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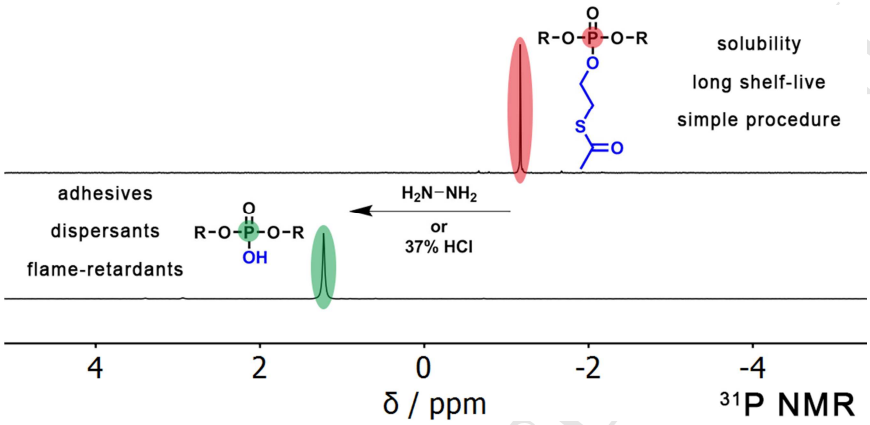

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The 2-acetylthioethyl ester group: A versatile protective group for P-OH-groups

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ABSTRACT

Phosphodiesters are bridging elements in nucleic acids. In nature and synthesis, their negative charge protects them from hydrolysis and controls their solubility profile. RNA is a promising material for gene technology but cellular uptake is low due to negative charges. Synthetic oligonucleotides were delivered into cells by a prodrug approach relying on the enzymatic release of the polyphosphodiester oligonucleotides. In synthetic chemistry, a protective group for the P-OH functionality is often necessary, e.g. due to solubility or chemical incompatibility. Several methods for P-OH protection were proposed, but often with low selectivity or harsh conditions. Here, we present the 2-acetylthioethyl group as a versatile protective group for low molecular weight or polymeric phosphodiesters, which can be cleaved under acidic conditions in water or by hydrazine in THF to release the P-OH-functionality, but olefins remain intact. This straightforward use allows designing various synthetic polyphosphodiesters, e.g. for flame-retardant or dispersants.

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1. Introduction

Phosphodiesters build the backbone of DNA or RNA. Their negative charge protects DNA from hydrolytic degradation.[2,3] In modern medicine, oligonucleotides are promising gene therapeutics, but due to the negative charge are only poorly internalized by cells.[1] To increase cellular uptake, prodrug approaches were developed.[1] One method used a 2-acetylthioethyl group, which masks the negative charge of the phosphate by creating a neutral phosphotriester. Those neutral oligonucleotides could be internalized by cells, but the thioacetate is cleaved enzymatically by intracellular carboxyesterases to release the free thiol-group, which then forms the phosphodiester under the release of thiirane.[1]

Besides biomedical applications, polyphosphodiesters are also interesting for synthetic chemistry, e.g. for the generation of potentially biocompatible and biodegradable materials[4-7] or to increase polymer hydrophilicity. However, the direct preparation of polyphosphodiesters is not possible. Polycondensation with phosphoryl chloride results in ill-defined branched or cross-linked structures or acyclic diene metathesis polymerization (ADMET) only produced oligomeric PPEs[8]. Also, the ring-opening polymerization of cyclic phospholanes does not tolerate free P-OH-groups and makes protective groups inevitable.[9] We recently used a diene monomer containing a P-Cl bond for ADMET, followed by hydrolysis to the respective polyphosphodiester.[8,10] However, the high electrophilicity of the P-Cl makes handling difficult and working under strictly dry conditions is essential to prevent short shelf life and cross-linking reactions during polymerization. Other protective groups could be removed by hydrogenation, e.g. benzylesters.[9] This method however, would also remove olefins in the desired polymers.[9] Other reports used dealkylation of an alkoxy sidechain by treating the phosphate with a strong nucleophilic reagent, which however is plagued by backbone degradation.[11-13] The third method utilized the different pH-stability of phosphoramidates and phosphates, in which the acid-labile phosphoramidate bond was cleaved under relatively mild acidic conditions.[14,15] Other routes rely on the oxidation of H-phosphonates with N_2O_4[16] or thermolysis of tert-butyl esters. [17]

We propose the 2-acetylthioethyl ester group as a protective group for the P-OH functionality in low molecular weight phosphates as well as polyphosphates. In contrast to the above-mentioned strategies, the protected materials are accessible by straightforward chemistry and possess long shelf lives with fast purifications. The cleavage was possible both in organic solvents as well as in water and did not interfere with olefins. All these factors render the 2-acetylthioethyl phosphoester an attractive protective group for synthetic (poly)phosphodiesters.

2. Results and Discussion

Phosphoryl chloride was first reacted with 2-bromoethanol followed by a reaction with hex-5-en-1-ol to yield 2-bromoethyl di (hex-5-en-1-yl)phosphate (**2**), which was then reacted with potassium thioacetate to give 2-acetylthioethyl di(hex-5-en-1-yl)phosphate (**3**, Figure 1). This compound was used as an unsaturated model compound for the cleavage of the 2-acetylthioethyl phosphoester to di(hex-5-en-1-yl) hydrogen phosphate (**4**).

Thioesters are often used as a protective group for the highly nucleophilic thiols, which can be cleaved under acidic or basic conditions.[18-20] However, if a 2-acetylthioethyl group is linked via a phosphoester, the hydrolysis of the thioester to the thiol will be followed by a complete removal of the alkyl chain from the phosphate and releases a phosphodiester.[21,1] This strategy was extensively used in solid-phase oligonucleotide synthesis, followed by enzymatic cleavage of the thioester.[21,1] We used either hydrazine (method a (Experimental)) or hydrochloric acid (method b (Experimental)) to successfully release the $PO(OR)_2OH$ (compounds **4** or **Poly-4**). The successful formation of the P-OH group was proven by $^{31}P\{H\}$ and ^{1}H NMR spectroscopy and mass spectrometry. The first indication for a successful removal of the 2-acetylthioethyl ester was the down-field shift in the ^{31}P NMR from -1.17 ppm in compound **3** to 1.30 ppm in compound **4** (Figure 1 f). The release of the P-OH-group was further supported by the integral of the methylene groups next to the phosphoesters at ca. 4 ppm in the ^{1}H NMR spectra, which corresponded to four instead of six (Figure 1 c – e). This indicates that there are only two alkyl chains attached to the

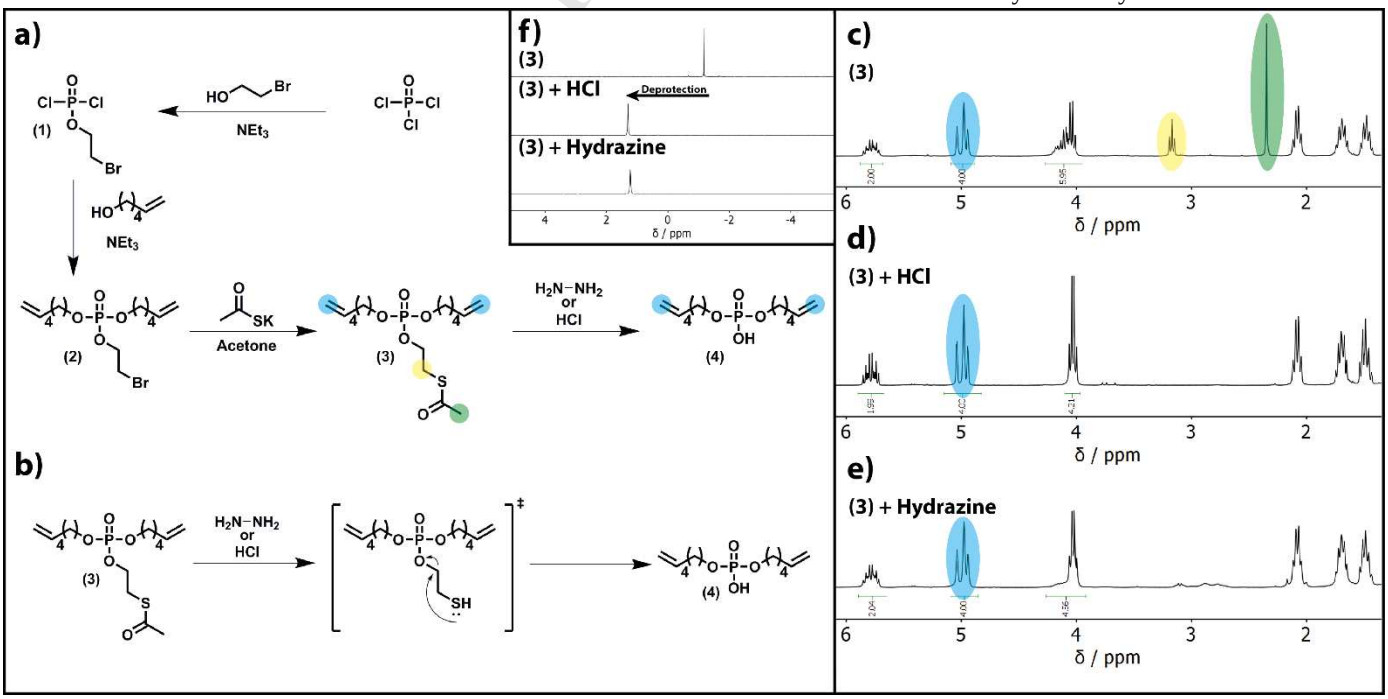

Fig. 1. a) Synthesis of 2-acetylthioethyl ester–protected unsaturated phosphate **3** and removal of the protective group to **4** according to b) the proposed mechanism.[1] c) ^{1}H NMR spectrum (300 MHz, $CDCl_3$, T = 298K) of 3. d, e) ^{1}H NMR (300 MHz, $CDCl_3$, T = 298K) of reaction of **3** with HCl (d) and hydrazine (e). f) ^{31}P NMR spectra (121 MHz, $CDCl_3$, T = 298K) of the 2-acetylthioethyl ester–protected compound (**3**) and the reaction product (**4**) after treatment with HCl or hydrazine.

phosphate. Furthermore, the resonances for the methylene groups next to the thioester at 3.15 ppm vanished and no new resonances for a free thiol group are detected (Figure 1 d, e). When we used K_2CO_3 in methanol to remove the 2-acetylthioethyl group (another typical procedure for the cleavage of thioesters[20]), we found partial formation of **4**, but also the formation of the methyl ester via transesterification with methanol. The ^{1}H NMR spectrum of the crude reaction mixture showed a typical doublet with a J=11.1 Hz for a methyl-ester ("P-O-CH3") due to the coupling with phosphorus (Figure S8).

The mechanism for the enzymatic removal of the 2-acetylthioethyl ester in synthetic oligonucleotides was reported to release the phosphodiesters.[21,1] After hydrolysis of the thioester to the thiol, a nucleophilic attack by the thiol on the methylene group next to the ester group was postulated to release the phosphodiester and thiirane, which probably will be hydrolyzed to mercaptoethanol or react with the nucleophilic hydrazine.[1,21]We propose the same mechanism on the synthetic phosphates as shown in Figure 1 b, which retains the olefins in the molecule for further functionalization. Mass spectrometry analysis of the crude reaction from **3** to **4** proves the formation of the product **4** and the cleaved side chain with further adducts with hydrazine (cf. Supp. Info.). The reaction was also monitored by real-time ^{1}H NMR spectroscopy as the disappearance of the thioacetate resonance gives a direct insight to the reaction kinetics (cf. Supp. Info. for further details). The successful formation of **4** was proven by the reaction with trimethylsilyldiazomethane to convert the hydroxyl group to a methyl ester. The typical doublet for the methyl ester due to coupling with phosphorus can be appreciated in the ^{1}H NMR spectrum (J=11.1 Hz, Figure S1).

common organic solvents.[8] Thus, a solubilizing protective group is a desirable intermediate. We used monomer **2** to prepare a polyphosphate by ADMET polymerization (**Poly-2**, M_n=5100 g mol^{-1} D=1.68) according to a modified literature procedure.[10] After the polymerization, the alkyl bromide in the pendant chains was reacted with potassium thioacetate (in acetone, r.t., 12h) to produce the 2-acetylthioethyl ester protected PPE (**Poly-3**). The product shows two characteristic resonances in the ^{1}H NMR spectrum at 2.28 ppm, which were attributed to the methyl group of the thioacetate, and the methylene group at 3.10 ppm next to the thioester. The polymer (**Poly-3**) was bench-stable at room temperature for at least several weeks. In the final step, the polymer was treated with hydrazine in THF at r.t. to release the polyphosphodiester (**Poly-4**). During the reaction, the polymer precipitated from solution. The crude product **Poly-4** was soluble in $CDCl_3$/MeOD and the removal of the 2-acetylthioethyl ester was proven as the resonance of the methylene group next to the thioester at 3.17 ppm vanished and the signal of the leaving group shifts to 1.73 ppm. In addition a down-field shift in the ^{31}P NMR from -1.16 ppm to 0.55 ppm was observed, which is indicative for the cleavage of the pendant ester. Purification of **Poly-4** was achieved by precipitation into diethyl ether and obtained as an off-white viscous liquid in 84% yield. The ^{1}H DOSY-NMR spectrum additionally proves the formation of **Poly-4** (Figure 2 e).

3. Conclusion

We demonstrated the use of the 2-acetylthioethyl ester group for the protection of (poly)phosphodiesters. The protective group guarantees solubility in common organic solvents and the compounds show high shelf-lives. The 2-acetylthioethyl ester was successfully removed either by hydrazine (in THF) or

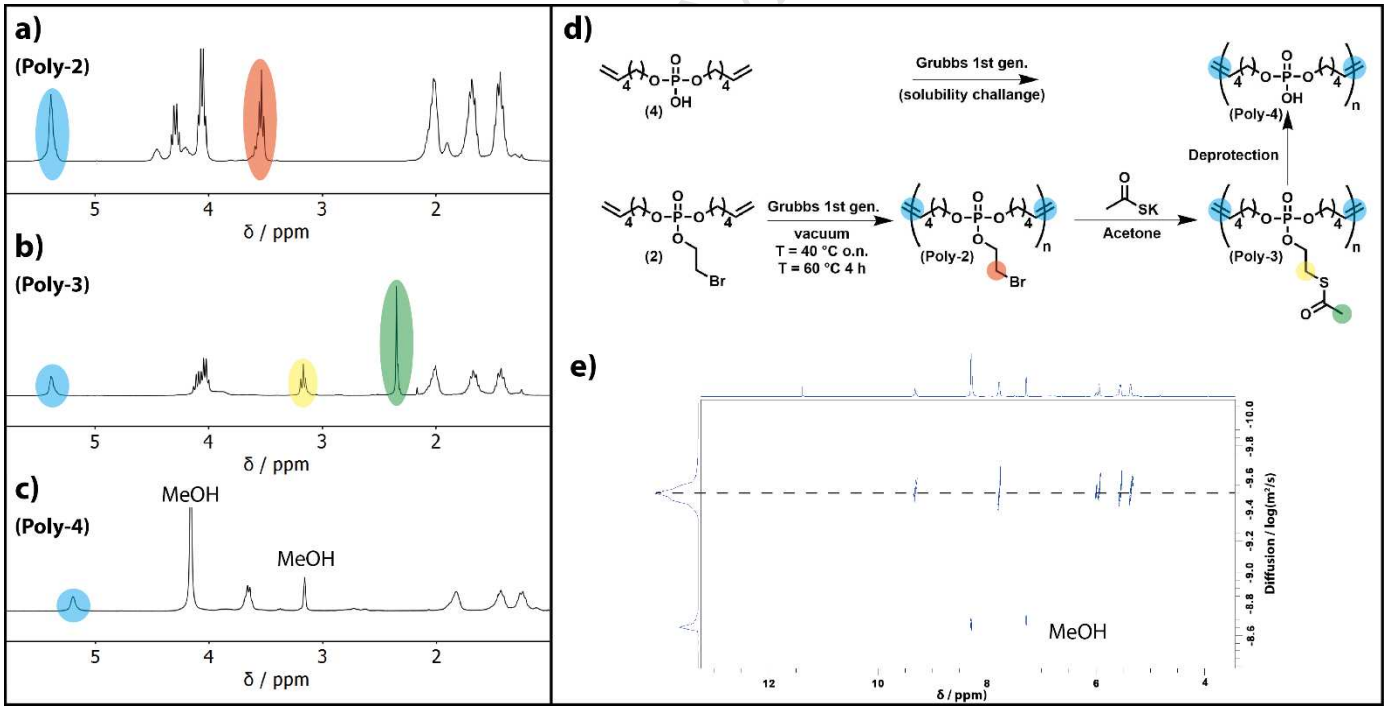

Fig. 2. Polymer Examples: a-c) ^{1}H NMRs (300 MHz, $CDCl_3$, T = 298K) of the polymers a) poly-2, b) poly-3 after introducing the thioacetate group, and c) of the polyphosphodiester (poly-4, measured at 300 MHz, in $CDCl_3$:MeOD 7:3, T = 298K). d) Reaction scheme. e) ^{1}H DOSY (700 MHz, $CDCl_3$:MeOD 7:3, T = 298K) spectrum of poly-4 underlining the successful removal of the pendant chains.

After the successful preparation of low molecular weight phosphodiesters, we studied the synthesis of polyphosphodiesters by the 2-acetylthioethyl ester protective group. The synthesis of polyphosphodiesters with a hydrophobic backbone is often plagued by the low solubility of oligomers or polymers in hydrochloric acid (in water) and additional double bonds in the (macro)molecule remained untouched during the procedure. The 2-acetylthioethyl ester can be used as a soluble precursor for P-OH-containing polymers which are appealing for adhesives, dispersants, or flame-retardants.

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5. Experimental

All chemicals were purchased from commercial suppliers as reagent grade and used without further purification. ^{1}H and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded on Bruker AV 300 at 300 MHz (^{1}H) and 121.50 MHz (^{31}P) or Bruker AV 700 spectrometers at 700 MHz (^{1}H). The temperature of measurement is indicated in the corresponding figure captions. Chemical shifts are reported in ppm using the residual non-deuterated solvent signals as an internal reference.

2-Bromoethyl phosphorodichloridate (1)

To a dried three-necked, 250 mL round-bottomed flask fitted with a dropping funnel, 61.35 mL of phosphoryl chloride (672.19 mmol; 7.0 eq.) were added under an argon atmosphere in 300 mL of dry toluene. Then 13.31 mL of triethylamine (96.03 mmol; 1.0 eq.) and 12 g of 2-bromoethanol (96.03 mmol; 1.0 eq.) dissolved in 50 mL of dry toluene were added dropwise to the above-mentioned flask at 0 °C. The reaction was allowed to stir overnight at room temperature. Afterward, the crude mixture was filtered to remove the triethylammonium chloride and concentrated at reduced pressure. Then, all by-products and starting material were removed at reduced pressure (R.T., $5 * 10^{-2}$ mbar). The product was used without further purification. 87% (20.2 g) yield.

2-Bromoethyl di (hex-5-en-1-yl)phosphate (2)

To a dried three-necked, 250 mL round-bottomed flask fitted with a dropping funnel, 7.00 g of 2-bromoethyl phosphorodichloridate (28.95 mmol; 1.0 eq.) were added under an argon atmosphere in 40 mL of dry toluene. Then 7.30 mL of hex-5-en-1-ol (60.79 mmol; 2.1 eq.) and 8.43 mL of triethylamine (60.79 mmol; 2.1 eq.) were dissolved in 20 mL of dry toluene and added dropwise to the mixture at 0 °C. The reaction was allowed to warm up to room temperature, stirred for an additional 12 h and filtered. The toluene was removed on the rotary-evaporator and the crude product was dissolved in toluene to wash it with 10% aqueous hydrochloric acid solution, a saturated solution of calcium carbonate and brine. The organic layer was dried over anhydrous magnesium sulfate, filtered, and dried at reduced pressure. 82% (8.6 g) yield.

^{1}H NMR (300 MHz, 298K, chloroform-*d*): δ [ppm] = 5.81 – 5.68 (m, 2H); 4.98 – 4.89 (m, 4H); 4.29 – 4.22 (q, 2H); 4.06 – 4.00 (q, 4H); 4.49 (t, 2H); 2.08 – 2.01 (td, 4H); 1.71 – 1.62 (tt, 4H); 1.50 – 1.40 (tt, 4H).

^{31}P {H} NMR (121 MHz, 298K, chloroform-*d*): δ [ppm] = -1.23 (s, 1P).

2-Acetylthioethyl di (hex-5-en-1-yl)phosphate (3)

The synthesis of 2-acetylthioethyl di (hex-5-en-1-yl)phosphate was carried out adopting a procedure previously described by Liras et al.[22]

To a 100 mL round bottom flask 500 mg of 2-Bromoethyl di (hex-5-en-1-yl)phosphate (1.35 mmol; 1.0 eq.) and 170.11 mg of potassium thioacetate (1.49 mmol; 1.1 eq.) were added under argon atmosphere in 5 mL of acetone and stirred for 20 h at room temperature.

Then, the mixture was filtered through Celite 501 to remove the salt. The acetone was removed by rotary evaporation. The crude mixture was dissolved in diethyl ether and washed three times with brine. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated on the rotary evaporator. The purified product was recovered as a viscous oil (yield: 0.4 g, 82%). The purity and chemical structure were determined by ^{1}H NMR and ^{31}P {H} NMR spectroscopy.

^{1}H NMR (300 MHz, 298K, chloroform-*d*): δ [ppm] = 5.81 – 5.68 (m, 2H); 5.02 – 4.93 (m, 4H); 4.16 – 3.99 (m, 6H); 3.15 (t, 2H); 2.33 (s, 3H); 2.08 – 2.01 (td, 4H); 1.71 – 1.62 (tt, 4H); 1.50 – 1.40 (tt, 4H).

^{31}P {H} NMR (121 MHz, 298K, chloroform-*d*): δ [ppm] = -1.17 (s, 1P).

Di(hex-5-en-1-yl) hydrogen phosphate (4)

a) **3** (1 g), 5 mL of 1M hydrazine solution in THF were mixed and 10 mL dichloromethane and stirred overnight at r.t. in an argon atmosphere. The solvent was removed at reduced pressure and the crude product redissolved in dichloromethane and the organic phase was washed with 10% HCl, saturated $NaHCO_3$ and again 10% HCl. The organic phase was dried over $MgSO_4$ and the solvent removed at reduced pressure to yield the product (yield: 736 mg, 83%).

b) **3** (200 mg) was dissolved in 0.66 mL methanol. Then 0.5 mL (10 eq.) of 37% HCl was added and the solution was stirred at 65°C overnight. The solvent was removed at reduced pressure and the crude product was dissolved in diethyl ether and washed two times with brine. The organic phase was dried over $MgSO_4$ to yield the pure product (yield: 0.2 g, 87%).

^{1}H NMR (300 MHz, 298K, chloroform-*d*): δ [ppm] = 5.85 – 5.71 (m, 2H; 5.04 – 4.95 (m, 4H, f); 4.06 – 4.00 (q, 4H); 2.12 – 2.05 (td, 4H); 1.74 – 1.65 (tt, 4H); 1.54 – 1.44 (tt, 4H).

^{31}P {H} NMR (121 MHz, 298K, chloroform-*d*): δ [ppm] = 1.30 (s, 1P).

Poly-2

In a Schlenk tube equipped with a stir bar, 500 mg of **2** with 10 mg (0.6 mol%) of Grubbs I catalyst were added. The reaction was allowed to procede at reduced pressure over night at 40 °C, then the temperature was increased to 60 °C for another 4 hours to give the product in quantitative yield (439 mg).

^{1}H NMR (300 MHz, 298K, chloroform-*d*): δ [ppm] = 5.39 (m, 2H); 4.28 (q, 2H); 4.05 (q, 4H); 3.53 (m, 2H); 2.02 (m, 4H); 1.68 (m, 4H); 1.43 (m, 4H).

^{31}P {H} NMR (121 MHz, 298K, chloroform-*d*): δ [ppm] = -1.26 (s, 1P).

Poly-3

Poly-2 (150 mg), 51.03 mg of potassium thioacetate (0.447 mmol; 1.1 eq.) and 2 mL of acetone were stirred overnight at r.t. under an argon atmosphere. The solution was filtered

through a syringe filter and the solvent was removed under reduced pressure to give the product in quantitative yield (148 mg)

^{1}H NMR (300 MHz, 298K, chloroform-*d*): δ [ppm] = 5.39 (m, 2H); 4.09 (q, 2H); 4.02 (q, 4H); 3.17 (m, 2H); 2.35 (s, 3H); 2.00 (m, 4H); 1.67 (m, 4H); 1.43 (m, 4H).

^{31}P {H} NMR (121 MHz, 298K, chloroform-*d*): δ [ppm] = -1.16 (s, 1P).

Poly-4

Poly-3 (150 mg), 0.6 mL 1M hydrazine solution in THF and 2.5 mL DCM were stirred overnight at room temperature under an argon atmosphere. The polymer precipitated during the reaction and the solvent was removed at reduced pressure. The polymer could be redissolved in chloroform:methanol 7:3. It was then precipitated into diethyl ether for purification. 84% (90.7 mg) yield.

^{1}H NMR (300 MHz, 298K, chloroform-*d:* methanol-d_4 7:3): δ [ppm] = 5.20 (m, 2H); 3.66 (m, 4H); 1.83 (m, 4H); 1.43 (m, 4H); 1.24 (m, 4H).

^{31}P {H} NMR (121 MHz, 298K, chloroform-*d:* methanol-d_4 7:3): δ [ppm] = 0.55 (s, 1P).

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