Eur. J. Org. Chem. **2016** · ISSN 1099–0682

SUPPORTING INFORMATION

DOI: 10.1002/ejoc.201501306
Title: Exploiting Acid Phosphatases in the Synthesis of Phosphorylated Monoalcohols and Diols
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General remarks

All chemicals were purchased from commercial suppliers and were used without purification. 4-Nitrophenyl phosphate disodium salt hexahydrate (*p*-NPP), sodium pyrophosphate dibasic (PP_i), polyphosphoric acid (polyP) and Immobead 150 were purchased from Sigma, Relizyme HA403/M and Sepabeads EC-HA/M were from Resindion, Purolite ECR4204M as well as phytase from *A. niger* were kind gifts from BASF. Eupergit C was from Röhm Pharma (currently Evonik), sodium tripolyphosphate (PPP_i) from Alfa Aesar. Ni-NTA column for His-tag purification was from GE Healthcare Life Sciences. NMR spectra were measured on a Bruker Avance III 300 MHz NMR spectrometer. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS or H_3PO_4 as a reference. GC-FID analysis was carried out on an Agilent 7890A equipped with FID using H_2 as a carrier gas (14.5 psi). HPLC analysis was carried out on a Dionex Ultimate 3000 system equipped with Shodex RI-101 refractory index detector (HPLC-RI) and on an Agilent 1260 Infinity system equipped with Agilent Q6120 quadrupole mass spectrometer using electrospray ionization (HPLC-MS).

Overexpression of enzymes

The genes encoding PhoN-Sf from *Shigella flexneri*[1] and PhoN-Se[2] from *Salmonella typhimurium* LT2 were ordered and synthetized by Life Technologies (UniProt accession Nos. O50542 and P26976, respectively). The genes coding for PiACP from *Prevotella intermedia* and AphA-St[3] from *Salmonella typhimurium* LT2 (UniProt accession No. O87188 and P58683, respectively) were obtained from DNA 2.0. All genes were cloned into pET-28a(+) and the expression vectors were subsequently transformed into *E. coli* BL21 (DE3) cells. All proteins were expressed as *N*-terminal histidine tagged proteins.

AphA-St, PhoN-Se and PiACP were produced as follows: 15 mL LB (lysogeny broth) containing 30 μg mL^{-1} kanamycin was inoculated with *E. coli* cells harboring the corresponding plasmid followed by shaking overnight at 37 °C and 120 rpm. Then the whole amount was transferred to 1 L LB medium containing 30 μg mL^{-1} kanamycin and the culture was grown at 37 °C and 120 rpm until an OD of ~0.8 was reached. Expression was initiated by induction with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) followed by shaking at 20 °C and 120 rpm overnight.

PhoN-Sf was expressed using autoinduction medium as described.[4] For 670 mL medium, the following sterilized mixtures were combined in a 2 L shaking flask: 618 mL aqueous solution of yeast extract (16.5 g L^{-1} final concentration) and tryptone (33 g L^{-1} final concentration), 13.4 mL aqueous solution of glycerol (25 w/v%), glucose (2.5 w/v%) and lactose (10 w/v%), 33.5 mL aqueous solution of NH_4Cl (1 M), Na_2SO_4 (0.1 M), KH_2PO_4 (0.5 M) and Na_2HPO_4 (0.5 M), 2 mL trace elements (20 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 2 mM $CoSO_4$, 2 mM $CuCl_2$, 2 mM

Na_2MoO_4, 2 mM H_3BO_3, 2 mM Na_2SeO_3, 2 mM $NiCl_2$ in 60 mM HCl) and 1.34 mL 1 M $MgSO_4$. Then 2 mL 50 mM $FeCl_3$ in 60 mM HCl and kanamycin (30 µg mL^{-1} final concentration) were added and the mixture was inoculated with 20 mL overnight culture and shaken for 3 d at 20 °C and 120 rpm. The cells were harvested by centrifugation (8000 rpm, 20 min, 4 °C), resuspended in 50 mM K-P_i pH 7.5 containing 300 mM NaCl and 10 mM imidazole (2 mL g^{-1} wet cells) and sonicated for 5 min (40% amplitude, 1 s pulse on, 4 s pulse off). After pelleting (15000 rpm, 20 min, 4 °C), the supernatant was purified on a Ni-NTA affinity column according to the supplier's protocol yielding 78.3, 12.8, 6.9 and 17.3 mg L^{-1} culture of purified PhoN-Sf, PhoN-Se, AphA-St and PiACP with a specific activity of 66.9, 19.3, 83.3 and 84.9 U mg^{-1}, respectively. The specific activity of phytase was 150 U mg^{-1}.

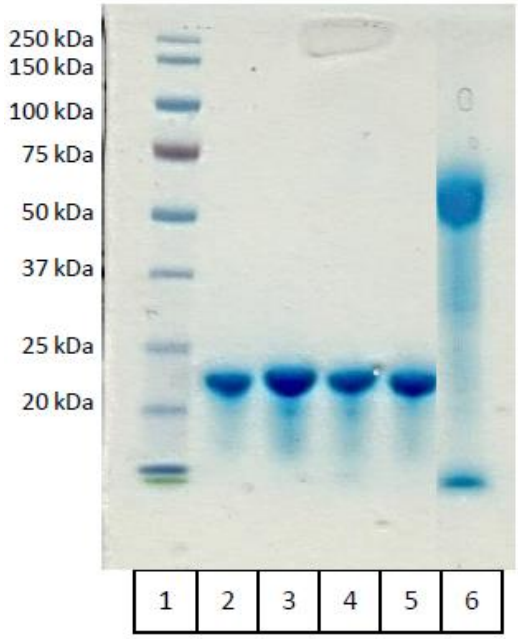

Fig. S1 SDS-PAGE of enzymes tested in this study. 1: protein ladder, 2: PhoN-Sf (27.2 kDa), 3: PhoN-Se (28.4 kDa), 4: PiACP (29.2 kDa), 5: AphA-St (26.3 kDa), 6: phytase (52.7 kDa, glycosylated)

Assay for phosphohydrolase activity (*p*-NPP assay)

Free and immobilized enzyme activities were assayed spectrophotometrically by measuring the dephosphorylation of 4-nitrophenyl phosphate (*p*-NPP) via release of *p*-nitrophenol. Soluble (100 µg mL^{-1}) or immobilized enzyme (10 µL settled beads) was added to maleate buffer pH 6.0 (100 mM final concentration, containing 1 mM $MgCl_2$ for AphA-St) to a final volume of 480 µL followed by the addition of 20 µL 250 mM *p*-NPP (10 mM final concentration in H_2O) and were mixed at 30 °C and 450 rpm. After 1 min incubation time, the reaction was quenched with 500 µL 1

M NaOH and the absorbance of 4-nitrophenol (*p*-NP) was recorded at 405 nm (ε = 18500 M^{-1} cm^{-1}). The activity tests were always performed in triplicates. One unit of phosphatase activity (U) corresponds to the amount of *p*-NP (micromoles) released per minute under assay conditions. Specific activity (A_{spec}) represents the phosphatase activity (U) of 1 mg protein or 1 g dry resin.

Magnesium supplementation study with AphA-St

The above described *p*-NPP assay was performed using 100 mM maleate or 250 mM PP_i buffer pH 6.0 supplemented with 0, 1 or 10 mM $MgCl_2$ and 50 μg mL^{-1} enzyme. Results are shown in Fig. S2.

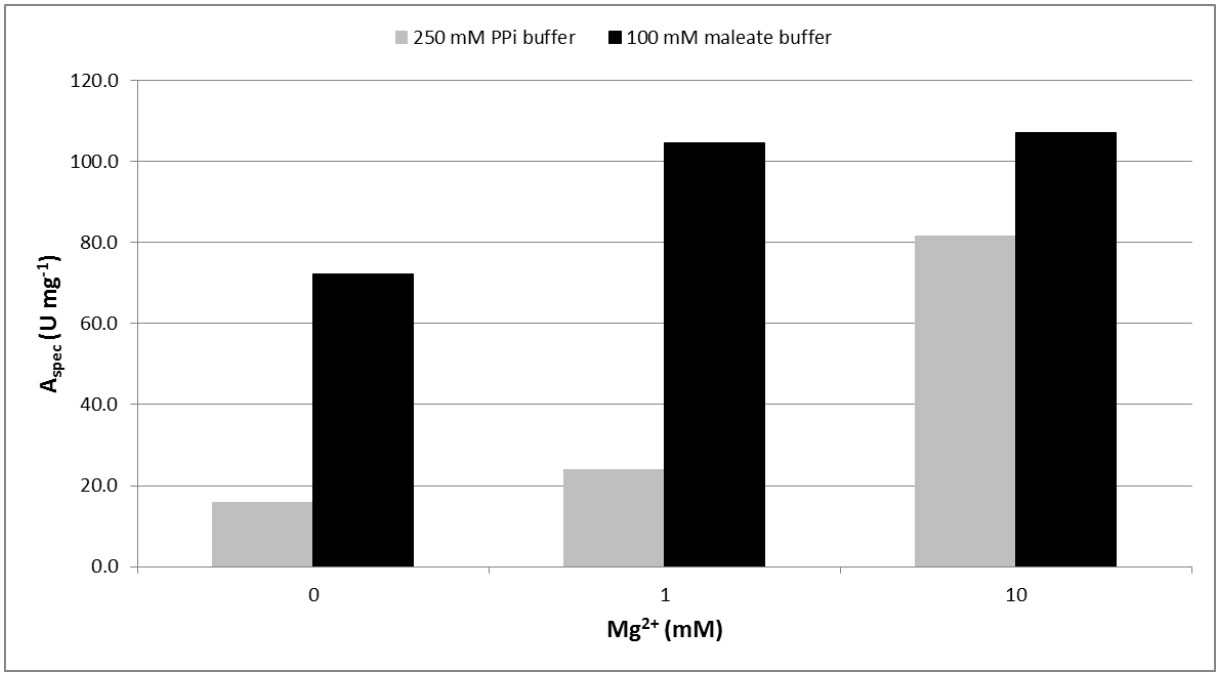

Fig. S2 Effect of Mg^{2+} on the hydrolytic activity of AphA-St in the *p*-NPP assay in maleate and PP_i buffer.

General conditions for enzymatic transphosphorylation

A standard reaction mixture contained substrate and P-donor in H_2O at a concentration indicated in the footnotes of tables and captions of figures and 1% DMSO as internal standard in 1 mL final volume at a given pH. The reactions were initiated by the addition of 50 μg mL^{-1} enzyme and were shaken at 30 °C and 600 rpm. 25 μL samples were taken at intervals and diluted to 500 μL with 5 mM H_2SO_4 followed by injection to HPLC-RI equipped with an Alltech OA-1000 or an Alltech IOA-2000 cation exchanger column (for conditions and retention times see Table S1).

With substrates **6** and **11-19** as well as to determine the regioselectivity on **8**, parallel experiments were run and at intervals one sample was quenched by adding 30 μL conc. HCl. 600 μL of the

mixture was added to 100 μL D_2O and ^{31}P-NMR spectrum was taken using inverse gated decoupling.

For the determination of enantiomeric excess of **10**, 25 μL samples were taken from the reaction mixture at intervals and extracted with 475 μL EtOAc followed by addition of 10 μL DMAP/pyridine 1:9 (w/v) and 10 μL Ac_2O. The organic layer was separated, dried with Na_2SO_4 and injected to GC-FID equipped with a Chrompack Chirasil-DEX CB β-cyclodextrin capillary column (25 m x 0.32 mm, 0.25 μm film), detector temperature 250 °C, injector temperature 250 °C, split ratio 50:1. Method: 100 °C, hold 15 min, 10 °C min^{-1} to 150 °C, hold 2 min (total: 22 min). Retention times (min): 19.7 for (1*S*,2*S*)-**10** and 20.1 for (1*R*,2*R*)-**10**.

Table S1 Conditions of HPLC analysis and retention times of compounds

compound	Alltech OA-1000[a]		Alltech IOA-2000[b]		Rezex OA[c]	
	retention time [min]		retention time [min]		retention time [min]	
	substrate	product	substrate	product	substrate	product
PP_i	4.3		3.6		19.7	
PPP_i	4.6		n/a		n/a	
polyP			n/a		n/a	
P_i	6.1		5.1		27.3	
DMSO	18.4		13.6		78.7	
1	12.4	5.4	n/a		n/a	
2	15.5	5.6 (4.5)[d]	n/a		66.0	25.7 (20.3)[d]
3	29.6	7.2 (4.7)[d]	n/a		n/a	
4	13.4	5.1 (4.3)[d]	n/a		58.0	23.6 (20.2)[d]
5	21.1	5.9	n/a		n/a	
7	n/a	n/a	30.2	7.5	n/a	
8	12.6	5.1/5.3	n/a		n/a	
9	21.9	6.8	n/a		n/a	
10	23.9	6.4	n/a		n/a	

[a] Eluent: 5 mM H_2SO_4; flow rate: 0.6 mL min^{-1}; 50 °C; injection volume: 40 μL; [b] eluent: 5 mM H_2SO_4; flow rate: 0.4 mL min^{-1}; 50 °C; injection volume: 40 μL; [c] eluent: 5 mM H_2SO_4; flow rate: 0.2 mL min^{-1}; 60 °C; injection volume: 40 μL; [d] bis-phosphorylated product in bracket; n/a = not applicable.

Product identification

Products were identified by HPLC-MS equipped with a Zorbax 300-SCX cation exchanger column [eluent: 0.1% (v/v) formic acid, flow rate: 1 mL min^{-1}; 40 °C; injection volume: 10 µL] and by ^{31}P-NMR. Representative ^{31}P-NMR spectra of the phosphorylation of a primary as well as a secondary alcohol are shown in Figures S3 and S4. Analogous products gave similar chemical shifts.

The ratio of mono- versus bis-phosphorylated product was determined by HPLC-RI equipped with a Rezex OA (for **2** and **4**) or an Alltech OA-1000 cation exchanger column (for **3**) according to area ratios on chromatograms (for conditions and retention times see Table S1).

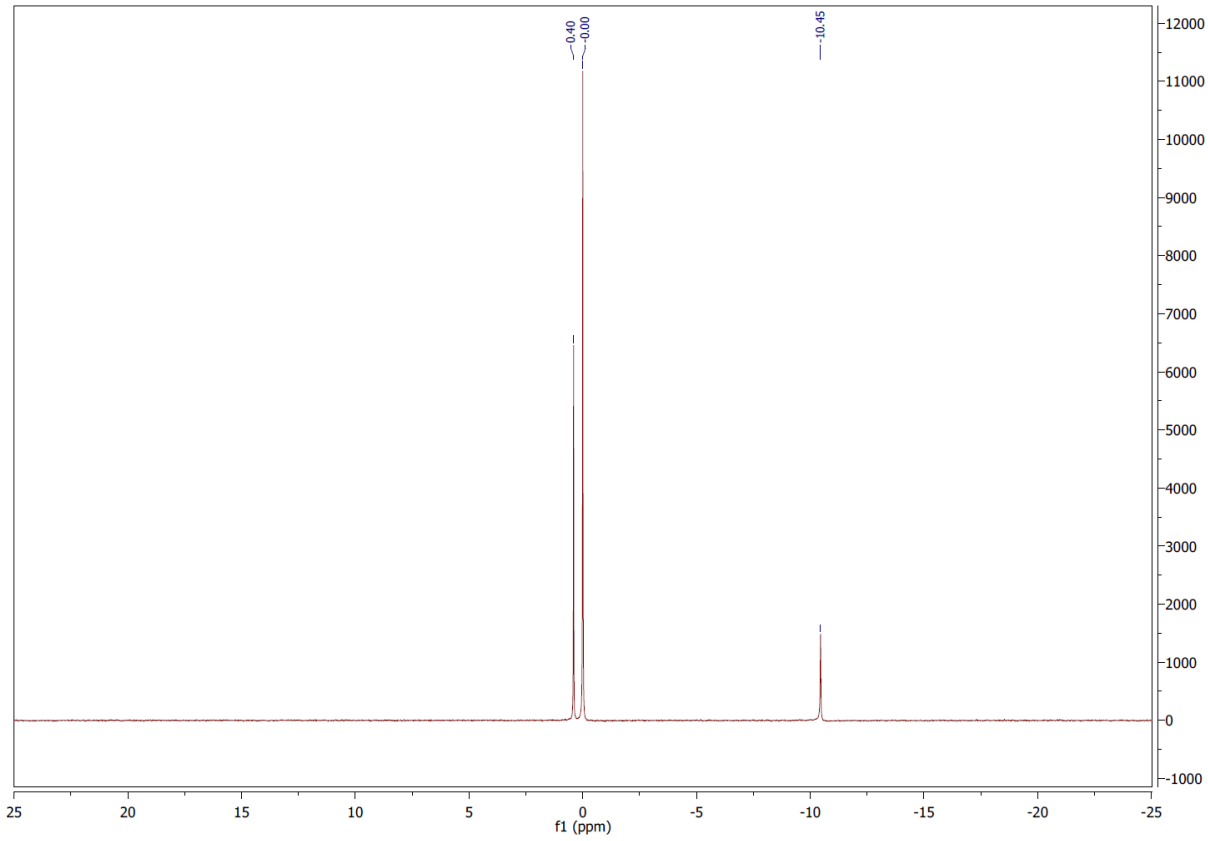

Fig. S3 ^{31}P-NMR spectrum of phosphorylation of 1,4-butanediol (**2**) (121 MHz, 15% D_2O): δ (ppm) = 0.40 (product), 0.00 (P_i), -10.45 (PP_i).

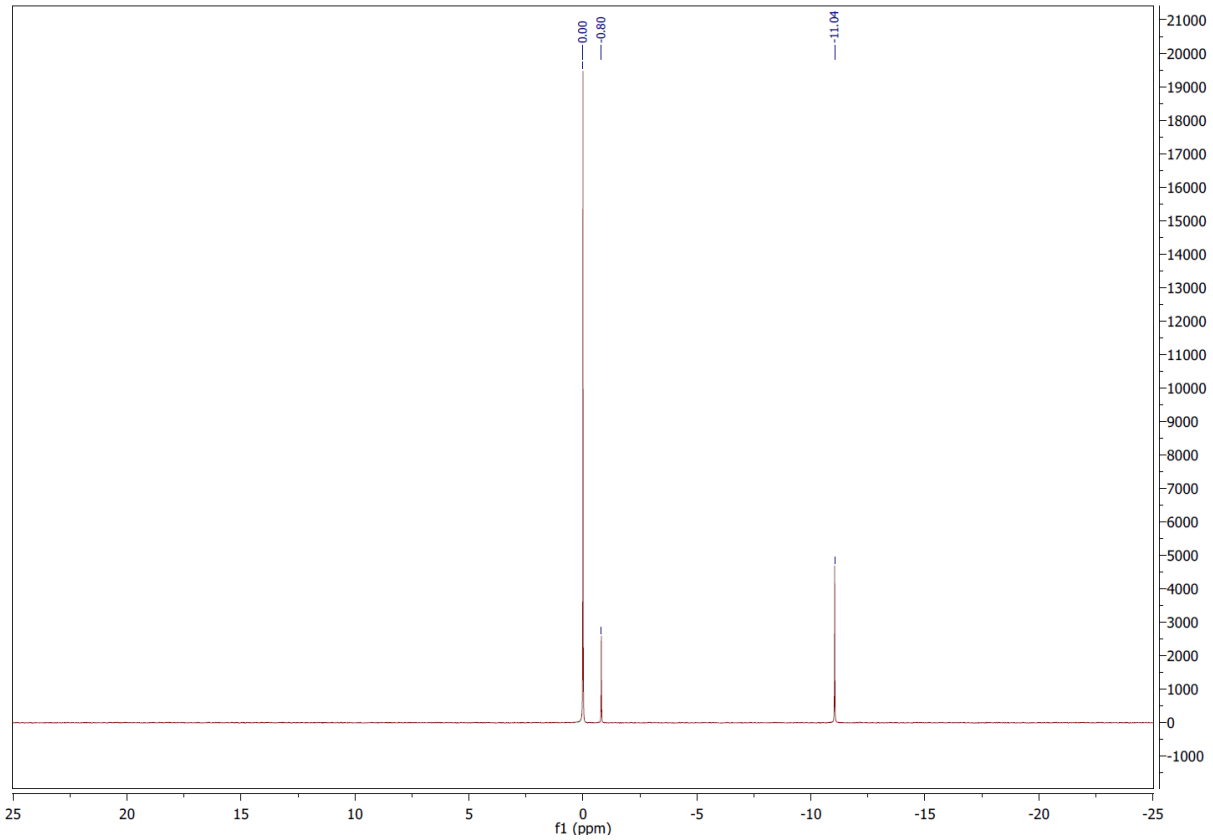

Fig. S4 ^{31}P-NMR spectrum of phosphorylation of cyclohexanol (**7**) (121 MHz, 15% D_2O): δ (ppm) = 0.00 (P_i), -0.80 (product), -11.04 (PP_i).

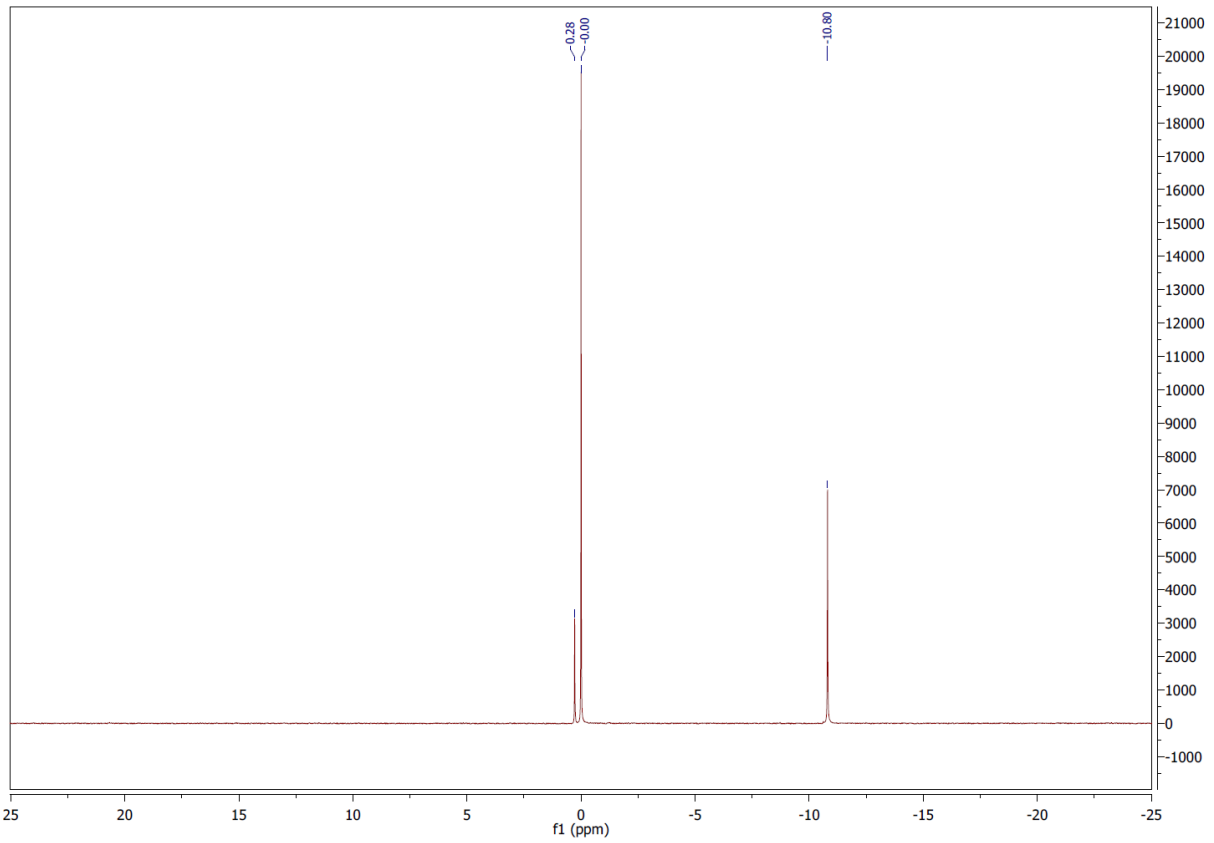

Fig. S5 ^{31}P-NMR spectrum of phosphorylation of 6-amino-1-hexanol (**6**) (121 MHz, 15% D_2O): δ (ppm) = 0.28 (product), 0.00 (P_i), -10.80 (PP_i).

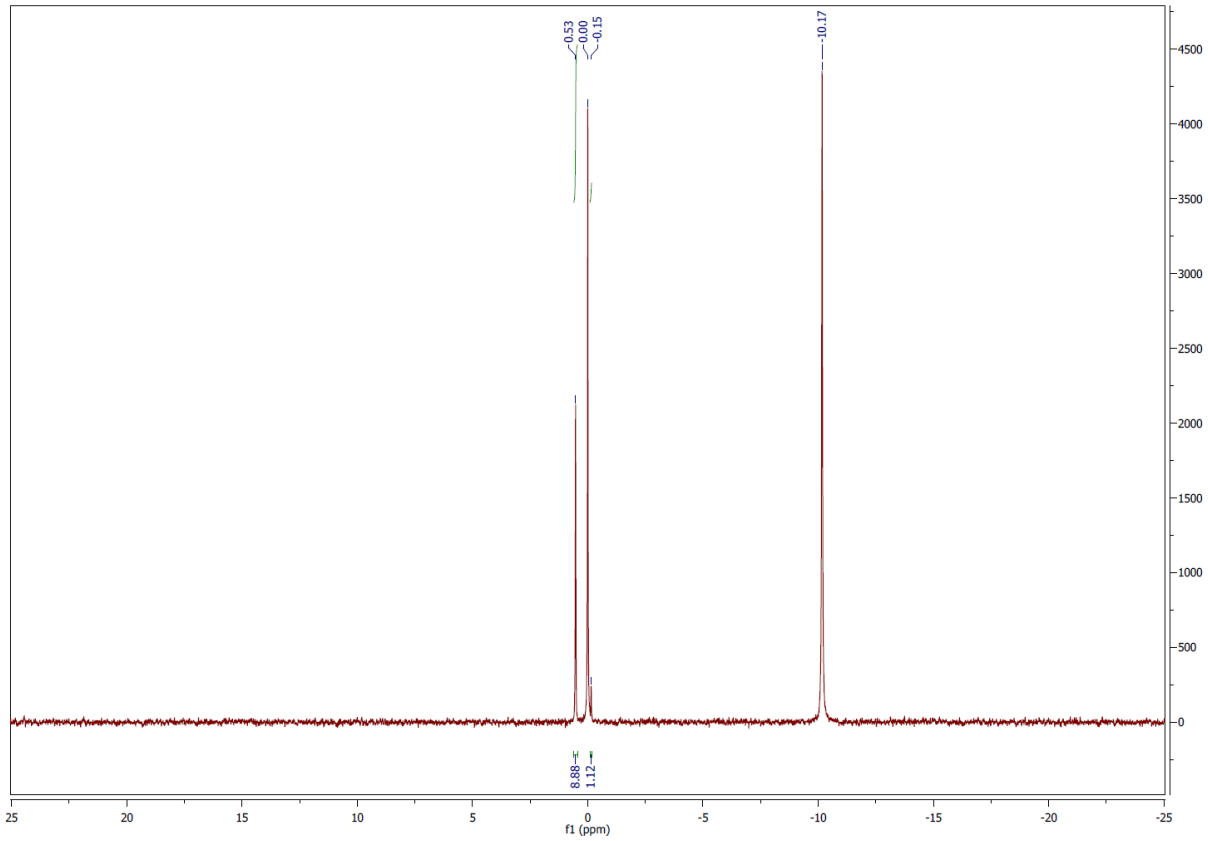

Fig. S6 ^{31}P-NMR spectrum of phosphorylation of 1,2-propanediol [(*R*)-**8**] (121 MHz, 15% D_2O): δ (ppm) = 0.51 (product, phosphorylation at *prim*-OH), 0.00 (P_i), -0.14 (product, phosphorylation at *sec*-OH), -10.16 (PP_i).

Stereoselectivity of PhoN-Se

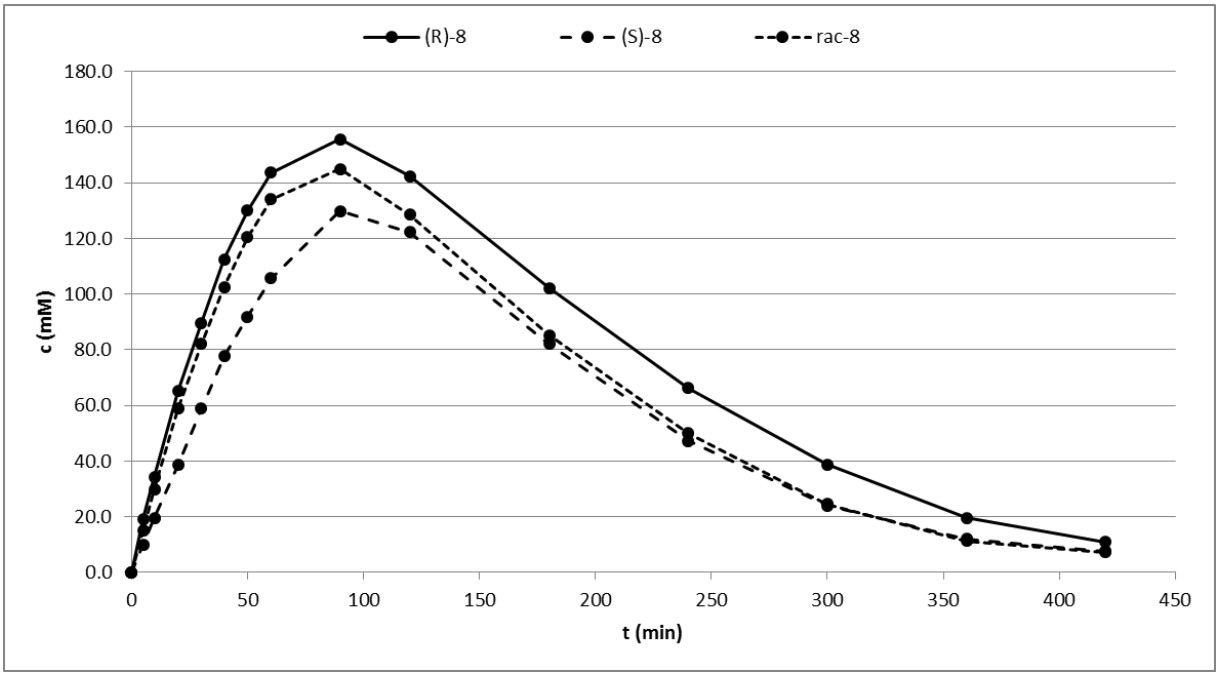

Fig. S7 Time course of the phosphorylation of *rac*-, (*R*) and (*S*)-**8** by PhoN-Se. Conditions: 500 mM **8**, 250 mM PP_i, 50 μg mL^{-1} PhoN-Se, pH 4.2, 1 mL volume, 1% DMSO as internal standard, 30 °C, 600 rpm shaking.

Table S2 Amount of product and P_i formed in acid phosphatase-catalyzed phosphorylation of **2** with various P-donors at various pHs over 16.5 h

		PP_i (500 mM)			**PPP_i (250 mM)**			**PPP_i (400 mM)**		
		PhoN-Sf	**PiACP**	**PhoN-Se**	**PhoN-Sf**	**PiACP**	**PhoN-Se**	**PhoN-Sf**	**PiACP**	**PhoN-Se**
pH 4.5	**product (mM)**	59	7	1	108	13	5	232	81	17
	P_i (mM)	936	866	911	548	686	694	1002	1165	1128
	product/P_i[a]	0.06	0.01	0.00	0.20	0.02	0.01	0.23	0.07	0.02
	Cons.$_{donor}$ (%)[b]	100	87	91	87	93	93	103	104	95
	Conv. (%)[c]	12	1	0	22	3	1	46	16	3
pH 4.8	**product (mM)**	53	6	0	104	16	6	17	7	9
	P_i (mM)	935	879	911	594	708	722	1178	1180	1153
	product/P_i[a]	0.06	0.01	0.00	0.18	0.02	0.01	0.01	0.01	0.01
	Cons.$_{donor}$ (%)[b]	99	89	91	93	97	97	100	99	97
	Conv. (%)[c]	11	1	0	21	3	1	3	1	2
pH 5.5	**product (mM)**	0	0	0	0	0	0	5	9	7
	P_i (mM)	968	840	891	690	692	692	1138	1119	1132
	product/P_i[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01
	Cons.$_{donor}$ (%)[b]	97	84	89	92	92	92	95	94	95
	Conv. (%)[c]	0	0	0	0	0	0	1	2	1

Reaction conditions: 500 mM **2**, phosphate donor as indicated, 50 μg mL^{-1} PhoN-Sf, pH as indicated, 1 mL volume, 1% DMSO as internal standard, 30 °C, 600 rpm shaking, 16.5 h. [a] Ideal (maximum obtainable) product to P_i ratio is 1:1 with PP_i and 2:1 with PPP_i; [b] cons.$_{donor}$: consumption of P-donor calculated as follows: $(c_{prod} + c_{Pi})/c_{theoretical\ max.\ Pi} \times 100$; [c] conv.: conversion of substrate with respect to theoretical maximum.

Influence of P-donor on the phosphorylation of 2

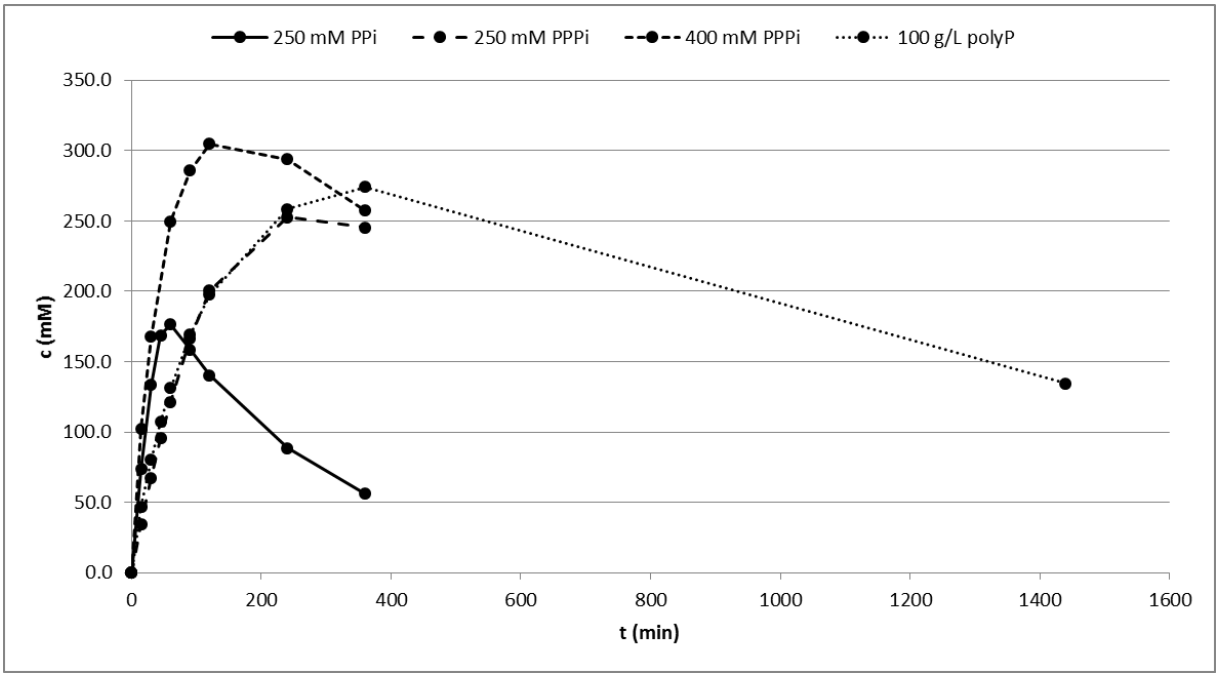

Fig. S8 Product formation in the phosphorylation of **2** with PhoN-Sf and different P-donors. Conditions: 500 mM **2**, 50 μg mL^{-1} PhoN-Sf, pH 4.2, 1 mL volume, 1% DMSO as internal standard, 30 °C, 600 rpm shaking.

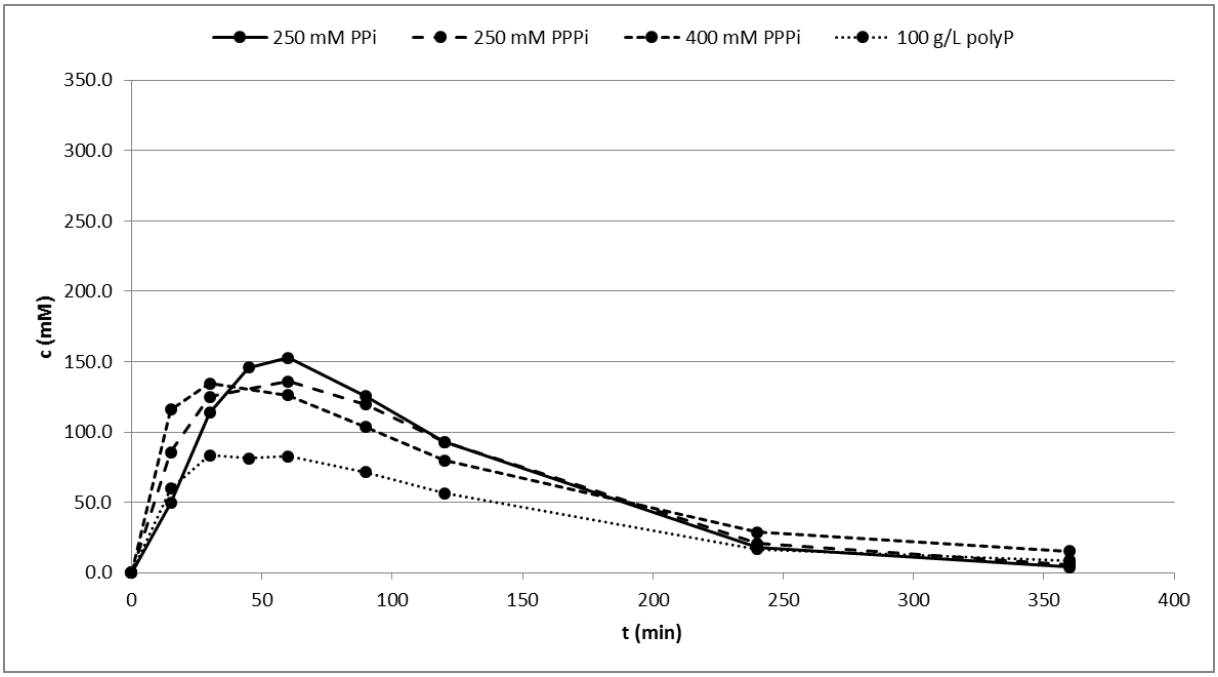

Fig. S9 Product formation in the phosphorylation of **2** with PhoN-Se and different P-donors. Conditions: 500 mM **2**, 50 μg mL^{-1} PhoN-Se, pH 4.2, 1 mL volume, 1% DMSO as internal standard, 30 °C, 600 rpm shaking.

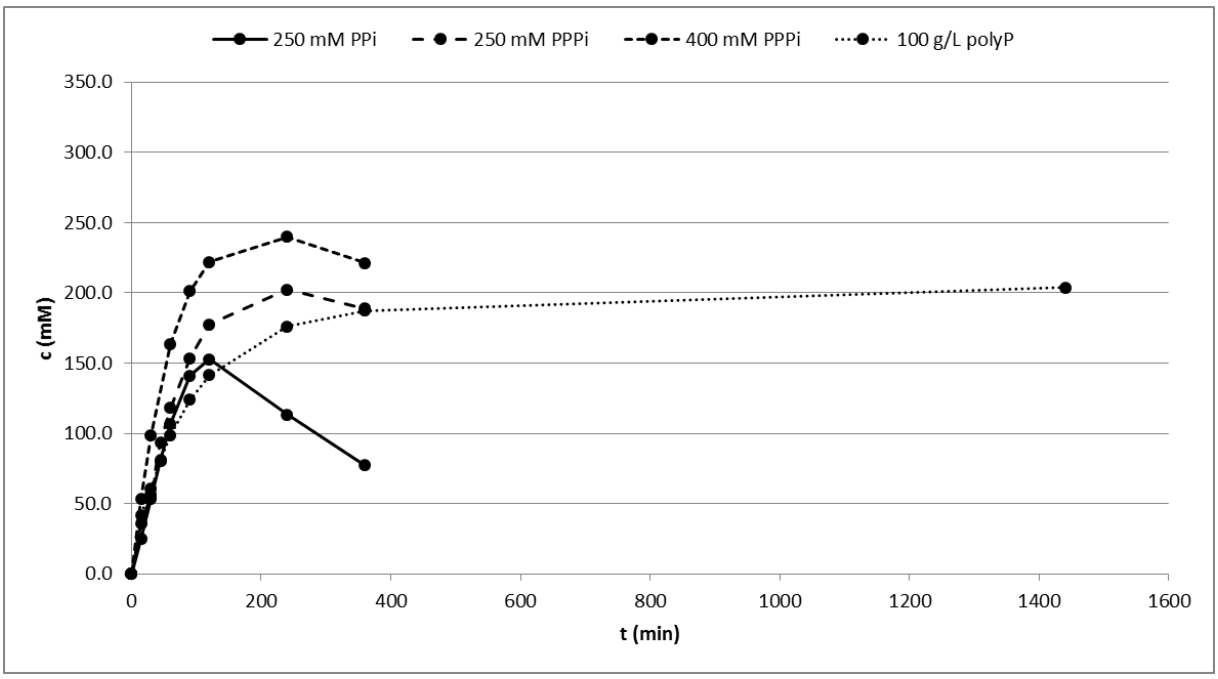

Fig. S10 Product formation in the phosphorylation of **2** with PiACP and different P-donors. Conditions: 500 mM **2**, 50 μg mL^{-1} PiACP, pH 4.2, 1 mL volume, 1% DMSO as internal standard, 30 °C, 600 rpm shaking.

Immobilization of PhoN-Sf and PiACP

Before immobilization, Sepabeads EC-HA/M and Relizyme HA403/M beads were activated via functionalization with glutaraldehyde. The beads (100 or 300 mg wet beads) were mixed with 20 mM K-P_i buffer pH 8 (1 or 3 mL) and 40 μL 25% glutaraldehyde (~0.4 mM). The mixture was shaken (120 rpm) for 1 h at room temperature. Afterwards the beads were washed with 1 mL of the buffer and shaken (120 rpm) for 30 minutes at room temperature. The latter step was repeated three times, then the beads were stored in 100 mM K-P_i buffer at pH 7 and 4 °C or used immediately for immobilization.

To the beads (100 or 300 mg wet beads) was added 1.25 M K-P_i buffer pH 8 (5 or 15 mL) and the enzyme (100 or 300 U). The mixture was shaken at room temperature and 120 rpm for 6 hours. The immobilization process was followed by measuring the activity of the supernatant (10 μL) at various time points. When no more decrease in the supernatant activity was observed, the beads were washed with 1 mL H_2O (2×) and 1 mL 2 M glycine buffer pH 8.5 (1×) and shaken in this buffer overnight at room temperature and 120 rpm. The supernatant was removed; the beads were washed with 1 mL H_2O (2×) and 1 mL 100 mM K-P_i buffer pH 7 (1×) and stored in this buffer at 4 °C.

Table S3 Results from immobilization of PhoN-Sf and PiACP.

Enzyme	Bead	Immobilization yield [%][a]	Immobilization efficiency [%][b]	Activity recovery [%][c]	A_{spec} [U × g dry beads]
PhoN-Sf	Immobead 150	97	9.4	9.1	100
	Eupergit C	88	10.5	9.2	101
	Purolite ECR4204/M	0	0	0	0
	Sepabead EC-HA/M	97	3.4	3.3	68
	Relizyme HA403/M	99	5.8	5.7	159
PiACP	Immobead 150	94	14.4	13.5	149
	Eupergit C	83	9.9	8.3	91
	Purolite ECR4204/M	33	11.6	3.8	66
	Sepabead EC-HA/M	96	4.2	4.1	85
	Relizyme HA403/M	85	9.1	7.7	215

[a] Total immobilized activity/total starting activity × 100; [b] total observed activity/total immobilized activity × 100; [c] total observed activity/total starting activity × 100.[5]

Determination of the weight of the settled wet beads

In order to determine the specific activity of the immobilized preparations, it is necessary to know the weight of the settled wet beads (Table S4). To this end, a suspension of the beads was prepared with H_2O. Various amounts (10, 20, 30, 40, 50 μL) of the settled beads were added into Eppendorf vials. The beads were freeze-dried and the weight of the dry beads was measured and plotted against the volume of the settled wet beads. Linear relationships were obtained and allowed determination of the dry bead weight (mg) per 10 μL.

Table S4

Bead type	Weight of dry beads corresponding to 10 μL wet settled beads (mg)
Immobead 150	0. 88
Eupergit C	1.47
Purolite ECR 4204/M	0.79
Sepabeads EC-HA/M	1.30
Relizyme HA403/M	0.53

Preparative-scale synthesis of 4-hydroxybutyl phosphate

An aqueous solution of 500 mM **2** and 250 mM PP_i at pH 4.2 was pumped through a column (50 × 4.6 mm) containing PhoN-Sf immobilized on Relizyme HA403/M resin (300 mg wet bead) with a

flow rate of 0.3 mL min^{-1} at 30 °C. To the recovered product mixture (220 mL, 150 mM 4-hydroxybutyl phosphate, 60% conversion of maximum transferable phosphate) was added 500 mM $Ba(OAc)_2$ and the pH was adjusted to 9. After stirring for an hour at room temperature, the mixture was filtered and 3 volumes of EtOH were added to the filtrate. The product was allowed to precipitate overnight at 4 °C. Filtration and drying at room temperature resulted in 6.86 g (22.5 mmol) of 4-hydroxybutyl phosphate barium salt (41% yield of maximum transferable phosphate). ^{1}H, ^{13}C and ^{31}P-NMR spectra of product recorded in CH_3COOD are shown in Fig. S11, S12 and S13, respectively and confirmed the purity of the product. ^{1}H-NMR (300 MHz, CH_3COOD): δ (ppm) = 1.52 (4H, m, $CH_2CH_2CH_2CH_2$), 3.51 (2H, m, CH_2CH_2OH), 3.76 (2H, m, $CH_2CH_2OPO_3^{2-}$). ^{13}C-NMR (75 MHz, CH_3COOD): δ (ppm) = 21.8, 26.3 (d, J_{POCC} = 6.9 Hz), 61.3, 65.4 (d, J_{POC} = 5.2 Hz). ^{31}P-NMR (121 MHz, CH_3COOD): δ (ppm) = 0.44.

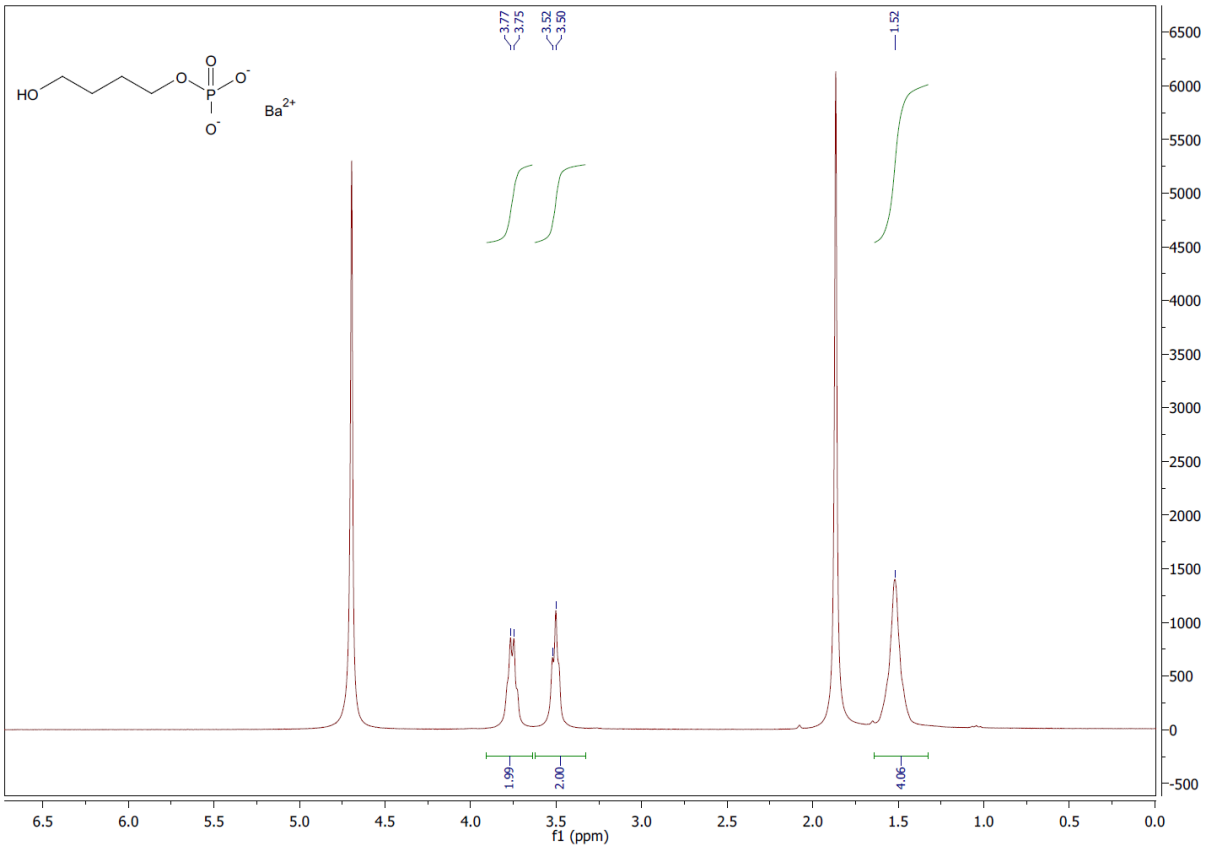

Fig. S11 ^{1}H-NMR spectrum of 4-hydroxybutyl phosphate

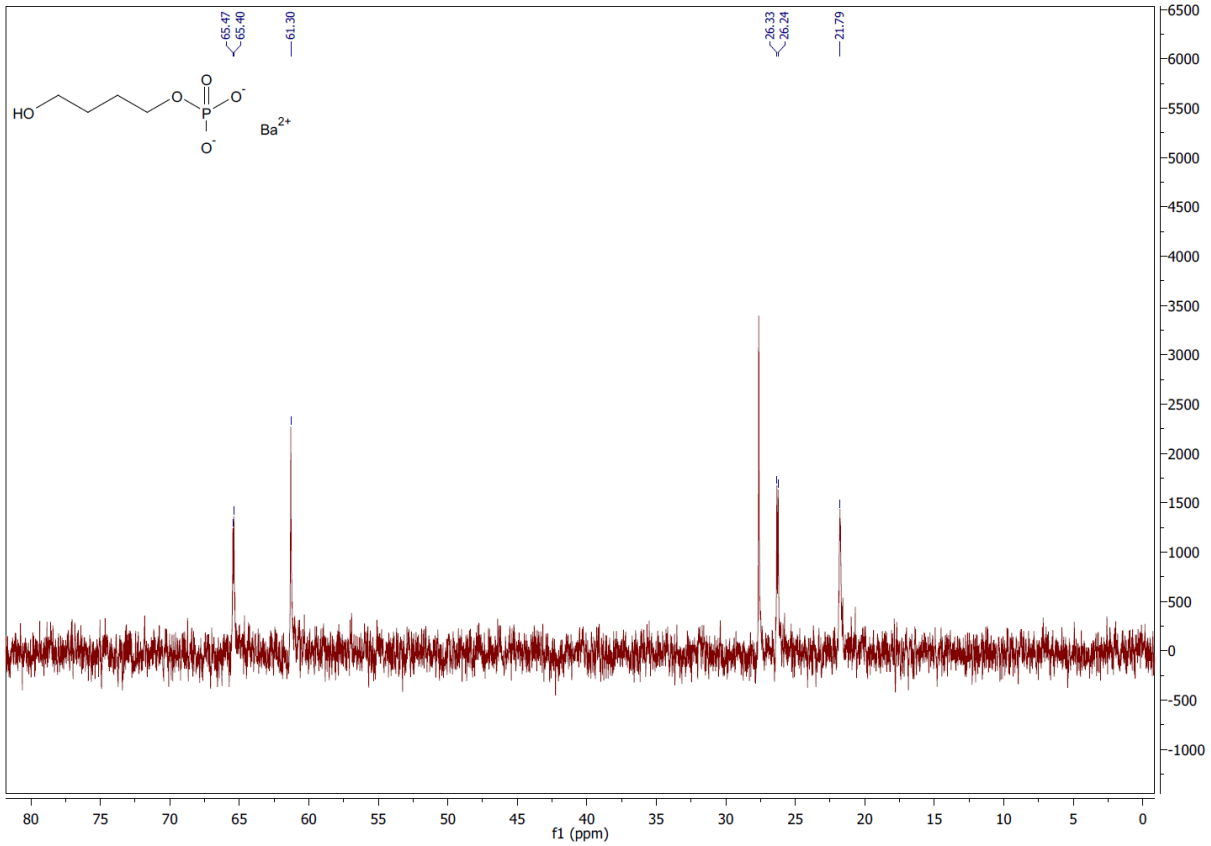

Fig. S12 ^{13}C-NMR spectrum of 4-hydroxybutyl phosphate

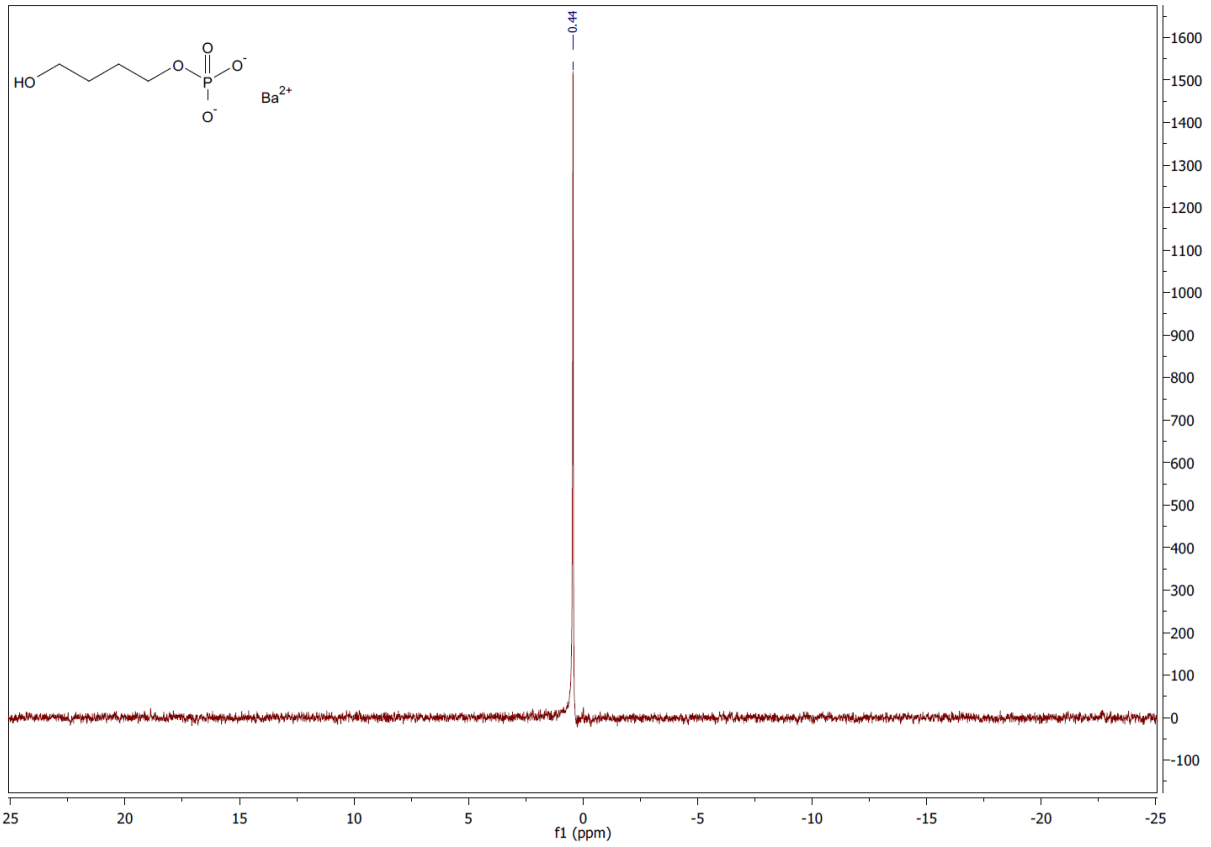

Fig. S13 ^{31}P-NMR spectrum of 4-hydroxybutyl phosphate

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