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5'-*O*-(2-Isopropoxyprop-2-yl)-protected Phosphoramidite Building Blocks in the Liquid Phase Oligonucleotide Synthesis

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5'-*O*-(2-isopropoxyprop-2-yl) (IIP)-protection was introduced to 5′-OH function of nucleosides in high yields by an acid-catalysed transacetalization with 2,2-diisopropoxypropane. The applicability of this temporal 5′-*O*-protecting group was demonstrated in the liquid phase oligonucleotide synthesis (LPOS) using the corresponding phosphoramidite building blocks (dA, dG, dC and dT) and a tetrapodal precipitative soluble support. Standard protecting groups were used on nucleobases. Tetrazole as an activator, followed by oxidation using *m*-chloroperbenzoic acid, was used for the coupling. The IIP was shown to be a capable choice to the 5′-*O* protection in solution phase synthesis. It could be readily removed with formic acid ($t_{1/2}<$ 10 s in 6% HCOOH in dichloromethane/methanol (2/1) at RT), resulting in volatile byproducts (acetone and isopropanol).

Introduction

Solid phase oligonucleotide synthesis (SPOS) is one of the breakthrough achievements of nucleic acids chemistry, which with established automated techniques allows a prompt and fast assembly of oligonucleotides (ONs) of the desired sequence and modifications on demand.[1] By SPOS, ONs can be produced in a process scale with high efficiency and minimized reactant consume, but it does not change the fact that the two-phase system is a non-ideal reaction environment that needs an extra excess of reagents to drive the reactions to the completion. Furthermore, the applicability of SPOS for real-time optimization is limited. The reaction parameters, presenting technical and chemical limitations, cannot be optimized beyond the linearity of the two-phase system, which has challenged SPOS in current sustainability demands. To make ON manufacturing more sustainable and process compatible,[2] robust synthesis strategies, which work entirely in solution (i.e., Liquid Phase Oligonucleotide Synthesis, LPOS) have attracted growing interest.[3–5] The advanced methods are based on soluble supports, which facilitate isolation of the growing ONs from the reaction media either by membrane filtration or precipitation.[6–16] In addition, a marked achievement has recently been done in a convergent approach, which ligates tetra- and pentameric fragments to gain full-length ON products.[17]

LPOS may need re-evaluation of the ON protecting group scheme. In addition to chemical tolerability, the optimized protecting groups should reduce the total process mass intensity and improve the solubility of the growing protected ON segments in the reaction media, which is a more obvious parameter in solution than on a solid phase. Furthermore, it would be beneficial if the deprotection results in volatile byproducts, which simplifies or reduces the purification steps during the synthesis. An additional concern relates to the temporal 5′-*O*-protection. Dimethoxytrityl (DMTr) is the protecting group of choice in SPOS, but it is not an ideal one in LPOS. The complete and fast acid catalyzed removal of DMTr is interfered by the reversible nature of the reaction, proceeding *via* the DMTr carbocation intermediate. Scavengers, e.g., silanes[16] and thiols,[17] are needed to drive the reaction to the completion. Furthermore, the DMTr derivatives formed in the cleavage process are lipophilic, and they must thus be removed from the protected oligomeric products by an additional purification step, which sometimes requires column chromatography.

To offer alternatives to the ON protecting group scheme, we have recently studied properties of acetone acetals as the 5′-*O*-protecting groups.[18] We propose that the above-mentioned disadvantages of DMTr group in LPOS could mostly be overcome by using a small sized acetal (ketal) group. The pseudo irreversible removal of the acetal groups can be run to completion under the mild synthesis conditions, and the residual byproducts (acetone and alcohol) can be readily removed by evaporation or extraction. Acetals are in general among the most conventional and widely used protecting

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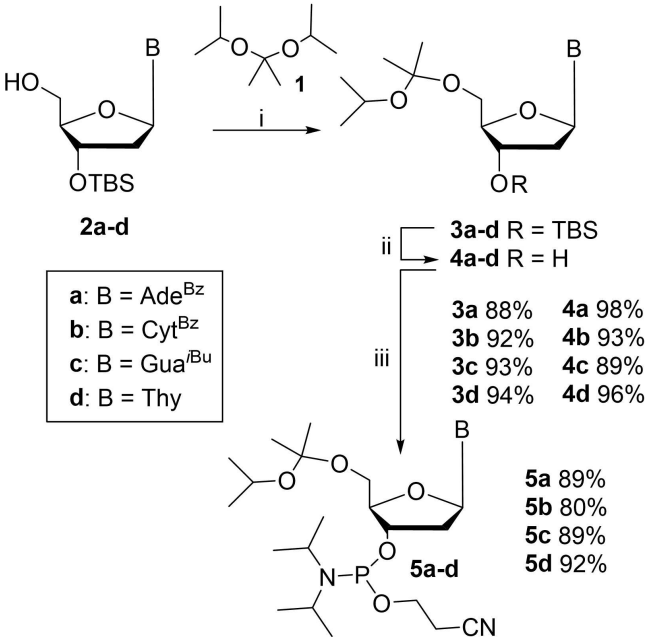
groups for hydroxyl groups but have not been commonly applied for 5′-*O* protection in ON synthesis, besides the examples reported with the 2-methoxyisopropyl (MIP) group.[19,20] The acid lability of the acetal protection can be steered by varying its structure. Based on the results of the earlier study[18] we chose to take the isopropoxyisopropyl group (IIP; 2-isopropoxyprop-2-yl) under more detailed examination. IIP appears to be faster removable in acid than MIP is (a 7.4-fold difference was found in hydrolysis rates).[18] The IIP also makes the nucleosides slightly more lipophilic, as compared to MIP protected ones, which is beneficial for the synthesis and isolation of the corresponding phosphoramidite building blocks.

In the present study, 5′-*O*-IIP protected 2′-deoxyribonucleoside phosphoramidite building blocks were synthesized and their applicability in LPOS on a tetrapodal soluble support was demonstrated. The advantage was the mild and fast IIP-deprotection step, which could be carried out by a mixture of nonhalogenated carboxylic acids. The results obtained in this study look promising concerning both the management and repeatability of the synthesis cycle.



Scheme 1. Preparation of 5'-*O*-IIP-protected 2'-deoxyribonucleoside phosphoramidites **5a**–**5d**; Conditions: i) 2,2-diisopropoxypropane (**1**), *p*TSA; ii) $Bu_4N^+F^-$, iii) 1-chloro-1-(2-cyanoethoxy)-*N*,*N*-diisopropylphosphinamine, DCM, TEA, at RT for 3 h.

Results and Discussion

Preparation of 5'-O-(2-Isopropoxyprop-2-yl)-2'-Deoxyribonucleoside Phosphoramidites

Previously, vinyl ethers have been used to introduce acetal protection to the 5′-*O* site.[18] A simple and efficient transacetalization method using 2,2-diisopropoxypropane (**1**) was applied in the present study (Scheme 1). This gives somewhat better yields minimizing the side products perceived to electrophilic addition of vinylethers.[18] The acetal reagent for the procedure, 2,2-di-isopropoxypropane (**1**), was prepared by a simple one step procedure[21] from acetone and isopropanol under acid catalysis in the presence of 5 Å molecular sieves. Product **1** could be prepared in 100 g scale in a reproducible manner in a moderate (21 %) yield.

The most conventional acyl protecting groups were employed at the exocyclic amino groups of the starting nucleosides (commercial products), and the *tert*-butyldimethylsilyl (TBS) group was introduced to the 3′-*O* sites by known methods[22] *via* the 3′,5′-*O*,*O*-bis-silylated intermediates. The 5′-*O*-unprotected nucleosides **2a**–**d** were then treated with acetal **1** using *p*-toluenesulfonic acid (*p*TSA) as an acid catalyst to give the fully protected nucleosides **3a**–**d** in high 80–90 % yields. Removal of the 3′-O-TBS by tetrabutylammonium fluoride results in nucleosides **4a**–**d** in 80–90 % yields, and phosphitylation of the exposed 3′-OH by 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite in the presence of triethylamine (TEA) in dichloromethane (DCM) gave the desired 5′-O-IIP-protected phosphoramidites **5a**–**d** in 80–92 % yields.

Acid-catalyzed methanolysis of 5'-O-protected 2'-deoxyribonucleosides

To suppress depurination, mixtures of halogenated carboxylic acids (TCA and DCA in the presence of silane and alkylthiol scavengers) have been carefully examined for the removal of 5′-O-DMTr in LPOS.[16,17] Even purine-rich sequences have successfully been assembled using these deprotection cocktails. However, a protecting group that can be removed by mixtures of non-halogenated natural carboxyl acids would make the ON assembly greener and may also be beneficial to find optimal conditions to minimized depurination during the assembly. The proper IIP deprotection conditions in LPOS were optimized using variable amounts of formic and acetic acid in different solvent systems. Considering the rate and reproducibility of the deprotection, along with solubility and precipitation efficiency of the soluble support constructs, 6 % formic acid in DCM/MeOH (2:1; *v*/*v*, RT) was selected for removing the IIP. To demonstrate the relative deprotection and depurination rates, acid-catalyzed methanolysis of N^6-benzoyl-2′-deoxy-5'-*O*-IIP-adenosine (**4a**), 2′-deoxy-5'-*O*-IIP-thymidine (**4d**), 2′-deoxy-5'-*O*-MIP-thymidine (**6**) and 2′-deoxy-5'-*O*-DMTr-thymidine (**7**) was studied in the same conditions. The progress of the reactions was followed by HPLC. The observed half times are collected in Table 1. The departure of IIP group from **4a** and **4d** ($t_{1/2} \approx 6$ and 10 s, respectively) takes place about 15 times faster than the departure of MIP group from **6** ($t_{1/2} = 125$ s). For comparison, the half time for detritylation of **7** under the same conditions is 560 s. Thus, the deprotection rate of IIP was proved superior compared to MIP and DMTr to avoid parallel depurination. The half time of depurination of **4a** was 25000 s, which is more than

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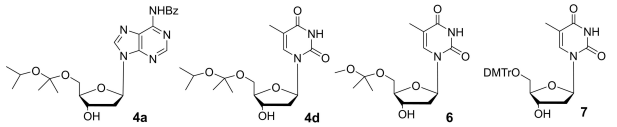
Table 1. Half times of deprotection of 5′-*O*-protected nucleosides.



Entry	nucleoside	5′-*O*-deprotection $t_{1/2}$/s	depurination $t_{1/2}$/s
1	**4a**	6 ± 1	25000 ± 110
2	**4d**	10 ± 2	NA
3	**6**	125 ± 6	NA
4	**7**	560 ± 54	NA

Reaction conditions: 6% formic acid in DCM/MeOH (2:1; *v/v*, RT).

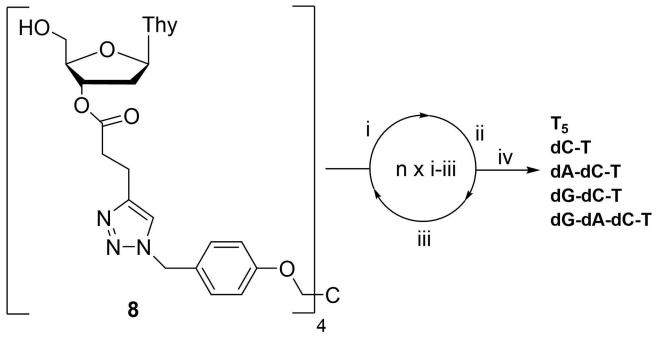
3 orders of magnitude longer than the half times of deacetalization of **4a** and **4d**.

Stability of 5′-O-IIP protecting group in phosphoramidite coupling conditions

To examine the stability of the IIP protecting group towards the acidic activators used in the phosphoramidite coupling, 5′-*O*-IIP-thymidine (**4d**) was used as a model compound and exposed to a stoichiometric amount of 1*H*-tetrazole (Tet*H*) or 4,5-dicyanoimidazole (DCI) in a mixture of DMF and MeCN (2:1, *v/v*) at RT. The potential IIP removal was monitored by HPLC (Figure S1). Compound **4d** proved relatively stable in the tetrazole solution (virtually intact after 2 h, and 10% cleavage after 24 h), whereas notable cleavage (46% after 2 h) of IIP was observed in the presence of DCI. The stability of IIP was against that may be expected according to the standard aqueous pK_a values of the activators (DCI: p*K*a 5.2, tetrazole: pK_a 4.89). However, in the aprotic solvents the situation is different, and even nucleophilicity of the conjugate base of the activator (DCI anion vs. tetrazolide anion) towards the released oxocarbenium cation may affect the observed rate of the acetal cleavage. Based on these results, we chose tetrazole for the assembly of short ON sequences by LPOS, despite the known harmful properties of this reagent.

LPOS using 5'-O-IIP protected phosphoramidites (5a–d)

To evaluate the applicability of **5a–d** in LPOS, short oligonucleotides were assembled on a thymidine-loaded pentaerytritol-derived soluble support (**8**) on a 0.2 mmol scale as demonstrated previously (Scheme 2).[6] A two-precipitation protocol in 2-propanol was followed, with one precipitation after the oxidation and the other one after the deacetalization. Nearly quantitative couplings were obtained using the protected phosphoramidite building blocks **5a–5d** (0.13 mol L^{-1}, 2.0 eq./ 5'-OH group) and tetrazole (2.0 eq./ 5'-OH group) in a mixture of DMF-MeCN (2:1, *v/v*) (for 2 h at RT). The phosphite esters obtained were oxidized to the phosphate triesters with 3-chloroperoxybenzoic acid (3.8 eq.) in 5 min, after which the product was precipitated in 2-propanol. The complete IIP-removal from the tetrapodal oligonucleotide products was achieved by dissolving the precipitates in a mixture of DCM and MeOH (2:1, *v/v*) containing 6% of formic acid. The reaction solution was neutralized by addition of pyridine (2 equiv. in relation to HCOOH) and the product was precipitated in 2-propanol. The steps of each extension cycle were monitored by HPLC. Aliquots of the precipitates were released/deprotected by aqueous 25% ammonia (5 h at 55 °C) and the efficiency of the chain elongation was evaluated by RP HPLC analysis (Figure 1). The average yield of the individual synthesis cycles, including two precipitations, was ca 86%.



Scheme 2. Synthesis procedure for di- tri-, tetra and pentanucleotide phosphodiesters using **5a–d** in LPOS. i) *Coupling:* **5a–5d**, TetH, DMF/MeCN (1:1, *v/v*), for 2 h at RT; ii) *Oxidation:* mCPBA, for 2–5 min, followed by precipitation in 2-propanol; iii) *Deprotection:* 6% HCOOH in DCM/MeOH (2:1, *v/v*) for 6 min at RT, followed by addition of pyridine (2 eq. in comparison to HCOOH) and precipitation in 2-propanol; iv) 25% aqueous ammonia, for 5 h at 55 °C.

Conclusions

5'-*O*-(2-isopropoxyprop-2-yl)(IIP)-protected 2′-deoxyribonucleoside phosphoramidites were prepared and used for the liquid

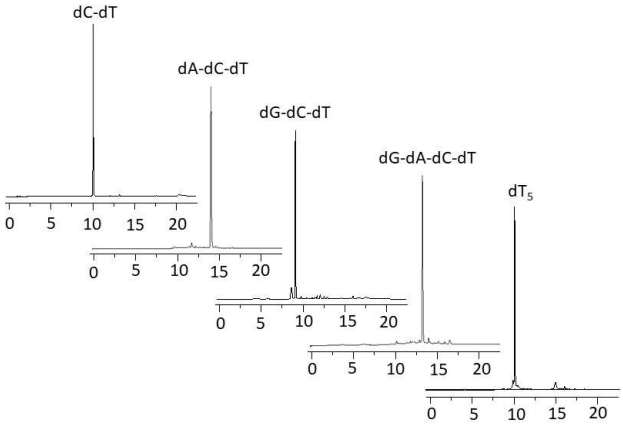


Figure 1. Examples of RP HPLC profiles of the crude product mixtures of the oligomers shown in Scheme 2. Chromatographic conditions: 2 min isocratic elution with 50 mM TEAA and MeCN (2%), followed by linear gradient from 2% to 23% of MeCN.

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phase oligonucleotide synthesis (LPOS) on a precipitative, tetrapodal soluble support. The 5′-*O*-IIP protecting group is introduced to nucleosides readily by an acid-catalyzed trans acetalization with 2,2-di-isopropoxypropane. The advantage of IIP in LPOS is its fast acid-catalyzed cleavage by a mild natural carboxylic acid treatment (6% formic acid in DCM-methanol, 2:1, *v/v*), in which volatile byproducts, acetone and isopropanol, are formed. The acid-catalyzed methanolysis of IIP group is more than three orders of magnitude faster than depurination under the same conditions. On the other hand, the high acid sensitivity of IIP sets limitations to coupling conditions, defining the activators that can be used for the phosphoramidite coupling. The 1*H*-tetrazole activator worked well in this study with **5a–d**. Studies under alternative greener coupling conditions are under way. We believe that the strategy may be tuned for a potential alternative for the preparation of short purine-rich 2′-deoxyribo oligonucleotides in solution.

Experimental Section

2,2-diisopropoxypropane (1): A published procedure[21] was applied. This allowed preparation of **1** on a large scale, even though the yield remained lower than those given in the reference. A mixture of anhydrous 2-propanol (4.84 mol, 370 ml), acetone (1.35 mol, 100 ml), *p*-toluenesulfonic acid (0.244 mol, 46.5 g), activated 5 Å molecular sieves (powder form, 1 kg) and cyclohexane (600 ml) was stirred under argon flow with a mechanical stirrer in a round bottom flask cooled in an ice-bath. After 1.5 h stirring, the ice-bath was removed, and after stirring for 45 min at RT the reaction was quenched with triethylamine (78 mL, 0.56 mol). The mixture was filtered through a glass sinter, and the molecular sieves were washed with pentane. The combined filtrate was washed with ice-cold 15% aqueous NaOH (2×250 ml) and water (2×250 ml). The organic phase was dried over K_2CO_3, and after filtration the solution was concentrated under reduced pressure. The pressure was kept above 120 mbar over the evaporation. The concentrate was then distilled under reduced pressure. The product was distilled at 69 °C/68 mmHg as a colorless liquid. Yield: 45.6 g (21%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.04 (septet, J = 6.15 Hz, 2H, isopropoxy $OCH(Me)_2$), 1.35 (s, 6H, acetal $OC(CH_3)_2O$)), 1.15 (d, J = 6.15 Hz, 12H, isopropoxy $OCH(CH_3)_2$); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 100.18 ($OC(Me)_2O$), 62.37 ($OCH(Me)_2$), 27.02 ($OC(CH_3)_2O$), 24.44 ($OCH(CH_3)_2$).

Preparation of the 5′-*O*-(2-isopropoxyprop-2-yl)-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxynucleosides 3a–3d: Typically, 18 mmol of the appropriate protected nucleoside (**2a–2d**) was dissolved in anhydrous dichloromethane (20 mL). The solution was effectively stirred, and 2,2-diisopropoxypropane **1** (120 mmol, 7 eq.) was added. To initiate the reaction, *p*-toluenesulfonic acid was added as a 1 M anhydrous THF solution. The acid was added gradually in 5 mol% increments and the proceeding of the reaction was monitored by TLC. As completed, the reaction was quenched with triethylamine (2 eq. as compared to the added acid catalyst). The solution was diluted with 50 mL of dichloromethane and washed with a 5% aqueous $NaHCO_3$ (20 mL) and with saturated NaCl (20 mL). The organic phase was dried over $MgSO_4$, filtered, and evaporated to dryness. The product was purified on a silica column eluted with a mixture of ethyl acetate and *n*-hexane. After evaporation of the solvents, the target nucleoside was obtained as a white foam in 88–97% yields.

Preparation of the 5′-*O*-(2-isopropoxyprop-2-yl)-2′-deoxynucleosides 4a–4d (removal the 3′-*O*-*tert*-butylsimethylsilyl group): The fully protected nucleoside **3** (20 mmol) was dissolved in ethyl acetate (110 mL) and tetrabutylammonium fluoride (40 mmol) was added into the stirred solution. As the reaction was completed (2.5 h at RT, monitored by TLC) the mixture was diluted with 110 mL of ethyl acetate, and then washed with 5% aqueous $NaHCO_3$ (2×70 mL) and saturated aqueous NaCl (70 mL). The aqueous phase was back-extracted with EtOAc (70 ml). The combined organic layers were dried over $MgSO_4$, and after filtration the solvent was evaporated under reduced pressure. The product was purified on a silica column eluted with a mixture of methanol (0–5%, *v/v*) and ethyl acetate, containing 1% triethylamine. After evaporation of the eluent the product was obtained as a white foam. Yields were varying between 80–90%.

Preparation of the protected 2′-deoxynucleoside

3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites 5a–d: Typically, 1.10 mmol of the 5′-*O*-(2-isopropoxypropan-2-yl) protected 2′-deoxynucleoside (**4a–d**) was dried with P_2O_5 overnight and dissolved in a mixture of anhydrous dichloromethane (DCM, 5.7 mL) and triethylamine (TEA, 0.22 mL, 1.58 mmol) under nitrogen atmosphere at room temperature. 2-Cyanoethyl *N*,*N*-diisopropyl-chlorophosphoramidite (0.29 mL, 1.32 mmol) was added. The phosphitylation was allowed to proceed for 3 h. The crude product was purified on a silica gel chromatography eluting with a mixture of EtOAc and hexane (2:1, *v/v*) containing 3% of TEA. The product obtained as a white foam was co-evaporated twice with DCM that contained 1% pyridine. The products **5a–5d** were obtained in 80–92% yields.

Liquid phase oligonucleotide synthesis using building blocks 5a–5d: The tetrapodal nucleoside support (**8**; 0.05 mmol)[6] and the appropriate nucleoside phosphoramidite **5a–d** (0.41 mmol) were dissolved in a mixture of anhydrous DMF (1.53 mL) and MeCN (0.62 mL), and tetrazole (0.45 mol L^{-1} in acetonitrile, 0.91 mL, 0.41 mmol) was added under nitrogen atmosphere at room temperature (0.13 mol L^{-1} concentration of **5a–d**). The reaction was allowed to proceed for 2 h, and 3-chloroperoxybenzoic acid (0.77 mmol, 3.8 eq./phosphite triester group) was added. After 2–5 min, the reaction mixture was added to cold 2-propanol (60 mL). The solution was centrifuged, the 2-propanol supernatant was decanted off and the precipitate was dried under vacuum. The precipitate was dissolved in a 6% solution of HCOOH (0.77 mmol) in a mixture of DCM and MeOH (2:1, *v/v*). The mixture was stirred for 6 min, neutralized by addition of pyridine (2 equiv. compared to HCOOH) and added to cold 2-propanol (12 mL). The solution was centrifuged, the 2-propanol supernatant was decanted off and the precipitate was dried under vacuum. By using this repeating synthesis cycle (n = 2–4), white powder of protected tetrapodal di-, tri-, tetra- and pentanucleotides were obtained [d(CT)-, d(ACT)-, d(GCT)-, d(GACT)- and dT_5-derivatives in 78%, 78%, 77%, 49% and 58% yields, respectively]. The precipitates were treated with concentrated (25%) aqueous ammonia (1.5 mL) at 55 °C for 5 h (or overnight). The precipitated traces of the soluble support, i.e. tetrakis[(4-{[4-(3-amino-3-oxopropyl)-1H-1,2,3-triazol-1-yl−]methyl} phenoxy)-methyl]methane,[6] were filtered off and the filtrates were evaporated to dryness. The residues were dissolved in water, washed with ethyl acetate, and subjected then to a RP HPLC analysis (Figure 1). The yields of the ammonolyses of the nucleotides as determined according to UV-absorbance at λ = 260 nm were 96% (dC-dT), 99% dA-dC-dT, 97% (dG-dC-dT), 82% (dG-dA-dC-dT) and 86% (dT_5). The overall yields (calculated from soluble support) for the dimer, trimers, tetramer and pentamer were 75%, 78%, 64%, 48% and 50% respectively. MS (ESI) *m/z* for dC-dT: calcd 530.1294 $[M–H]^-$; found 530.1294, for dA-dC-dT: calcd 843.1870 $[M–H]^-$; found 843.1956, for dG-dC-dT: calcd 859.1819 $[M–H]^-$;

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found 859.1911, for dG-dA-dC-dT: calcd 1172.2322 [M–H]$^-$; found 1172.2239 and for dT_5: calcd 728.1299 [M-2H]$^{2-}$, found 728.1230; ^{31}P NMR (200 MHz, 0.2 M pH 7.4 sodium cacodylate buffer/ D_2O (4:1, *v/v*)): $\delta = -1.026$ ppm (dC-dT), −1.046 and −1.149 (dA-dC-dT); −1,038 and −1.164 (dG-dC-dT); −1.120, −1.192, and −1.272 (dG-dA-dC-dT); $\delta = -1.180$, −1.207, −1.236 and −1.282 (dT_5).

Acid-catalyzed methanolysis of 5'-*O*-protected 2'-deoxyribonucleosides: The protected nucleosides **4a**, **4d**, **6** and **7** were dissolved in 6% HCOOH in a mixture of DCM/MeOH (2:1; *v/v*) in an Eppendorf tube (0.1 mol L^{-1}, substrate concentration), and the progress of the methanolyses (at RT) was followed by RP-HPLC At appropriate time intervals, 3 to 8 samples of 2 μL were taken. The samples were quenched by addition of triethylamine (2 equiv. compared to HOOCH), cooled in an ice bath, and diluted with MeCN/H_2O (200 μL, 1:1, *v/v*). The composition of the samples was analyzed by RP HPLC. The half lifes given were calculated by applying the first order rate equation to the diminution of the peak area of the starting compound. The reaction products were identified by spiking with the authentic samples.

Supporting Information

The data that support the findings of this study are available in the supplementary material of this article.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: acetals · liquid phase oligonucleotide synthesis (LPOS) · phosphoramidites · protecting groups · soluble support · sustainable chemistry

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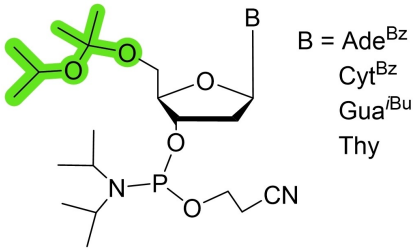
RESEARCH ARTICLE

Liquid phase oligonucleotide synthesis calls for improved methodologies. We have tested an acetal modification for protection of the 5′-*O* site of nucleotide building blocks. Good yields were obtained in synthesis of short 2′-deoxyoligonucleotides by phosphoramidite chemistry on a soluble support. The advantages of the title group include fast cleavage, easily removable volatile residues, and small size improving the atom economy.



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5'-*O*-(2-Isopropoxyprop-2-yl)-protected Phosphoramidite Building Blocks in the Liquid Phase Oligonucleotide Synthesis

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