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Communication

Phosphorylation

Exploiting Acid Phosphatases in the Synthesis of Phosphorylated Monoalcohols and Diols

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Abstract: A set of phosphatases was evaluated for their potential to catalyze the regio- and stereoselective phosphorylation of alcohols using a high-energy inorganic phosphate donor, such as di-, tri- and polyphosphate. Parameters such as type and amount of phosphate donor and pH of the reaction were investigated in order to minimize the thermodynamically favored hydrolysis of the phosphate donor and the formed phosphate ester. Diols were monophosphorylated with high selectivities. This biocatalytic phosphorylation method provides selectively activated and/or protected synthetic intermediates for further chemical and/or enzymatic transformations and is applicable to a large scale (6.86 g) in a flow setup with immobilized phosphatase.

Introduction

Functional groups often require activation in order to enhance their reactivity in synthetic protocols. The OH functionality is commonly activated by attachment of a good leaving group, e.g., by conversion into an ester derivative of a strong acid (tosylate, mesylate, triflate), usually employing Mitsunobu or Appel conditions.[1] This classic methodology suffers from poor atom economy and creates large amounts of waste, thus rendering these processes inefficient and unattractive, although remarkable progress towards catalytic processes was made in the past decade.[2] In nature, activation of hydroxy groups often proceeds through enzymatic phosphorylation by kinases using activated phosphate (adenosine triphosphate, ATP) or sulfation catalyzed by mammalian sulfotransferases in conjunction with the respective cofactor, i.e., 3′-phosphoadenosine-5′-phosphosulfate (PAPS).[3] Nonspecific bacterial arylsulfotransferases are also able to sulfate hydroxy groups by using organic sulfate donors.[4] The requirement for molar equivalents of these cofactors in synthetic applications is problematic from a scale-up perspective. Although enzymatic ATP recycling is feasible with reasonable efficiency (up to several hundred TTNs),[4a,5] PAPS cannot be recycled efficiently so far.[4] Most importantly, ATP-dependent kinases and PAPS-dependent sulfotransferases are typically involved in bio*synthetic* pathways and signal messaging and thus are often highly substrate-specific and hence do not allow broad synthetic applications.

An efficient alternative to kinases is the use of ATP-independent phosphatases using cheap inorganic polyphosphates (pyrophosphate, PP_i; triphosphate, PPP_i; polyphosphoric acid, polyP) as activated phosphate donor (P-donor), which circumvents the need for cofactor recycling.[6] Additionally, due to their occurrence in bio*degradation* pathways, phosphatases usually display a broad substrate spectrum, and inorganic phosphate formed as by-product is environmentally benign. A major drawback resides in the reversibility of phosphate transfer, causing the undesired hydrolysis of the newly formed phosphate ester, which requires a kinetically tightly controlled system (Figure 1).

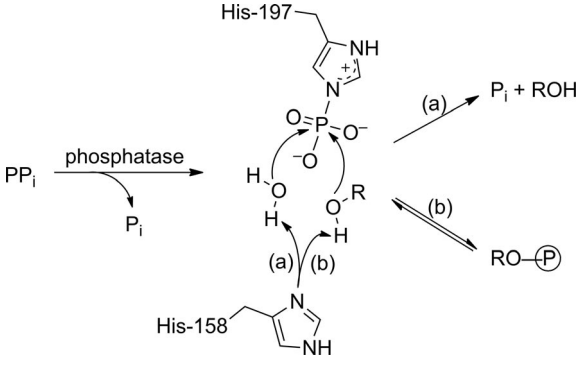

Figure 1. Mechanism of (a) competing hydrolysis and (b) transphosphorylation involving a covalent phospho-enzyme intermediate catalyzed by PhoNSe.[26]

The use of phosphatases for synthetic applications dates back to the late 1940s, when Axelrod and Appleyard achieved

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transphosphorylation using phosphatase-enriched extracts and *p*-nitrophenyl phosphate (*p*-NPP) or phenolphthalein phosphate as donor.[7] Later, Nordlie identified glucose-pyrophosphotransferase activity of *E. coli* alkaline phosphatase;[8] however, the method was applied on a preparative-scale only decades later.[9] Asano et al. applied acid phosphatases from enteric bacteria to the phosphorylation of nucleosides and designed enzyme mutants with reduced hydrolytic activity.[10] Acid phosphatases from *Shigella flexneri* (PhoN-Sf) and *Salmonella enterica* ser. *typhimurium* LT2 (PhoN-Se) were found to have a broad substrate spectrum[11] and were applied in combination with aldolases to synthesize natural and unnatural sugar molecules on a gram-scale.[12]

In drug discovery, phosphorylation is a common prodrug strategy to enhance the accessibility of drug candidates within the body by enhancing their polarity and solubility.[13] In some cases, the phosphorylated form may be the active species.[14] In synthetic chemistry, phosphate di- and triesters are widely applied as Brønsted acid catalysts for various asymmetric transformations[15] and as leaving groups in transition-metal-catalyzed coupling reactions[16] as well as in organolithium[17] and alkene synthesis.[18] In contrast, phosphate monoesters have attracted so far little attention as tools for functional-group modification in classic chemical processes, most likely as a consequence of their insolubility in organic solvents resulting from their negative charge. Current chemical routes to phosphate monoesters often proceed via the corresponding phosphate di- or triesters and require harsh reagents and conditions leading to hardly separable product mixtures.

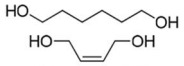
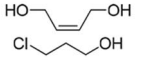
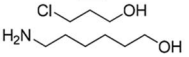
In this study, we aimed to evaluate a set of phosphatases as potential candidates for synthetic applications. Various techniques were investigated to enhance product titers of phosphorylated alcohols and to minimize the thermodynamically favored hydrolytic reaction.

Results and Discussion

Enzyme candidates were selected from the family of acid phosphatases, which have been shown to transfer efficiently a phosphate moiety from a high-energy inorganic di/tri/polyphosphate onto various hydroxylated compounds. Nonspecific acid phosphatase PhoN-Se from *Salmonella typhimurium* and acid phosphatase PhoN-Sf from *Shigella flexneri* have been reported to convert various primary and secondary alcohols and sugars[11] and were implemented in cascade reactions involving glucose-6-phosphate[19] and dihydroxyacetone phosphate as intermediates.[12] Acid phosphatase phytase catalyzes the hydrolysis of phytate, a phosphorylated natural cyclitol by liberating inorganic phosphate and is therefore commonly used as animal feedstock additive.[20] Acid phosphatase AphA-St from *Salmonella typhimurium* LT2[21] was selected due to its phosphotransferase activity on alcohols using *p*-NPP as donor.[22] PiACP from *Prevotella intermedia*[23] was selected based on high sequence similarity with robust PhoC-Mm developed for nucleoside phosphorylation.[24] All enzymes except phytase were overexpressed in *E. coli* and subsequently purified to homogeneity by using His-tag or Strep-tag techniques (Figure S1).

A common mechanistic feature of phosphate-transferring phosphatases is the formation of a covalent phospho-enzyme intermediate, which undergoes either nucleophilic attack by water (hydrolysis) or accepts a suitable nucleophile (transphos-

Table 1. Substrate scope of PhoN-Sf, PhoN-Se and PiACP in transphosphorylation.

Substrate		Substrate [mM]	P-donor [mM]	Selectivity	Maximal product level [mM][a,b] PhoN-Sf	PhoN-Se	PiACP
	**1**	500	PP_i (250)	only monophosphate	119 (120)	96 (90)	83 (60)
	**2**	500	PP_i (250) PPP_i (250) PPP_i (400) polyP (100)[c]	mono/bisphosphate 90:10	176 (60) 254 (240) 305 (120) 274 (360)	153 (60) 136 (60) 134 (30) 83 (30)	153 (120) 202 (240) 240 (240) 187 (360)
	**3**	500	PP_i (250)	mono/bisphosphate 85:15	187 (90)	146 (30)	141 (60)
	**4**	300	PP_i (250)	mono/bisphosphate 90:10[d]	105 (90)	120 (45)	76 (30)
	**5**	300/500	PP_i (250)	n.a.	78 (240)[e]	113 (60)	69 (120)[e]
	6	500[f]	PP_i (250)	only *O*-phosphorylation	84 (180)	85 (90)	71 (60)
	**7**	200/300	PP_i (250)	n.a.	<1[g]	48 (60)[e]	<1[g]
	(*R*)-**8**	500	PP_i (250)	*prim/sec*-phosphate PhoN-Se & PiACP >99:<1 PhoN-Se 90:10	58 (240)	142 (60)	51 (90)
	(*S*)-**8**	500	PP_i (250)	n.a.	58 (240)	126 (60)	47 (90)
	(*R*)-**9**	500	PP_i (250)	n.a.	n.d.	60 (180)	n.d.
	(*S*)-**9**	500	PP_i (250)	n.a.	n.d.	65 (180)	n.d.
	*rac-trans*-**10**	250	PP_i (250)	only monophosphate nonstereoselective	n.d.	52 (60)	n.d.

[a] Reaction conditions: pH = 4.2, 50 μg mL^{-1} enzyme, 1 mL of volume, 1 % DMSO as internal standard, 30 °C, 600 rpm shaking. PP_i: pyrophosphate; PPP_i: triphosphate; polyP: polyphosphoric acid. n.d. = not determined, n.a. = not applicable. Product levels were determined by consumption of substrate using HPLC with RI detector (control experiments used as reference). [b] Time [min] needed to reach maximal product concentration in brackets. [c] In g L^{-1}. [d] Limit of detectability of bisphosphate is ca. 5–10 %. [e] 300 mM substrate used. [f] Monitored by ^{31}P NMR spectroscopy. [g] Only hydrolysis of P-donor observed.

phorylation) (Figure 1). To this end, most enzymes use histidine as catalytic residue (PhoN-Sf, PhoN-Se, PiACP and phytase) except AphA-St which uses aspartic acid.[25] Due to the competition between hydrolysis and transphosphorylation in aqueous medium, high substrate loads are required to achieve high product titers, while kinetic control is crucial to keep the phosphorylation/hydrolysis ratio as high as possible.

In order to verify the activity of all recombinant proteins, a spectrophotometric assay based on the hydrolysis of *p*-NPP was used. A range of alcohols was investigated, including primary and secondary mono- and dihydroxy compounds, as well as cyclic substrates (Table 1). Products were identified by ^{31}P NMR spectroscopy and HPLC-MS (see Supporting Information). Although phytase has been successfully employed in a cascade reaction to convert glycerol into glycerol-1-phosphate in a non-stereoselective fashion,[27] under our assay conditions, the enzyme showed no transphosphorylation activity at pH = 2.5 and 4.2 on any substrate. Strong hydrolytic activity on inorganic phosphate donors was still observed, confirming active protein (data not shown). AphA-St also contains Mg^{2+} (in addition to the catalytically active nucleophile aspartate[21]), whose activity is affected by inorganic polyphosphates by metal chelation. Although the addition of 10 mM $MgCl_2$ could partly restore the hydrolytic activity on *p*-NPP in the presence of 250 mM PP_i (Figure S2), only traces of product and marginal hydrolytic activity were obtained in the transphosphorylation of 1,4-butanediol (500 mM) by using 100 mM PP_i as donor at pH = 4.2 and 5.5, independent of the $MgCl_2$ concentration (10, 50, 100 mM) (data not shown). This suggests that inorganic oligophosphates (as opposed to *p*-NPP) are not ideal phosphate donors for metal-containing phosphatases, such as AphA-St. On the contrary, PhoN-Sf, PhoN-Se and PiACP all proved to be efficient catalysts in the phosphorylation of various alcohols (Table 1).

Phosphorylation of *prim*-diols proceeded to various degrees depending on the chain length: While short-chain ethylene glycol (**1**) was selectively monophosphorylated, 1,4-butanediol (**2**) and the unsaturated (*Z*) analog **4** yielded the corresponding mono/bisphosphorylated products in a ratio of 90:10. Extending the chain length even further (1,6-hexanediol, **3**) gave 85:15 of mono/diphosphate as determined by LC-RI chromatography (see Supporting Information). It is obvious, that the presence of a (negatively charged) phosphate group in the proximity of the reacting hydroxy moiety determines whether an α,ω-diol undergoes bisphosphorylation: While short-chain ethylene glycol is exclusively monophosphorylated, long-chain 1,6-hexanediol yields the bisphosphate in up to 15 %.

Haloalcohol **5** was also converted: PhoN-Se tolerated higher substrate concentrations than PhoN-Sf and PiACP (500 mM vs. 300 mM) resulting in the formation of 113 mM product in 60 min. To probe the chemoselectivity of the enzymes for *O*- vs. *N*-nucleophiles, 6-amino-1-hexanol (**6**) was tested. The three enzymes exclusively furnished the *O*-phosphate monoester at very similar product levels (ca. 71–85 mM), while the amino moiety remained untouched. This was proven by ^{31}P NMR spectroscopy resulting in a chemical shift typical for signals of phosphorylated primary hydroxy groups (Figure S5).[28] In line with this observation, 1,6-diaminohexane (**12**) was not converted, only P-donor hydrolysis was observed (data not shown), proving that these enzymes do not accept amines in place of alcohols. Sterically demanding secondary alcohol **7** was transformed only with PhoN-Se, which is in line with the previously observed preference of this enzyme for *sec*-alcohols.[11] Similar observations were made with 1,2-propanediol (**8**), which was exclusively phosphorylated at the *prim*-alcohol by PhoN-Sf and PiACP, while PhoN-Se showed reduced selectivity (ca. 90:10 *prim*/*sec*-phosphate, determined by ^{31}P NMR spectroscopy; Figure S6).

In order to elucidate the enantioselectivity of phosphatases, the two enantiomers of **8** were tested separately. In case of PhoN-Sf and PiACP, similar activities on both enantiomers were observed indicating non-enantioselective phosphorylation. With PhoN-Se, a marginal enantiopreference for (*R*)-**8** was detected (Figure S7). To further investigate the enantioselectivity of PhoN-Se, both enantiomers of 2-butanol (**9**) were subjected to screening conditions; however, no significant difference in the reaction course was observed. Furthermore, racemic cyclic *trans*-diol **10** was tested, and beside a moderate amount of monophosphorylated product, no stereoselectivity could be observed (as determined by measurement of the *ee* value of remaining **10** by GC after derivatization of both OH functionalities; see Supporting Information).

Substrates containing *N*- or *S*-nucleophiles or charged groups were not converted (Figure 2), while PP_i was still hydrolyzed indicating that the enzymes were still active (data not shown). The absolute chemoselectivity of *O*-phosphorylation vs. *N*- and *S*-nucleophiles was corroborated by the non-acceptance of substrates **11** and **12**. Mevalonolactone (*rac*-**13**) and charged substrates, like lactic (*rac*-**14**) and tartaric acid (**15**) (both enantiomers tested separately) were not converted. Although *O*-phospho-D,L-serine could be stereoselectively hydrolyzed by a mutant of PhoN-Se,[29] the wild-type enzyme was not active in the phosphorylation of *rac*-serine (**16**). Finally, oximes **17**–**19** did not undergo phosphorylation.

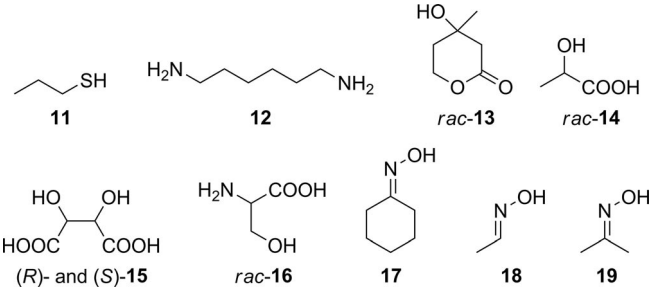


Figure 2. Substrates not converted by PhoN-Sf, PhoN-Se, PiACP and phytase [300 mM substrate concentration except for *rac*-**14** (200 mM) and **17** (15 mM)].

Due to the reversibility of the reaction (Figure 1), kinetic control of the reaction is necessary to ensure optimal product titers by avoiding product depletion by (reverse) hydrolysis and to ensure optimal product titers. This may be achieved by adjustment of reaction parameters such as pH or selection of oligophosphate donor.[30] Therefore, 1,4-butanediol (**2**) was selected as a model substrate, and enzymes were tested with tri- and polyphosphate as alternative to PP_i (Table 1 and Figures S8–10).

The amount of 4-hydroxybutyl phosphate formed could be significantly increased by replacing PP_i by high amounts of PPP_i and polyP in the reaction catalyzed by PhoN-Sf and PiACP, allowing up to 61 % conversion (305 mM product) with respect to the theoretical maximum. The difference in the slope in product disappearance clearly shows that product hydrolysis is decreased in the presence of tri- and polyphosphate compared to PP_i. In contrast, these donors had a negative influence on the performance of PhoN-Se, where a product maximum was obtained with PP_i. Moreover, HPLC monitoring of the reaction indicated incomplete consumption of polyphosphoric acid. This suggests that this enzyme accepts only short chain oligophosphates as reported for calf intestine alkaline phosphatase.[9]

Since PhoN-Sf generally delivered the highest product titers with primary alcohols as acceptors, the enzyme was selected for further investigations with 1,4-butanediol concerning the effect of pH (range 3.8–4.8) by using different P-donors (Figure 3). The pH had no significant impact on the reaction using PP_i. On the other hand, PPP_i and polyP usually yielded higher product concentrations at lower pH, due to slower product hydrolysis. However, the poorly defined oligomeric composition of the polyphosphate donor renders precise analysis as well as product isolation challenging, and polyP was not investigated further.

The results depicted in Figure 3 using PhoN-Sf suggest that a high number of transferable high-energy phosphate moieties as well as low pH values are crucial to achieve and maintain high product titers. Inspired by this observation, reactions were conducted at variuos pH values using high amounts of PP_i and PPP_i overnight (Table 2). It should be noted that within the incubation time of 16.5 h both transphosphorylation and hydrolysis of product and phosphate donor will occur, and the balance between the two processes is determined by the activity of the enzymes and the pH. High product levels were found at pH = 3.5–4.2. Above this pH range, hydrolysis usually dominated (Table S2). Although identical amounts of transferable phosphate moieties are available from 500 mM PP_i and 250 mM PPP_i, significant differences in product formation were obtained after 16.5 h reaction time. Using 500 mM PP_i as a donor, product yields of **2** for PhoN-Sf and PiACP were similar at the three pH values. Using PP_i, up to 292 mM product (up to 58 % conversion) could be obtained accompanied by nearly total consumption of PP_i (> 90 %), and the product/P_i ratio was ca. 1:2 (the ideal ratio is 1:1 in the absence of product hydrolysis and complete phosphate transfer). However, under these conditions PhoN-Se furnished only inorganic monophosphate, indicating total hydrolysis of PP_i and product. In the presence of 250 mM PPP_i the picture changed: The transphosporylation activity at pH = 3.5 of PhoN-Sf dropped considerably, that of PiACP also dropped, but surprisingly considerable phosphorylating activity of PhoN-Se was maintained (164 mM, 33 % conversion and 54 % P-donor consumption). At higher pH values this enzyme was still active, but only hydrolysis was observed, while PhoN-Sf and PiACP delivered moderate to good amounts of product at full consumption of the P-donor. At 400 mM PPP_i and pH = 4.2 both PhoN-Sf and PiACP showed the highest phosphorylating activity resulting in up to 393 mM product (79 % conversion, product/P_i ratio ca. 4:5) at 75 % P-donor consumption.

In general, higher PPP_i concentrations translate into higher product concentration and lower P_i concentration, indicating more efficient phosphate transfer due to reduced product hydrolysis. From the data it is clear that using these phosphorylating enzymes in synthesis effectively requires a careful study of the donor concentrations, the type of donor, and the pH; in batch processes also the time of incubation should be carefully chosen. A long incubation time will result in low yields or near complete hydrolysis of the phosphorylated product.

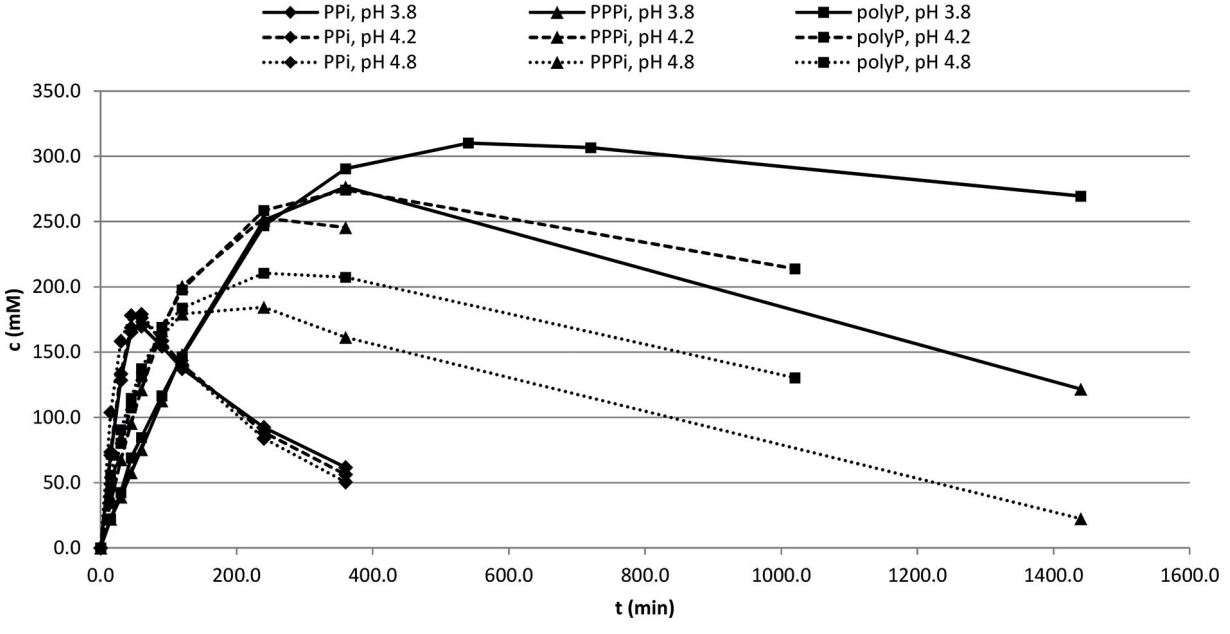


Figure 3. Effect of P-donor type and pH on the phosphorylation of **2** (product formation over time). Conditions: 500 mM **2**, 250 mM PP_i or PPP_i or 100 g L^{-1} polyP, 50 µg mL^{-1} PhoN-Sf, 1 mL volume, 1 % DMSO as internal standard, 30 °C, 600 rpm shaking.

Table 2. Amount of product and P_i formed in acid phosphatase catalyzed phosphorylation of **2** with PP_i or PPP_i as donor, at three pH values with PhoN-Sf, PiACP and PhoN-S.

		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 = 3.5	product [mM]	236	253	0	15	131	164	8	24	48
	P_i [mM]	743	655	940	22	106	244	13	13	46
	product/P_i[a]	0.32	0.39	0.00	0.68	1.24	0.67	0.62	1.85	1.04
	$Cons._{donor}$ [%][b]	98	91	94	5	32	54	2	3	8
	Conv. [%][c]	47	51	0	3	26	33	2	5	10
pH = 3.8	product [mM]	226	292	0	190	122	4	276	393	83
	P_i [mM]	760	611	934	540	613	747	238	507	1099
	product/P_i[a]	0.30	0.48	0.00	0.35	0.20	0.01	1.16	0.78	0.08
	$Cons._{donor}$ [%][b]	99	90	93	97	98	100	43	75	99
	Conv. [%][c]	45	58	0	38	24	1	55	79	17
pH = 4.2	product [mM]	110	105	0	101	31	3	315	265	21
	P_i [mM]	893	818	924	603	669	703	805	768	1154
	product/P_i[a]	0.12	0.13	0.00	0.17	0.05	0.00	0.39	0.35	0.02
	$Cons._{donor}$ [%][b]	100	92	92	94	93	94	93	86	98
	Conv. [%][c]	22	21	0	20	6	1	63	53	4

[a] 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. Ideal (maximum obtainable) product/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.

Consequently, kinetic control of the phosphorylation is very important, and physical separation of the phosphatase by employing a continuous flow-reactor has proven to be an efficient tool to maintain high product levels (by minimizing hydrolysis) and scale-up the reaction.[12c,31] Under optimized conditions, the contact time between enzyme and substrate(s) is controlled by the flow rate to ensure that (rapid) phosphorylation is complete, but (slow) product hydrolysis cannot take place after the product has left the enzyme-containing column reactor. This requires the enzyme to be immobilized on a suitable support. We evaluated several epoxy- and amino-functionalized polymeric beads for the covalent immobilization of PhoN-Sf and PiACP (Table S3). The activity of the beads was tested in the *p*-NPP assay. Relizyme HA403/M, an amino-functionalized resin, resulted in the highest specific activity for both enzymes (159 and 215 U g^{-1} dry beads for PhoN-Sf and PiACP, respectively). PhoN-Sf (300 U) immobilized on Relizyme HA (300 mg) was applied to the preparative-scale phosphorylation of **2**, yielding 6.86 g (22.5 mmol) of the corresponding barium salt of 4-hydroxybutyl phosphate (41 % isolated yield, for details see Supporting Information).

Conclusions

The enzymatic phosphorylation of primary and secondary alcohols in aqueous medium using cheap inorganic di-, tri- and polyphosphate as P-donors catalyzed by three acid phosphatases proceeded with absolute chemoselectivity for *O*- vs. *N*- and *S*-nucleophiles and with high regioselectivity for *prim*- over *sec*-hydroxy groups. The use of mild reaction conditions allowed the formation of phosphate monoesters as sole products, yielding environmentally innocuous inorganic monophosphate and unreacted phosphate donor as by-products. PhoN-Se could phosphorylate secondary alcohols, although no chiral induction was observed. The thermodynamically favored hydrolysis of phosphate esters could be minimized by controlling various reaction parameters, thus allowing the use of phosphatases in the synthetic direction. Scale-up was demonstrated by the synthesis of 4-hydroxybutyl phosphate (6.86 g, 41 % yield) using an immobilized enzyme in flow mode.

Experimental Section

For experimental details, see the Supporting Information.

Acknowledgments

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Keywords: Phosphorylation · Phosphatase · Biocatalysis · Regioselectivity · Inorganic phosphate donors

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