A Novel Oxime-Derived Solid Support for the Synthesis of 3-Phosphorylated Oligonucleotides

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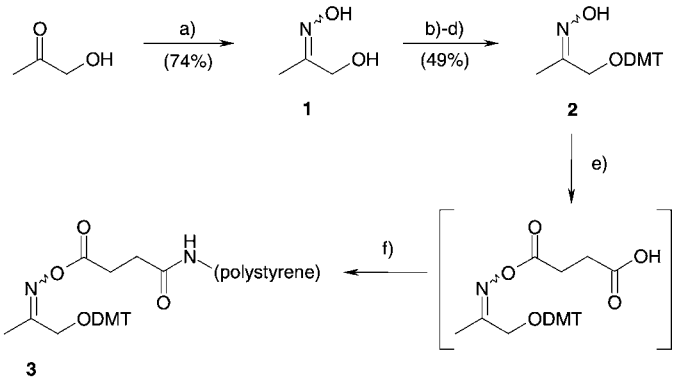
Oligonucleotides containing terminal phosphate groups serve as useful intermediates for different applications in molecular and cell biology, as well as for diagnostic purposes. The chemical synthesis of these derivatives has been an important topic of oligonucleotide research. We report here a novel polystyrene-based solid support bearing an α-hydroxy oximate linker that allows the synthesis of 3′-phosphorylated oligodeoxynucleotides as well as oligoribonucleotides in high quality. The formation of the phosphorylated oligonucleotides likely proceeds through an eliminative pathway with concurrent nitroso-ene production.

Introduction. – The use of phosphorylated oligonucleotides in today's research is widespread. They are used as substrates in enzymatic (see, *e.g.*, [1] [2]) and chemical [3] ligation reactions, as well as for diagnostic purposes [4]. Furthermore, the existence of a terminal phosphate or thiophosphate group allows the attachment of reporter groups of other chemical moieties, altering the properties or the functions of oligonucleotides [5]. Various chemical methods exist that allow the attachment of a terminal phosphate or phosphorothioate group onto oligonucleotides in the process of oligonucleotide synthesis on solid support [6–21]. Due to the well-known behavior of phosphate as a leaving group, many of the existing methods make use of a β-eliminative process, yielding the desired phosphorylated derivative [6]. When it comes to the synthesis of 3′-phosphorylated oligoribonucleotides, only few reports exist in the literature describing the chemical preparation of 3′-phosphorylated RNAs [11][22][23]. In the course of our research, we observed that the base treatment of oligonucleotides bearing a 3′-phosphate-linked α-hydroxy oxime resulted in the clean formation of the corresponding 3′-phosphorylated oligonucleotides. We report here the preparation and evaluation of a novel type of solid support, bearing an α-hydroxy-oximate linker for the synthesis of 3′-phosphorylated oligodeoxynucleotides as well as oligoribonucleotides.

Results and Discussion. – Synthesis of the α-hydroxy-oximate-derived support was achieved in a short series of chemical steps starting from the commercially available 1-hydroxypropan-2-one (see *Scheme 1*). The latter was converted into oxime **1** by treatment with $NH_2OH \cdot HCl$ in a 74% yield. To obtain the monoprotected derivative **2**, oxime **1** was first treated with BzCl followed by 4,4′-dimethoxytrityl chloride. Subsequent removal of the intermediate Bz protecting group from the oxime moiety

gave the desired mono-protected **2** in 49% overall yield from **1**. Compound **2** was further reacted with succinic anhydride, and the succinate obtained was coupled onto amino-derivatized polystyrene in the conventional way (see *Exper. Part*). The capacity of the thus obtained oximate-derivatized PS support **3** was found to be 30.0 μmol/g.

Scheme 1. *Synthesis of Polystyrene-Based Solid Support Bearing an Oximate Linker*



a) $NH_2OH \cdot HCl$, AcONa, H_2O/EtOH, reflux. b) PhCOCl, pyridine. c) 4,4′-Dimethoxytrityl chloride (DMT-Cl). d) 2M aq. NaOH. e) Succinic anhydride, pyridine. f) 4-Nitrophenol, DCC; amino-derivatized polystyrene.

The oximate-derived polystyrene support **3** was subsequently used for the preparation of the 3′-phosphorylated oligonucleotides **4**–**8** (*Table*). Oligonucleotide synthesis was carried out by the standard automated procedure [24] with commercially available phosphoramidite building blocks. For oligodeoxynucleotide **4**, the synthesis involved 5′-DMT-protected deoxynucleoside phosphoramidites. In the case of oligoribonucleotides **5**–**8**, 2′-*O*-[(triisopropylsilyl)oxy]methoxymethyl (TOM)-protected ribonucleoside phosphoramidite building blocks were used. The 2-TOM protecting group has been shown to give very high coupling yields, and allows a very clean and reliable deprotection after oligoribonucleotide synthesis [25]. The oximate linkage was completely stable under the conditions of oligonucleotide synthesis. Coupling efficiencies were comparable to those obtained with the commercially available 2′-deoxynucleotide-derived *controlled pore glass* (*CPG*) or *polystyrene* (PS) supports[1]).

Table. *Sequences of 3′-Phosphorylated Oligodeoxynucleotide* **4** *and Oligoribonucleotides* **5**–**8** *Synthesized on Oxime-Derived Polystyrene Support* **3** *(***p** *indicates a 3′-phosphate group)*

	Sequence
4	5′ d(CTG AAT CGA CGT TGC AGT)**p**
5	5′ r(GGC CAU CCA CAG UCU UCU G)**p**
6	5′ r(CAG AAG ACU GUG GAU GGC C)**p**
7	5′ r(GGC CAG CCA CAU UCG UCU U)**p**
8	5′ r(AAG ACG AAU GUG GCU GGC C)**p**

[1]) In a different context, an oximate-derived resin (*Kaiser* resin) has been reported for the synthesis of DNA–peptide conjugates in moderate-to-good yields [26].

After assembly of the oligonucleotides, the support-bound material was exposed to basic conditions. This step, typically used for DNA and RNA synthesis, results in the cleavage of the oligonucleotides from the support, as well as the removal of the phosphate- and base-protecting groups. The basic treatment involved the use of concentrated aqueous NH_3 at 55° for 16 h in the case of oligodeoxynucleotide **4**. For oligoribonucleotides **5**–**8**, deprotection and cleavage were performed by treatment with aqueous NH_3/ethanolic $MeNH_2$ (1:1) at 65° for 30 min. The resulting solutions were then evaporated to dryness and subsequently treated with a 1M Bu_4NF solution in THF for 2 h at room temperature. The resulting 3′-phosphorylated oligonucleotides **4**–**8** were obtained in excellent quality. The *capillary-gel-electrophoresis* traces of the crude 3′-phosphorylated oligoribonucleotides **5** and **6** are shown in the *Figure*. The correct identity of all compounds was confirmed by mass spectrometry.

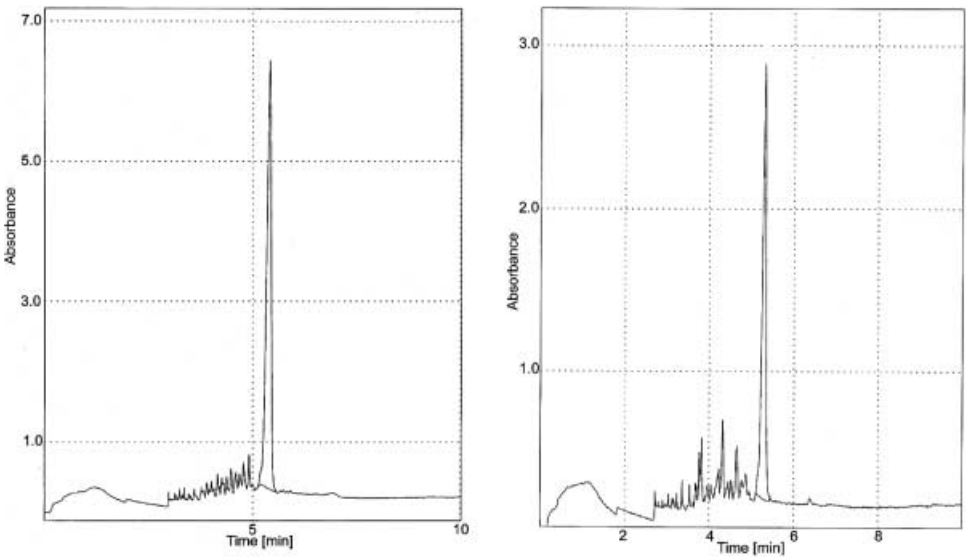
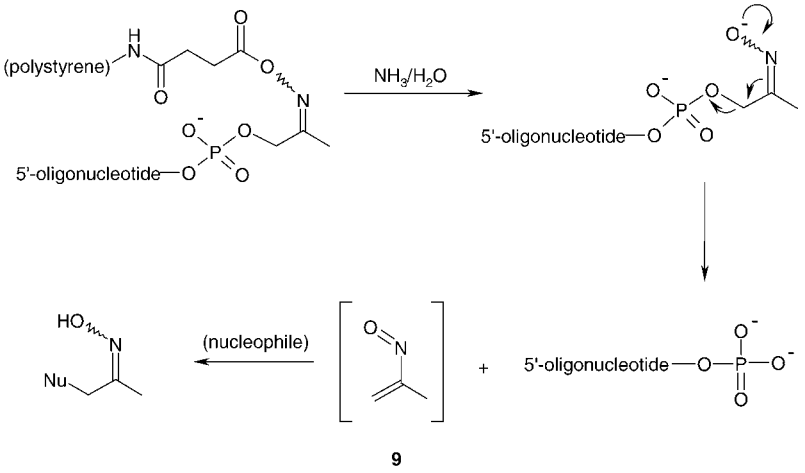


Fig. 1. *Capillary-gel-electrophoresis traces of crude 3′-phosphorylated oligoribonucleotides* **5** *and* **8**

The presumed mechanism of phosphate elimination is shown in *Scheme 2*. After basic cleavage of the oximate, the formed oximate anion undergoes phosphate elimination through formation of the corresponding nitroso-ene **9**. An alternative pathway would consist in the direct substitution of the 3′-phosphate monoester by either NH_3 or hydroxide. Evidence from the literature, however, is in favor of the eliminative pathway shown in *Scheme 2*. Nitroso-ene intermediates have been postulated as intermediates in the nucleophilic substitution of α-halo oximes [26][27]. More recently, *Boggs*, *Mahoney* and co-workers [28] have reported a detailed investigation of the mechanism and kinetics of the release of tetrazoles from α-oximes under alkaline conditions. Their findings strongly support an eliminative process *via* nitroso-ene formation. Thus, elimination of the 3′-phosphorylated oligonucleotides from the oximate anion is likely to proceed *via* the same mechanism. The intermediate nitroso-ene is completely trapped by either NH_3 or hydroxide since we could not detect,

Scheme 2. *Cleavage of Oximate-Linked Oligonucleotide from Solid Support and Subsequent Formation of 3′-Phosphorylated Oligonucleotide.*



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by mass spectrometry, any formation of side product(s) resulting from the addition of the nitroso-ene to the oligonucleotides.

Conclusions. – We have synthesized a polystyrene-based solid support bearing an α-hydroxy oximate linker for oligonucleotide synthesis. The support is compatible with automated DNA and RNA synthesis, yielding the 3′-phosphorylated derivatives in high quality. In accordance with data from the literature, formation of the phosphorylated oligonucleotides proceeds most likely *via* an eliminative pathway leading to 3′-phosphate monoester and nitroso-ene.

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Experimental Part

General. Abbreviations: DMT: 4,4′-dimethoxytrityl, DCC: *N,N′*-dicyclohexylcarbodiimide, DMAP: 4-(dimethylamino)pyridine. Chemicals, solvents, and reagents for reactions were generally from *Acros*, *Aldrich* or *Fluka*, and were of the highest quality available. Solvents for extraction and chromatography were of technical grade and distilled prior to use. Polystyrene support was from *Amersham Pharmacia Biotech* (*amino-derivatized polystyrene support*, high load, 50–70 μm). 5′-DMT Deoxynucleoside phosphoramidites and other reagents for automated oligonucleotide synthesis were from *Transgenomic Cruachem Ltd.,* Glasgow. 5′-DMT-2′-*O*-[(triisopropylsilyl)oxy]methyloxymethyl (TOM) ribonucleoside phosphoramidites were from *Xeragon AG*, Zürich. TLC: Silica-gel *60* F_{254} glass plates (*Merck*); visualisation by UV and/or *A*) by dipping in a soln. of H_2SO_4/H_2O/EtOH 14 : 4 : 1 or *B*) cerium(IV)sulfate (3 mM)/ammonium molybdate (250 mM) in aq. H_2SO_4 (10%), followed by heating. Column chromatography (CC) was performed on silica gel *60* (40–63 μm, 230–400 mesh, *Fluka*) at low pressure. In case of acid-sensitive compounds, the silica gel was pretreated with solvents containing 0.5–1% 4-methylmorpholine. HPLC Runs were carried out with *Merck Chromolith RP-18* on a *Merck-Hitachi* liquid-chromatography system formed by a *L-3000 Photo Diode Array Detector*, a *L-6200A Intelligent-Pump System*, and a *D-2500 Chromato-Integrator*. Capillary gel electrophoresis was performed on a *Beckman P/ACE 5010*. ^{1}H- and ^{13}C-NMR: *Bruker AC-300*, δ values in ppm (solvents signals as internal standard), *J* in Hz. ESI-MS: *VG Platform* single quadrupole ESI-mass spectrometer or *Waters ZMD2000* single

quadrupole ESI mass spectrometer. Disc dialysis involved use of nitrocellulose-based filter discs (*Millipore,* 0.025 μm).

Experiments Referring to Scheme 1. *1-Hydroxypropan-2-one Oxime* (**1**). A suspension of 10 ml (139 mmol) of 1-hydroxy-propan-2-one, 23.48 g (338 mmol) of $NH_2OH \cdot HCl$, 28.18 g (344 mmol) of AcONa in 240 ml of EtOH and 100 ml of H_2O was heated under reflux (80°) for 3 h. The mixture was stirred for 1.5 h at r.t., then sat. aq. $NaHCO_3$ soln. was added, and the mixture was extracted (4 ×) with CH_2Cl_2/i-PrOH 3 : 1. The org. phase was dried ($NaHCO_3$) and evaporated. The resulting oil was distilled under reduced pressure ($1 \cdot 10^{-3}$ mbar; 40°) to furnish 9.12 g (74%) of **1**, which formed a white solid upon cooling. ^{1}H-NMR ((D$_6$)DMSO, 300 MHz): 1.73 (*s*, 3 H); 3.86 (*s*, 2 H); 4.97 (*s*, 1 H); 10.44 (s, 1 H). ^{13}C-NMR ((D$_6$)DMSO, 75 MHz): 11.1; 63.2; 155.7. HR-ESI-MS (pos.): 90.0556 ($[M+H]^+$; calc. 90.0555).

1-[Bis(4-methoxyphenyl)phenylmethoxy]propan-2-one Oxime (**2**). A soln. of 6.0 g (67.3 mmol) of **1** in 60 ml of dry pyridine was placed under Ar. BzCl (7.8 ml, 67.3 mmol) was added over 10 min at 5°. The mixture was stirred for 3.5 h at 0°, then 22.8 g (67.3 mmol) of DMT chloride was added over 10 min at r.t. The mixture was stirred overnight and then added to a soln. of 150 ml of 2M aq. NaOH and 150 ml of EtOH, and stirred for 2 h at r.t. The mixture was extracted (2 ×) with Et_2O, and the org. phase was washed with H_2O and brine, dried (Na_2SO_4), and evaporated under reduced pressure. The resulting oil was purified by CC (silica gel; AcOEt/hexane 1 : 4 → 1 : 2 (+2% Et_3N)). The fractions were combined, evaporated, and dried under high vacuum to furnish 12.8 g (49%) of **2**. White foam. ^{1}H-NMR ((D$_6$)DMSO, 300 MHz): 1.82 (*s*, 3 H); 3.49 (*s*, 2 H); 3.72 (*s*, 6 H); 6.8–7.0 (*m*, 4 H); 7.2–7.4 (*m*, 9 H); 10.73 (*s*, 1 H). ^{13}C-NMR ((D$_6$)DMSO, 75 MHz): 11.8; 55.0; 65.3; 85.7; 112.9; 113.3; 123.9; 126.8; 127.6; 127.9; 129.7; 130.1; 135.3; 135.4; 136.1; 144.8; 149.6; 152.9; 154.6; 158.1; 170.3. HR-ESI-MS (pos.): 414.1683 ($[M+Na]^+$; calc. 414.1681).

Oxime-Derived Polystyrene Solid Support (**3**). A soln. of 4.5 g (11.5 mmol) of **2** in 50 ml of dry pyridine was placed under Ar; then 1.72 g (17.2 mmol) of succinic anhydride was added, and the mixture was stirred at r.t. After 5 d, 100 ml of sat. aq. NH_4Cl soln. was added, and the soln. was extracted (2 ×) with AcOEt, the combined org. phases were washed with H_2O and brine, dried (Na_2SO_4), and evaporated under reduced pressure. The crude product (1.0 g, 2.0 mmol) was dissolved in 8 ml of dry DMF and 0.33 ml of dry pyridine, and placed under Ar. 4-Nitrophenol (0.425 g, 3.0 mmol) and DCC (0.460 g, 2.2 mmol) were added, and the mixture was stirred for 3.5 d at r.t. The soln. was filtered. The amino-derivatized solid support (1.03 g) and 100 μl of Et_3N were added to the filtrate, and the mixture was shaken for 28 h at r.t. The resin was collected by filtration and washed successively with DMF, MeOH, and Et_2O.

For the capping of unreacted amino groups, the support was suspended in 10.8 ml of pyridine, 1.2 ml of Ac_2O, and 1.2 g of DMAP, and the suspension was shaken for 2.5 h at r.t. The resin was filtered, washed successively with pyridine, DMF, H_2O, MeOH, and Et_2O, and dried under high-vacuum. The loading of the support was determined by the following procedure: 10.6 mg of the resin was treated (4 ×) with 100 μl of a TsOH soln. (1.91 g (10 mmol) $TsOH \cdot H_2O$ in 100 ml MeCN), and the UV absorbance was measured at 498 nm ($\varepsilon = 70000$). The loading density of the oxime-derived polystyrene support **3** was found to be 30.0 μmol/g.

Synthesis and Deprotection of Oligonucleotides. Oligodeoxynucleotides were synthesized on a *392 DNA/RNA Synthesizer* (*Applied Biosystems*) by standard phosphoramidite chemistry [24]. Deprotection of the assembled oligomers involved treatment with conc. aq. NH_3 for 17 h at 55°. After removal of the polystyrene, the NH_3 soln. was concentrated. The residue was re-dissolved in H_2O (200 μl) and disk-dialyzed against H_2O for 25 min. Oligoribonucleotides were synthesized by standard phosphoramidite chemistry with TOM protecting groups [29]. In these cases, deprotection of the assembled oligomers involved treatment with aq. NH_3/ethanolic $MeNH_2$ 1 : 1 at 65° for 30 min, followed by evaporation to dryness and treatment with a 1M Bu_4NF soln. in THF for 2 h at r.t. Identities of all oligonucleotides were confirmed by electrospray mass spectrometry. The molecular masses were found to be within 0.03% of the expected value.

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