure S18: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·PV** in the range of 415-500 nm. **1·PV** (20 μM each in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphate containing analytes.

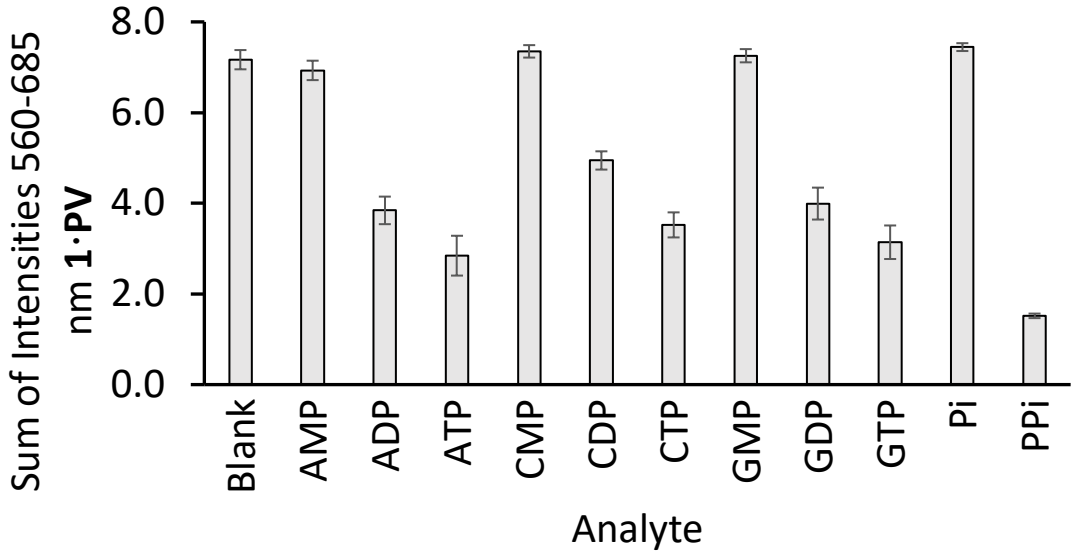


Figure S19: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·PV** in the range of 560-685 nm. **1·PV** (20 μM each) in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphoanions.

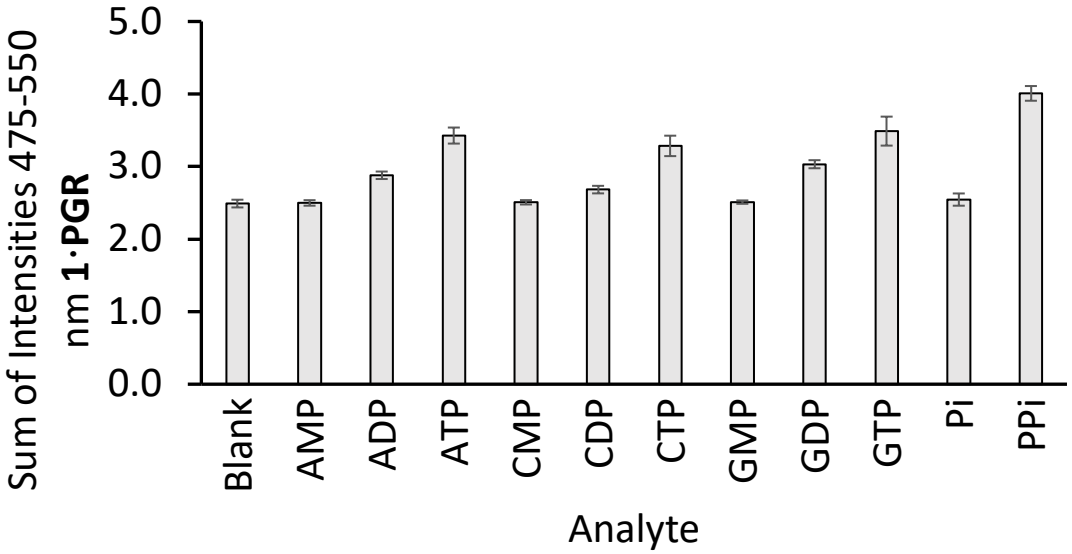


Figure S20: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·PGR** in the range of 475-550 nm. **1·PGR** (20 µM each) in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphoanions.

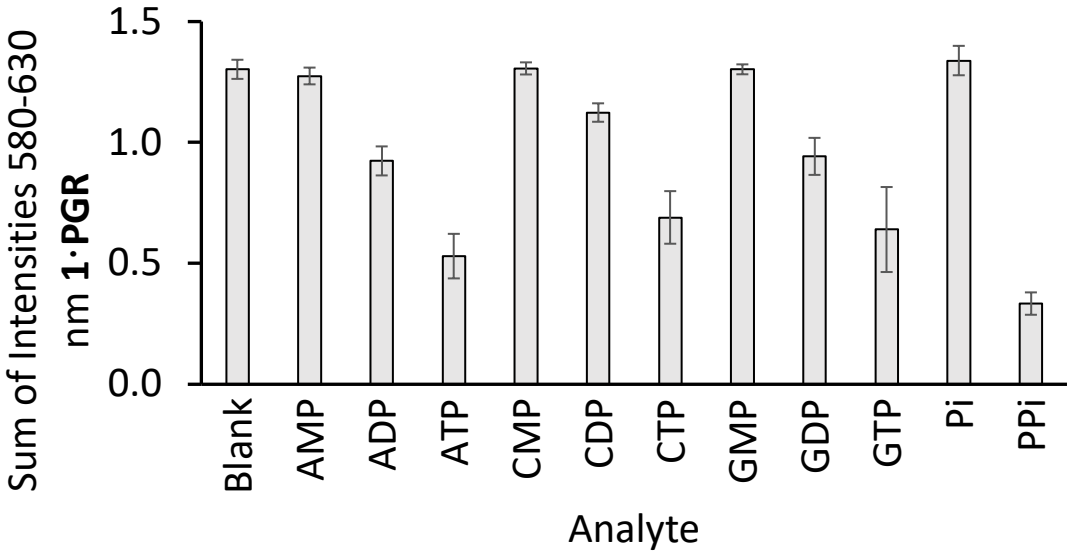


Figure S21: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·PGR** in the range of 580-630 nm. **1·PGR** (20 µM each) in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphoanions.

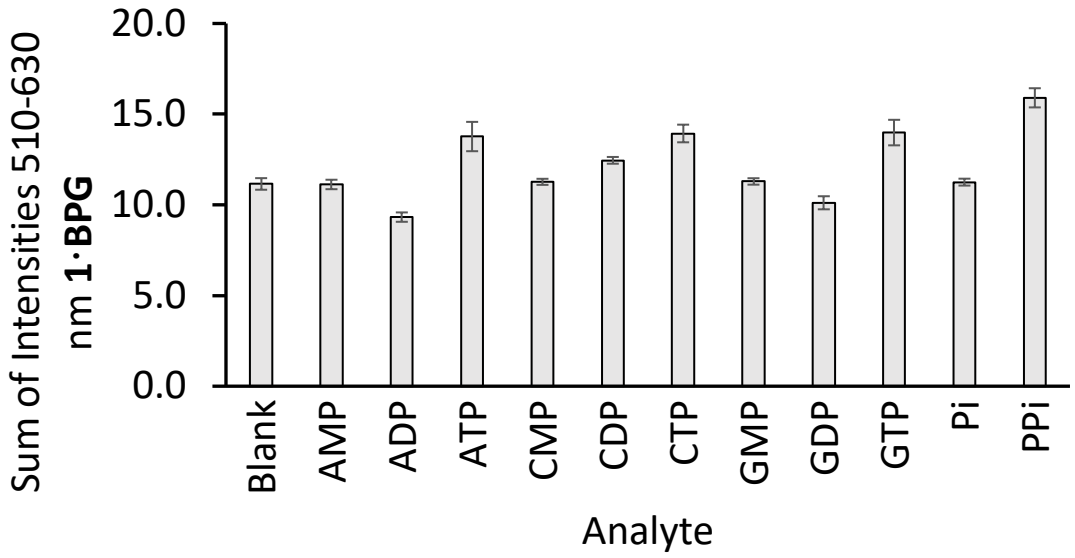


Figure S22: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·BPG** in the range of 475-550 nm. **1·BPG** (20 μM each) in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphoanions.

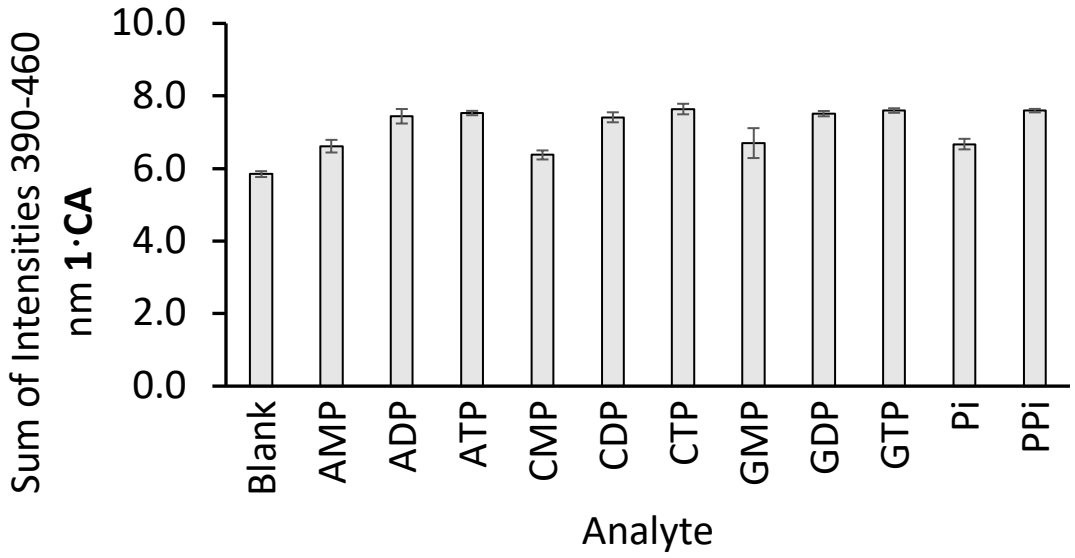


Figure S23: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·CA** in the range of 390-460 nm. **1·CA** (20 μM each) in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphoanions.

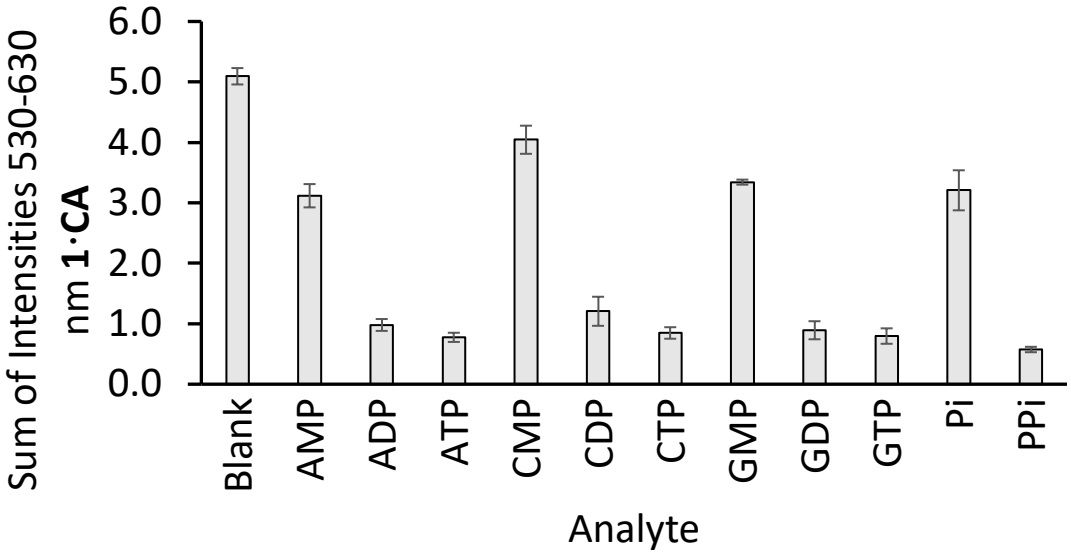


Figure S24: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·CA** in the range of 530-630 nm. **1·CA** (20 μM each) in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphoanions.

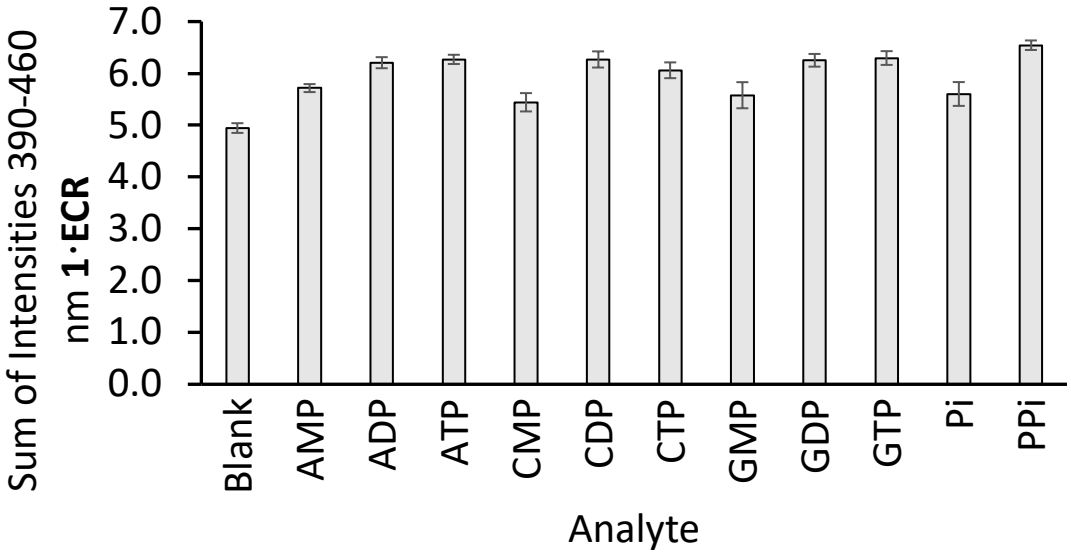


Figure S25: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·ECR** in the range of 390-460 nm. **1·ECR** (20 μM each) in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphoanions.

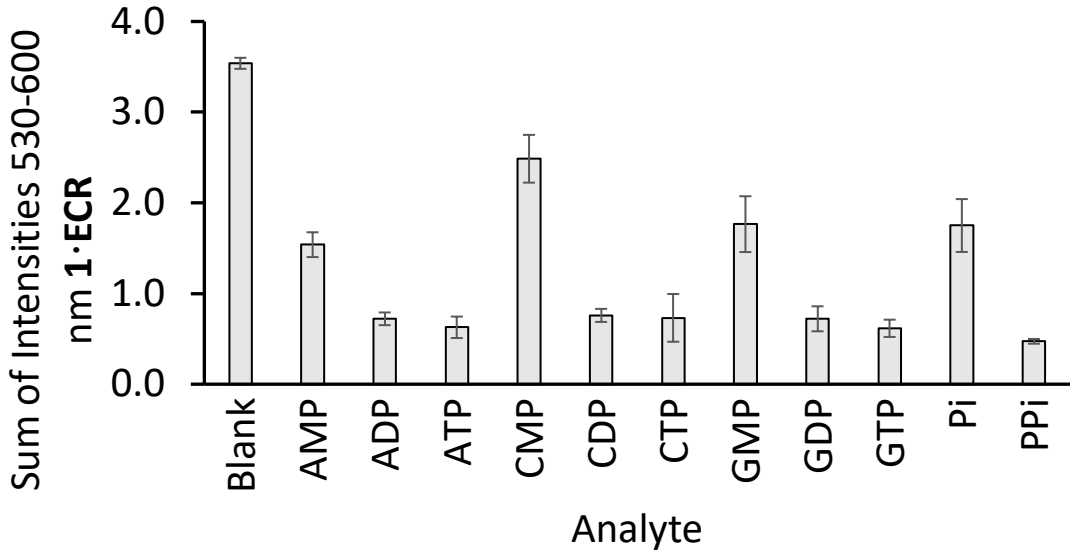


Figure S26: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·ECR** in the range of 530-600 nm. **1·ECR** (20 μM each) in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphoanions.

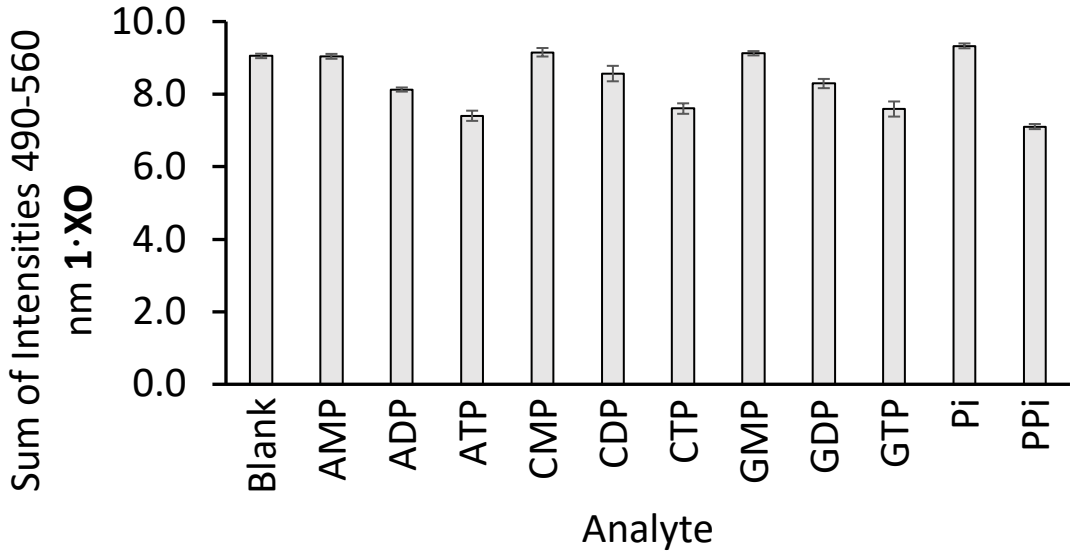


Figure S27: Effect of the addition of the addition of phosphoanions on the sum of the intensities of the absorbance of **1·XO** in the range of 490-560 nm. **1·XO** (20 μM each) in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) upon the addition of ten equivalents of a range of phosphoanions.

PCA

Table S2: KMO and Bartlett's Test, output from SPSS

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		0.9
Bartlett's Test of Sphericity	Approx. Chi-Square	2089.406

	df	45
	Sig.	0

Table S3: Communalities extracted using PCA, output from SPSS

Indicator	Wavelength range /nm	Extraction
PV	415-500	0.948
PV	560-685	0.947
PGR	475-550	0.962
PGR	580-630	0.96
BGP	510-630	0.834
CA	390-460	0.923
CA	530-630	0.983
ECR	390-460	0.916
ECR	530-600	0.942
XO	490-560	0.949

Table S4: Total variance explained by the components extracted using PCA, output from SPSS.

Component	Initial eigenvalues		Extraction Sums of Squared Loadings		Rotation Sums of Squared Loadings	
	Total	% of Variance	Total	% of Variance	Total	% of Variance
1	8.291	82.909	8.291	82.909	5.252	52.519
2	1.072	10.724	1.072	10.724	4.111	41.114
3	0.321	3.212				
4	0.123	1.23				
5	0.065	0.653				
6	0.048	0.48				
7	0.036	0.362				
8	0.02	0.201				
9	0.013	0.132				
10	0.01	0.097				

Table S5: Component Matrix extracted using PCA, output from SPSS

		Component	
Indicator	Wavelength range /nm	1	2
PV	415-500	-0.972	0.053
PV	560-685	0.972	-0.044
PGR	475-550	-0.926	0.322
PGR	580-630	0.949	-0.245
BGP	510-630	-0.665	0.625
CA	390-460	-0.902	-0.33
CA	530-630	0.941	0.311
ECR	390-460	-0.902	-0.319

ECR	530-600	0.882	0.404
XO	490-560	0.952	-0.204

LDA

Table S6: The eigenvalues extracted during LDA, output from SPSS.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	118.531a	90.2	90.2	0.996
2	9.506a	7.2	97.4	0.951
3	2.108a	1.6	99.1	0.824
4	1.248a	0.9	100	0.745

Table S7: Classification Results. 100.0% of original grouped cases correctly classified. 100.0% of cross-validated grouped cases correctly classified. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case. Output from SPSS where classes are as follows; 1=Blank, 2=AMP, CMP, GMP, Pi, 3=ADP, CDP, GDP, 4=ATP, CTP, GTP, and 5=PPi

		Class	Predicted Group Membership					Total
			1	2	3	4	5	
Original	Count	1	8	0	0	0	0	8
		2	0	32	0	0	0	32
		3	0	0	24	0	0	24
		4	0	0	0	24	0	24
		5	0	0	0	0	8	8
	%	1	100	0	0	0	0	100
		2	0	100	0	0	0	100
		3	0	0	100	0	0	100
		4	0	0	0	100	0	100
		5	0	0	0	0	100	100
Cross-validated	Count	1	8	0	0	0	0	8
		2	0	32	0	0	0	32
		3	0	0	24	0	0	24
		4	0	0	0	24	0	24
		5	0	0	0	0	8	8
	%	1	100	0	0	0	0	100
		2	0	100	0	0	0	100
		3	0	0	100	0	0	100
		4	0	0	0	100	0	100
		5	0	0	0	0	100	100

Classification with some analytes removed

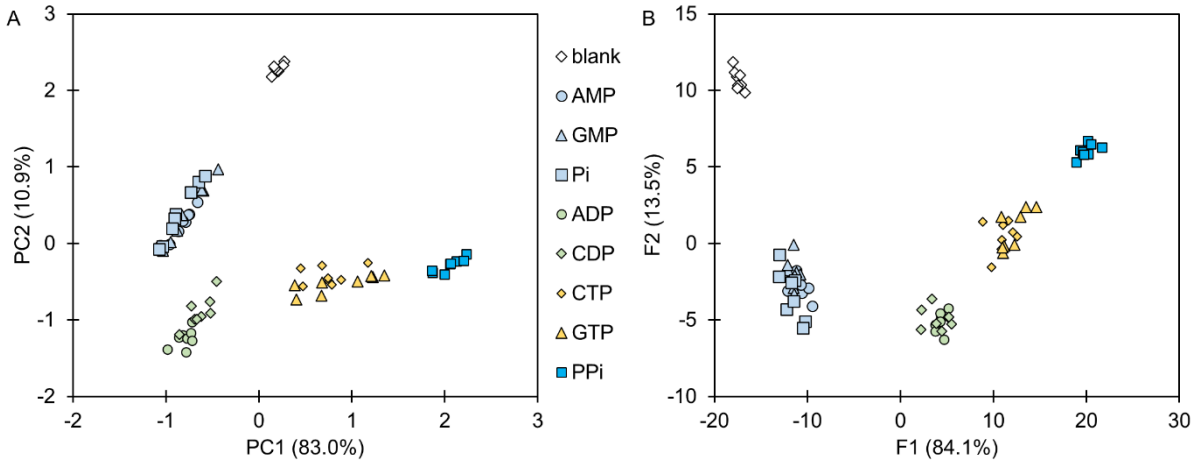


Figure S28: (A) Two-dimensional PCA plot for the analysis of the data obtained from the colorimetric array with eight replicates excluding CMP, GDP and ATP. **(B)** Two-dimensional LDA score plot generated from the colorimetric array data, classified into mono-, di- and triphosphates, PPi, and the blank excluding CMP, GDP and ATP.

Table S8: Classification results of the removed analytes (CMP, GDP, ATP) using the model produced by LDA of the remaining analytes. 2 samples of CMP misclassified as blank. Classes are as follows; 1=Blank, 2=AMP, CMP, GMP, Pi, 3=ADP, CDP, GDP, 4=ATP, CTP, GTP, and 5=PPi.

	Class	Predicted Group Membership				
		1	2	3	4	5
Count	CMP	2	6	0	0	0
	GDP	0	0	8	0	0
	ATP	0	0	0	8	0

References

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