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Supporting Information

Efficient labelling of enzymatically synthesized vinyl-modified DNA by an inverse-electron-demand Diels-Alder Reaction

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CONTENT

General Procedure

All reagents were used without further purification. Dry solvents were obtained from Sigma-Aldrich and used without further purification. Anion-exchange chromatography was performed on a ÄktaPurifier (GE Healthcare) with a DEAE Sephadex™ A-25 (GEHealthcare Bio-SciencesAB) column using a linear gradient (0.1 M – 1.0 M) of triethylammonium bicarbonate buffer (TEAB, pH 7.5). Reversed phase high pressure liquid chromatography (RP-HPLC) for the purification of compounds was performed using a Shimadzu unit having LC8a pumps and a Dynamax UV-1 detector. A VP 250/21 NUCLEODUR C18 HTec, 5µm (Macherey-Nagel) column and a gradient of acetonitrile in 50 mM TEAA buffer (pH 7.0) were used. All compounds purified by RP-HPLC were obtained as their triethylammonium salts after repeated freeze-drying. Analytical RP-HPLC was performed using a Shimadzu Prominence system. A VP 250/4 NUCLEODUR C18 HTec, 5µm (Macherey-Nagel) column and a gradient of acetonitrile in 50 mM TEAA buffer (pH 7.0) were used. NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer and a Bruker AVIII 600 MHz spectrometer. ^{1}H and ^{13}C chemical shifts are reported relative to the residual solvent peak and are given in ppm. HR-ESI-MS spectra were recorded on a Bruker Daltronics microTOF II and ESI-MS spectra were recorded on a Bruker Daltronics amaZonSL

Nucleotide Synthesis

d^{vin}ATP

7-deaza-7-vinyl-2'-deoxyadenosine was synthesized according to published procedures.[1] 93 mg (0.34 mmol) of the nucleoside were coevaporated 3 times with 3 mL acetonitrile each. 107 mg (0.5 mmol) proton sponge was added. The mixture was dissolved in 3 mL previously distilled trimethylphosphate. 52 µL (86 mg, 0.4 mmol) $POCl_3$ were added with ice cooling. The reaction was monitored by reverse phase TLC in isopropanol/water/ammonia3:1:1. After 10 min 3.36 mL 0.5 M pyrophosphate solution (1.68 mmol) in DMF and 0.81 mL of tributylamine (630 mg, 3.4 mmol) were added simultaneously. After 40 min, the reaction was quenched by addition of 10 mL 0.1 M triethylammoniumhydrogencarbonate buffer (pH 7). The aqueous solution was extracted 3 times with ethyl acetate. The aqueous solution was concentrated under reduced pressure. The residue was dissolved in 4 mL water and subjected to ion exchange chromatography, followed by a reverse phase HPLC using 0.05 M triethylammoniumacetate buffer (pH 7, buffer A) and acetonitrile (buffer B) to afford 0.07

mmol (22 %) d^{vin}ATP. Retention time: 21.1 min (gradient: 10 min 5% B, 40 min: linear gradient 5%-40% B)

^{1}H-NMR (400 MHz, d_4-MeOH): δ (ppm) 7.11 (s, 1H, *H2*), 7.70 (s, 1H, *H8*), 7.01 (dd, J= 11.0 Hz, 17.4 Hz, 1H, *vinyl-H*), 6.68 (t, J= 6.9 Hz, 1H, *H1'*), 5.72 (dd, J= 17.4 Hz, J=1.1 Hz, 1H, *vinyl-H*), 5.28 (dd, J= 11.0 Hz, J= 1.1 Hz, 1H, *vinyl-H*), 4.37-4.15 (m, 2H, *H3', H4'*), 4.15-4.08 (m, 1H, *H4'*), 2.69-2.58 (m, 1H, *H2'*), 2.39-2.30 (ddd, J= 13.4, J= 6.2, J= 3.5, 1H, *H2'*).

^{31}P-NMR (162 MHz, d_4-MeOH): δ (ppm) -10.3 (d, J= 21.3 Hz, 1P, *P-γ*), -11.2 (d, J= 21.4 Hz, 1P, *P-α*), -23.7 (t, J= 21.3 Hz, 1P, *P-β*).

HRMS: found: 515.0138

calculated for $[C_{13}H_{18}N_4O_{12}P_3]^-$ 515.0129

Determination of second-order rate constants of the DARinv

The second-order rate constant of the DARinv of d^{vin}A and d^{vin}U with tetrazine **2** (Fig. S1) was determined as described before.[2] Briefly, 5 mM of the nucleoside and the tetrazine **2** were dissolved in 100 mM acetate buffer at pH 4.8. The reaction was monitored by UV spectroscopy (λ=522 nm, ε=437.45 l/(mol·cm). 1/c was plotted versus the time and the rate constant was determined from the slope of the linear regression (Fig. S1). Each experiment was performed in triplicate.

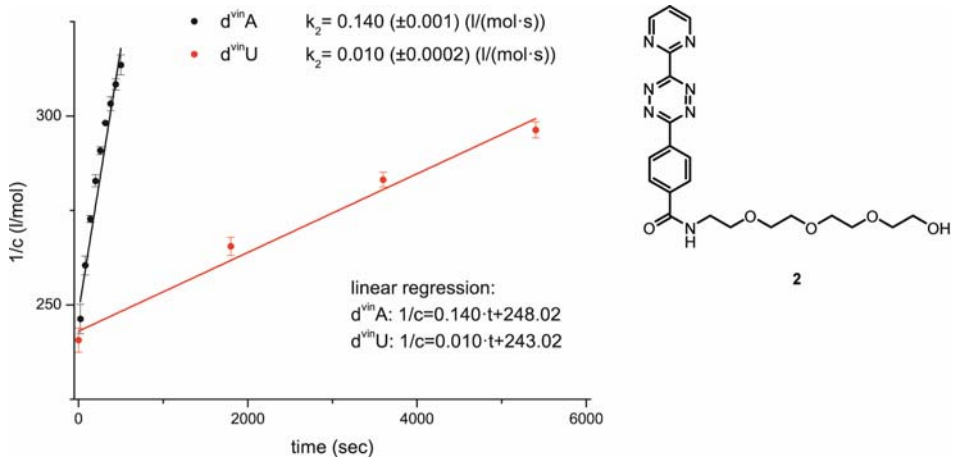

Fig. S1 Determination of second-order rate constants k_2 of DARinv of d^{vin}A and d^{vin}U with tetrazine.

DFT-calculations of the frontier orbitals

All quantum chemical calculations are based on Density Function Theory (DFT) and have been carried out using the GAUSSIAN09 program package.[3] The model geometries of these systems were optimized using Lanl2DZ basis set with the Becke three parameters hybrid exchange and the Lee–Yang–Parr correlation functional (B3LYP).[4] Quantum chemical

studies were performed without any symmetry constraints. The closed-shell systems were calculated by the restricted Kohn-Sham approach (RKS).[5] DFT calculations were carried out with B3LYP functional using Lanl2DZ effective core potential basis set in water using the Polarized Continuum Model (PCM).[6] Orbital energies were analyzed using GaussSum software.[7]

Due to symmetry, the LUMO+1 interacts with the HOMOs of the corresponding nucleobases (Fig S2). The energy-difference between the $LUMO+1_{Tetrazine}$ and $HOMO_{dvinU}$ is 0.55 eV larger than the energy difference between $LUMO+1_{Tetrazine}$ and $HOMO_{dvinA}$. Thereby, the reaction of $d^{vin}A$ and the tetrazine proceeds faster than the reaction of $d^{vin}U$ and the tetrazine.

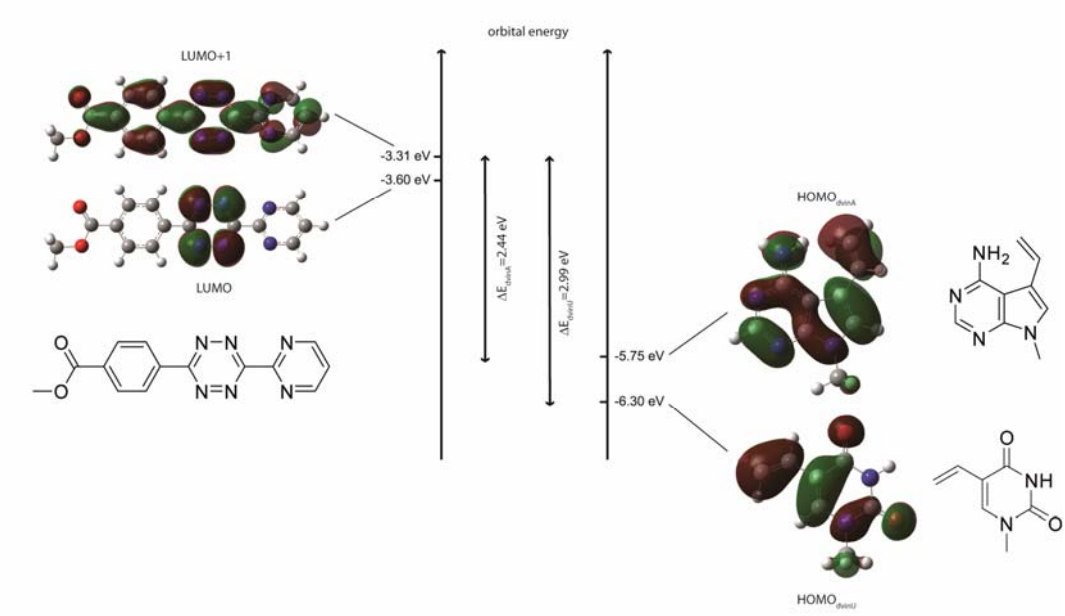

Fig. S2 Energies oft he frontier orbitals. Due to symmetry, the LUMO+1 of the tetrazine ($LUMO+1_{Tetrazine}$) interact with either the HOMO of $d_{vin}A$ or $d_{vin}U$.

Primer extension experiments and labelling by Diels-Alder reaction with inverse electron demand (DARinv)

0.9 µM (total concentration of radioactively labelled and unlabeled primer) primer (5'-GAC CCA CTC CAT CGA GAT TTC TC-3'), 1.2 µM template (5'-GCG CTG GCA CGG GAG AAA TCT CGA TGG AGT GGG TC-3'), 200 µM d^{vin}ATP, TTP, dCTP and dGTP each and 200 nM *KlenTaq* DNA polymerase in 20 µL buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, and 2 mM $MgCl_2$) were incubated at 37 °C for 30 min. The reaction was stopped by gel filtration. 10 µL of either a 5 mM tetrazine **1** solution or water were added and the samples were incubated for 4 h. The reaction was stopped by gel filtration. 1 µL of the samples were incubated with either 19 µL streptavidin (1 mg mL^{-1}) or 19 µL water and incubated for further

30 min. 20 µL loading dye (80% [v/v] formamide, 20 mM EDTA, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol) were added and 2 µL of each samples was loaded for PAGE analysis. Visualization was performed by phosphoimaging.

Time dependency of the labelling of DNA by DARinv

Primer extension reaction was performed as described above. To 20 µL of the PEX product 10 µL of a tetrazine **1** solution (5 mM, 1900x; 1.3 mM, 500x; 260 µM, 100x; 66 µM, 25x) was added at different time points for the different aliquots, so that the indicated reaction times were applied. 30 µL loading dye (80% [v/v] formamide, 20 mM EDTA, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol) were added to all samples simultaneously and the samples were loaded immediately for PAGE analysis. Visualization was performed by phosphoimaging.

Determination of the rate constant of the DARinv on dsDNA

Primer extension experiments were performed as described above. To 20 µL of the PEX product 10 µL of a tetrazine **1** solution (1.35 mM) was added. The reaction was stopped by addition of 30 µL stopping solution (2 mM norbornene 80% [v/v] formamide, 20 mM EDTA, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol). The samples were analyzed by PAGE. Visualization was performed by phosphoimaging.

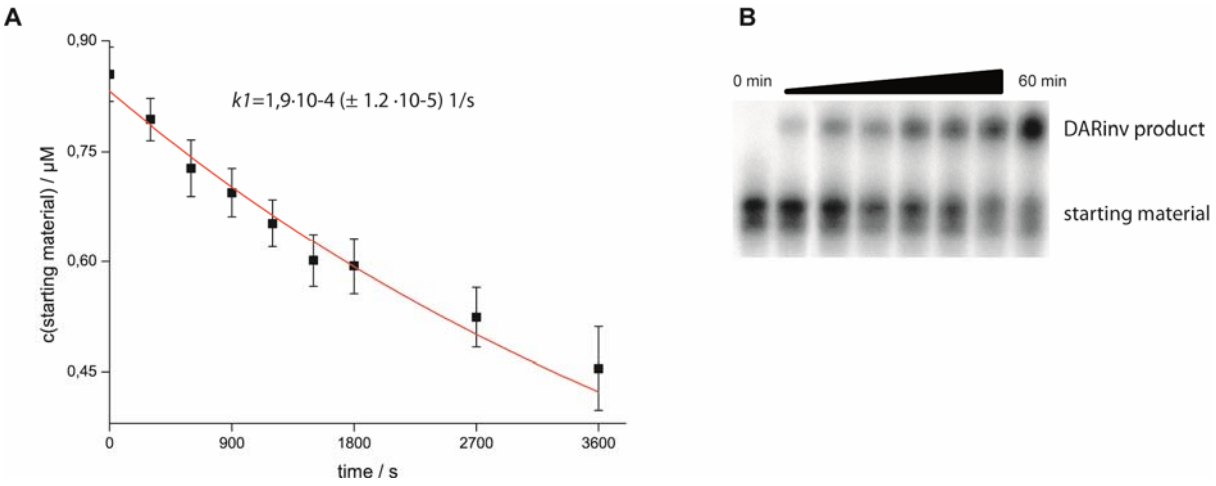

Fig. S3 Determination of the rate constant of the DARinv on dsDNA. A: Plot of the remaining starting material over time. Fitting of an exponential decay gave the kinetic rate constant pseudo first order of k= $1{,}9 \cdot 10^{-4}$ ($\pm$ $1.2 \cdot 10^{-5}$)1/s. Fitting was performed with Prigin v8.0724 (OriginLab Corp.). As the experiment was performed at 450 µM tetrazine concentration, the rate constant was devided by this concentration to give the rate constant of second order k_2=0.42 ($\pm$ 0.03) (L/(mol·s)

Labelling of a PCR product by DARinv

50 pM template, 0.5 µM of each primer and 200 µM of dNTPs (with dATP completely exchanged by d^{vin}ATP) in 25 µL reaction buffer (10 mM Tris-HCl , pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$, 0.08 vol% Nonidet P40) were cycled with following program:

1) 95 °C for 10 min
2) 95 °C for 30 sec
3) 55°C for 30 sec
4) 72°C for 30 sec to 2) for 29 times
5) 72°C for 10 min

The PCR product was purified by ethanol precipitation. The pellet was dissolved in 20 µL of water and either 10 µL of tetrazine **1** (5 mM) or water was added. The mixture was incubated for 1 h at r.t. and then loaded onto a 0.8 % agarose gel. The gel was run at 120 V and stained with ethidium bromide (EtBr). Visualization was perfomed by recording the fluorescence with a camera at an excitation wavelength of 200 nm.

Template: pET-21b
Forward primer:
5'-GCA GAG CGC AGA TAC CAA AT-3'
Reverse primer:
5'-GTT TCC CCC TGG AAG CTC-3'

Digest of labelled PCR product to nucleosides and HPLC analysis

The PCR product was prepared and modified by DARinv as described above. 4 aliquots (25 µL each) were pooled and incubated with 40 µl of a 1.5 mM tetrazine **1** solution for 1 h at room temperature. As control, 100 µL water was treated the same way. The samples were subjected to PD-10 G25 gel filtration (GE Healthcare). The collected sample was concentrated to 100 µl. The solution was heated for 5 min at 95 °C and was quickly cooled with liquid nitrogen. The solution was incubated with 250 U benzonase (Sigma-Aldrich), 300 mU snake venom phosphodiesterase (Worthington) and 20 U calf intestinal phosphatase (NEB) in a total of 500 µl of 20 mM Tris-HCl, pH= 7.9, 100 mM NaCl, 20 mM $MgCl_2$. The solution was incubated for 14 h at 37 °C. The solution was concentrated to dryness and

resobilized in 100 µL water. The samples were analyzed by HPLC. Peaks were identified by coinjection of the nucleosides.

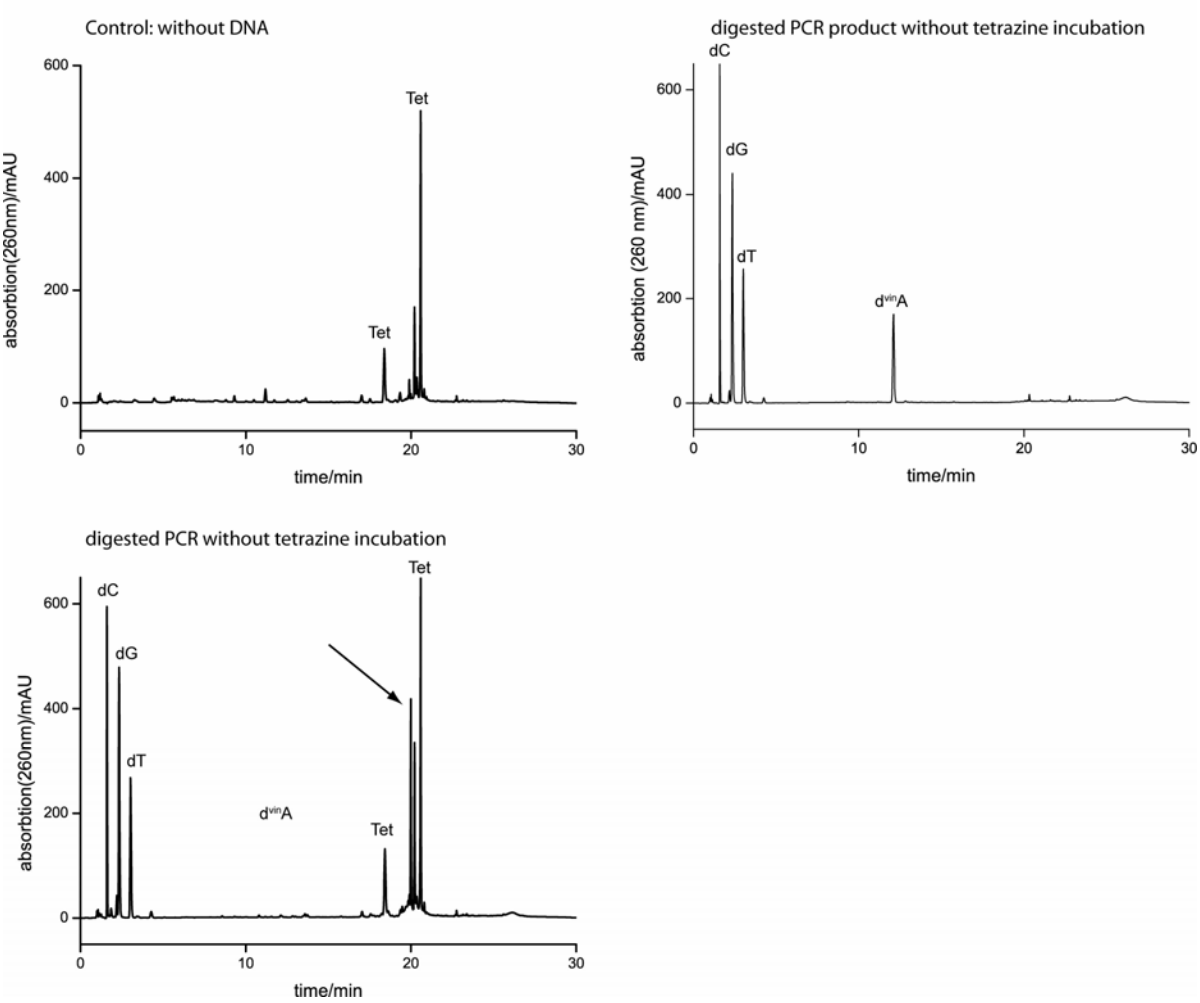

Fig. S3 HPLC-analysis of the digested PCR product. Peaks corresponding to the nucleobases are indicated. Peaks arising from the excess of tetrazine **1** are indicated by "Tet". Upon DARinv, a new peak appears in the chromatogram (indicated by an arrow). After DARinv, $d^{vin}A$ cannot be detected anymore, indicating complete conversion in the DNA duplex.

NMR spectra of d^{vin}ATP

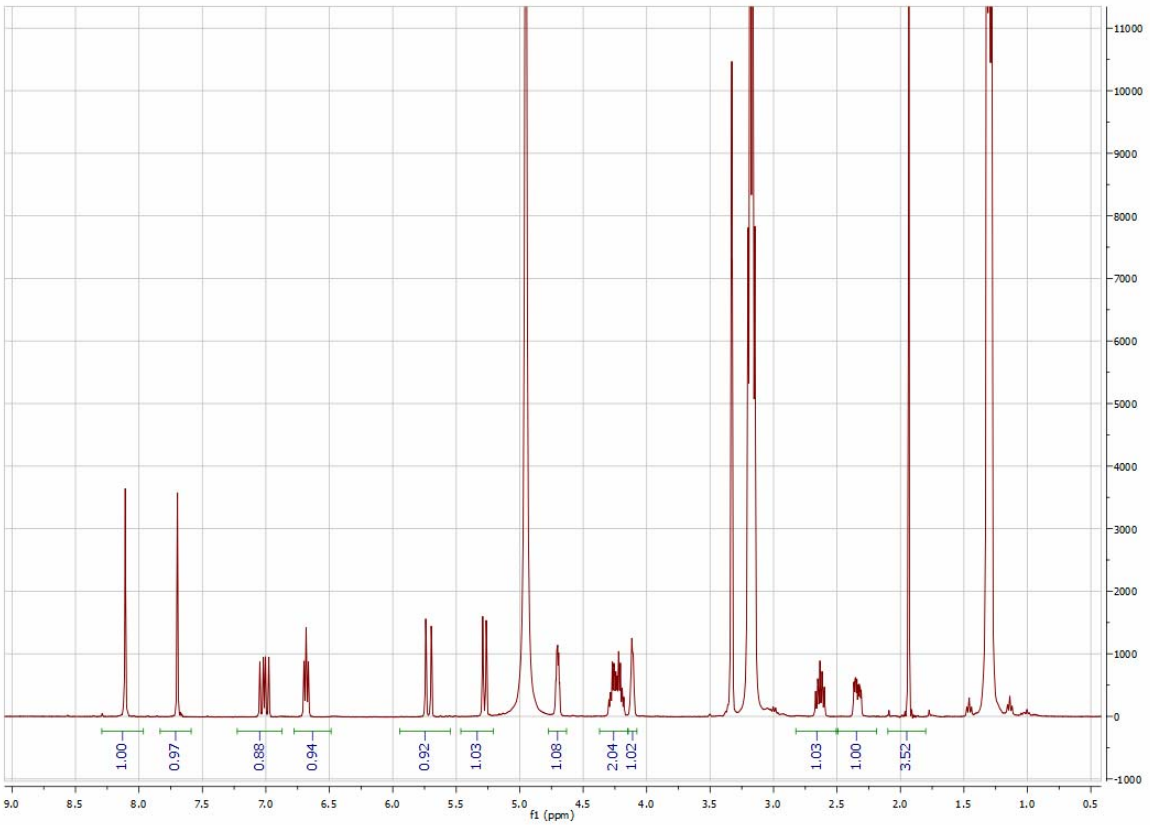

Fig. S5 ^{1}H-NMR (400 MHz) of d^{vin}ATP

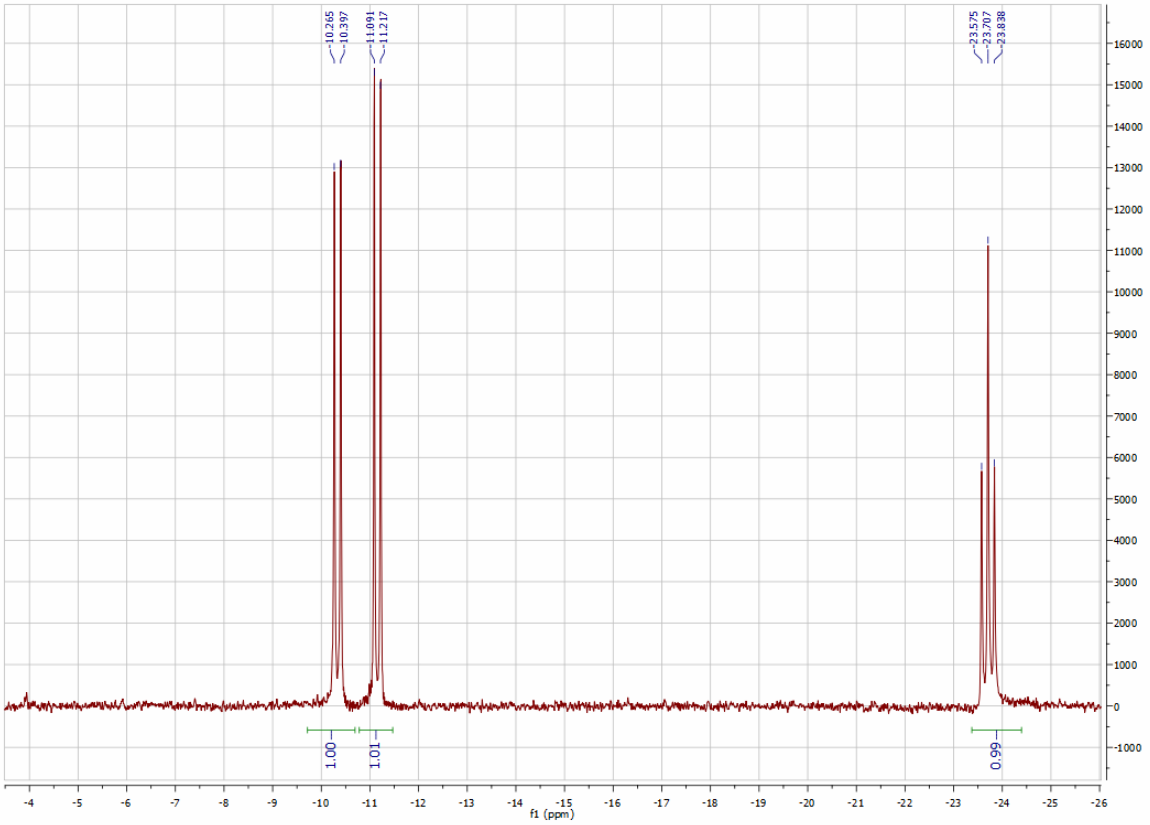

Fig. S5 ^{31}P-NMR (162 MHz) of d^{vin}ATP

References

1. F. Seela, M. Zulauf, M. Sauer and M. Deimel, *Helv. Chim. Acta*, 2000, **83**, 910.
2. A. Niederwieser, et al., *Angew. Chem., Int. Ed.*, 2013, **52**, 4265.
3. M. J. Frisch, et al., *Gaussian 10*, (2010) Gaussian, Inc., Wallingford, CT, USA.
4. K. Christopoulos, K. Karidi, A. Tsipis and A. Garoufis, *Inorg. Chem. Commun.*, 2008, **11**, 1341.
5. B. Machura, J. Mroziński, R. Kruszynski and J. Kusz, *Polyhedron*, 2008, **27**, 3013.
6. X.-L. Zeng, H.-J. Wang and Y. Wang, *Chemosphere*, 2012, **86**, 619.
7. N. M. O'Boyle, A. L. Tenderholt and K. M. Langner, *J. Comput. Chem.*, 2008, **29**, 839.