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Special Issue

Acid-Stable Nucleobase Protection for a Strongly Pairing Pyridone C-Nucleoside Suitable for Solid-Phase Synthesis of Oligonucleotides

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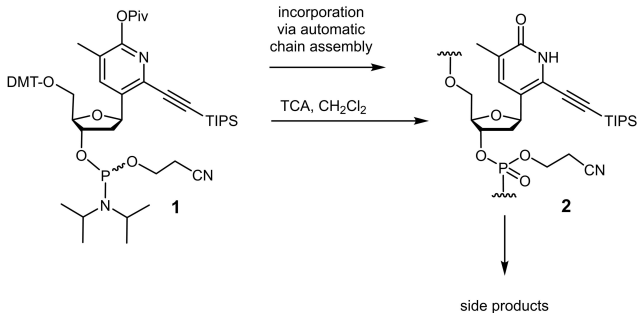
Oligonucleotides are indispensable tools in diagnostics, therapeutic applications and molecular biology. The low base pairing strength of thymine with adenine complicates their use. Ethynylpyridone *C*-nucleosides are analogs of thymidine that pair more strongly and with improved base selectivity, and sequences containing these analogs show improved target affinity and selectivity, but their routine use is hampered by diminished yields of solid-phase syntheses with the known building blocks. A partial loss of base protecting groups during the acidic deblocking step of chain extension cycles was identified as the cause of lower yields. Here we report the synthesis of an improved phosphoramidite building block featuring a pivaloyloxymethyl (POM) base protecting group. This building block gives oligonucleotides containing the strongly pairing ethynylmethylpyridone *C*-nucleoside in high yield and purity via solid-phase synthesis.

Introduction

Synthetic oligodeoxynucleotides (ODNs) are invaluable in diagnostics, therapeutics, molecular biology, bioorganic chemistry, and nanostructuring. Their predictable binding to complementary regions of target strands makes them useful as hybridization probes, primers, antisense[1] or antigene agents,[2] and building blocks for designed three-dimensional assemblies,[3] to name just a few of their many applications.[4] Solid-phase synthesis has made ODNs with canonical bases readily available at very reasonable costs. Sequences with modified bases are often more difficult to prepare, though, making them interesting targets from a synthetic standpoint.

One motivation for developing syntheses of modified oligonucleotides is to increase target affinity. The most weakly binding base of the four canonical nucleobases of DNA is thymine,[5] and A-rich target sequences can be difficult to bind with oligonucleotides containing unmodified bases.[6] This has prompted a quest for replacements of T that interact more strongly with A than the natural deoxynucleoside.[7–9] In this context, *C*-nucleosides have recently come into focus as surrogates with increased affinity for adenine in target strands. In particular, ethynylpyridones have shown potential to overcome the poor target affinity and fidelity of thymine or uracil.[10–14]

While several methods for setting up the *C*-nucleoside framework are known,[15,16] the incorporation in oligonucleotides usually relied on phosphoramidite or *H*-phosphonate-based coupling[10] on controlled pore glass as solid support as the predominant method. In automated syntheses, phosphoramidites are the preferred building blocks for chain assembly.[17] This is why a phosphoramidite building block of the 6-ethynyl-3-methylpyridone *C*-nucleoside, abbreviated "W", which pairs with adenine in target strands approximately as strongly as deoxycytidine pairs with deoxyguanosine was developed.[18] This pyridone building block (**1**, Scheme 1) has been commercialized,[19] leading to more extensive use in the synthesis of ODNs. While syntheses of strands containing one or several W residues were successful, and duplex stabilization was observed for each sequence tested,[18] side products were detected in crudes in a follow-up study.[20] The side reaction causing them was attributed to partial loss of pivaloyl (Piv)



Scheme 1. Removal of dimethoxytriyl (DMT) protecting groups with trichloroacetic acid can lead to loss of pivaloyl (Piv) protecting groups of ethynylpyridone *C*-nucleosides during automated DNA synthesis, resulting in side products. Piv = pivaloyl, TCA = trichloroacetic acid, TIPS = tri*iso*propylsilyl.

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groups during removal of dimethoxytrityl (DMT) 5′-protecting group in the so-called 'deblock' step of chain extension cycles, which uses trichloroacetic acid in anhydrous dichloromethane.

The resulting free pyridone (**2**, Scheme 1) then coupled to some of the the next incoming phosphoramidites, resulting in detectable levels of branched products that were difficult to hydrolyze fully to the desired linear strands.[20] Apparently, protonation of the pyridine nitrogen makes the pivaloyl ester so labile that even trace water in the deblock solution suffices to induce hydrolysis. This motivated a search for a less acid labile protecting group scheme for pyridone *C*-nucleosides. Here we report the results of our study that resulted in a building block with base protection that suppresses side reactions during ODN synthesis.

Results and Discussion

The report of an industrial group[20] indicated that milder Deblock reagents, such as dichloroacetic acid, can reduce the loss of Piv groups, but deviating from the established standard conditions of automated DNA synthesis was undesirable. Instead, a more stable protecting group for the pyridone unit was sought. Because protonation of the pyridinic nitrogen was the most likely cause of lability under acidic conditions, *N*- rather than *O*-protection was considered. However, it was known from thymidine that even *N3*-Piv protected nucleosides are unstable under acidic conditions.[21] Next, we tested other acyl groups. Unfortunately, both acetyl and benzoyl groups were unstable under the conditions of the Heck reaction installing the pyridone. Derivatives with sterically more shielded groups, such as the adamantoyl group employed for other non-canonical nucleosides[22] or the mesitoyl group, were also unstable or produced difficult-to-separate mixtures of *N*- and *O*-protected products when introduced to the pyridone. Carbonate and carbamate protecting groups, such as the fluorenylmethoxycarbonyl (Fmoc)[23] or diphenyl carbamoyl (DPC)[24] group also did not give the stability required to survive all steps of the established synthetic route to the pyridone *C*-nucleosides.[10,18]

Subsequently, ether protecting groups groups were tested. The *O*-methyl derivative proved resistant to cleavage under several conditions compatible with our nucleosides. The benzyl protecting group is well established for the synthesis of *C*-nucleosides,[10,25] and can be removed under Lewis acidic conditions or by hydrogenolysis. Neither of the Lewis acids was compatible with oligonucleotides, though, and hydrogenolysis led to partial reduction of the ethynyl group. The more redox-labile *para*-methoxybenzyl (PMB) group also could not be readily removed after DNA synthesis. A diphenyl methyl ether, similar to the one used for other nucleosides[26], was tested as a less stable version of the benzyl ether, but attempts to obtain the protected *C*-nucleoside in sufficient yield were unsuccessful. Finally, an allyl ether was found to be incompatible with the Heck reaction, due to the reactivity of its olefinic moiety.

As no appropriate ether protecting group was found, silyl protecting groups were investigated. The widely used *tert*-butyldimethylsilyl (TBDMS), *tert*-butyldiphenylsilyl (TBDPS) and tri*iso*propylsilyl (TIPS) ethers of the pyridone were either too unstable or incompatible with at least one step of the synthetic route. Next, a composite silyl protecting group with a two-step deprotection mechanism was considered. One example for this is the tri*iso*propylsilyloxymethyl (TOM) protecting group used for masking 2′-hydroxy groups during RNA syntheses. [27,28] Cross reactivity with the silyl group of the glycal employed as educt in the Heck reaction-based glycosylation was found to be problematic, though.

A more suitable composite protecting group was then identified that can be cleaved under basic conditions at the end of automated DNA syntheses, concomitantly with the removal of the acyl protecting groups on the nucleobases, the cyanoethyl group protecting the phosphodiesters, and the succinyl linkage to the solid support. This was the pivaloyloxymethyl (POM) protecting group, comprised of a formyl acetal and the pivaloyl group capping the distal hydroxy functionality. The POM protecting group is known from the phosphonate prodrug adefovir dipivoxyl,[29,30] as well as from protected forms of uridine, thymidine,[31–34] imidazole *C*-nucleosides, and pseudouridine.[35,36] In the latter cases, the nucleobases are *N*-protected, though, and it was unclear what the chemoselectivity would be for ethynylpyridones. Based on the high *O*-reactivity of the uracil[10] and thymine analogs,[18] we opted for phosphoramidite **3** as the preferred target molecule for our syntheses.

Two routes to **3** are shown in Scheme 2. The most obvious approach, introducing the POM group at the beginning of the aglycone synthesis, was abandoned because the diazonium salt intermediate used for the penultimate step in the elaboration of the aglycone did not precipitate, and the crude reaction mixtures gave significant side products upon conversion to the iodide. Instead, the first route introduced the POM group on the nucleoside level. For this, deoxynucleoside **4**[18] was treated with ammonia to obtain free pyridone *C*-nucleoside **5**. Then, the POM group was introduced using POM–Cl and carbonate in DMF. Both *O*-alkylated **6** and *N*-protected **7** were isolated, at a ratio of 3 : 1, and a combined yield of 56 %, as well as smaller quantities of Piv-protected species resulting from transacylation rather than alkylation. The structure of the *N*- and *O*-alkylated pyridone C-nucleosides was confirmed by 2D NMR (see Supporting Information). Both POM-protected pyridone *C*-nucleosides, **6** and **7** were subjected to deblock conditions (3 % TCA in CH_2Cl_2) and the release of free nucleotide **5** was monitored by UV absorption at the characteristic 323 nm maximum of the free ethynylmethylpyridone.

Figure 1 shows the results of this stability study, together with the kinetics for the corresponding *O*-pivaloyl protected **4**. Both POM-protected compounds passed the stability test, whereas **4** showed the expected instability under these conditions (no attempt to exclude residual water was made). Among the two *C*-nucleosides with composite protecting group, **7** appeared even more resistant to acidic cleavage, remaining unchanged for 14 d, but **6** was chosen for further elaboration to **3**, via 5′-protected **8**, as the more readily accessible compound.

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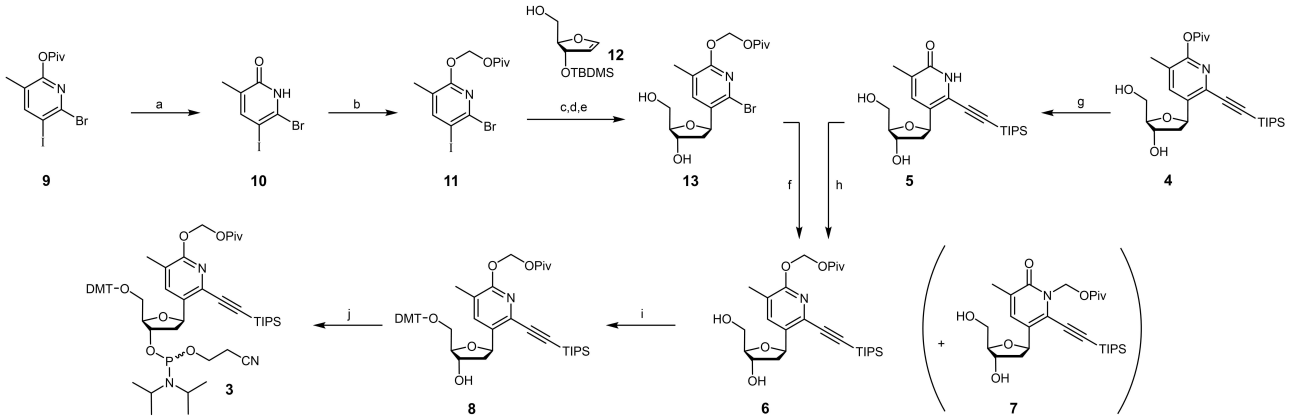

Scheme 2. Synthesis of the POM protected phosphoramidite **3**, starting from Piv protected aglycone **9** or *C*-nucleoside **4**. Conditions: (a) NH_4OH/MeCN, 99%; (b) POM–Cl, K_2CO_3, DMF, 82%; (c) glycal **12**, $Pd(OAc)_2$, $P(PhF_5)_3$, Ag_2CO_3, CH_3CN; (d) 3HF·NEt_3, THF; (e) $NaBH(OAc)_3$, CH_3CN, 64% over 3 steps; (f) tri*iso*propylsilyacetylene, $[Pd(PPh_3)_2Cl_2]$, CuI, NEt_3, DMF, 61%; (g) NH_4OH/MeCN, 86%; (h) POM–Cl, K_2CO_3, DMF, 44%; (i) DMT–Cl, pyridine, 67%; (j) $(iPr_2N)_2P(OC_2H_4CN)$, DIPAT, CH_3CN, 93%; DIPAT = diisopropylammonium tetrazolide, DMT = 4,4′-dimethoxytrityl, Piv = pivaloyl, POM = pivaloyloxymethyl, TIPS = tri*iso*propylsilyl.

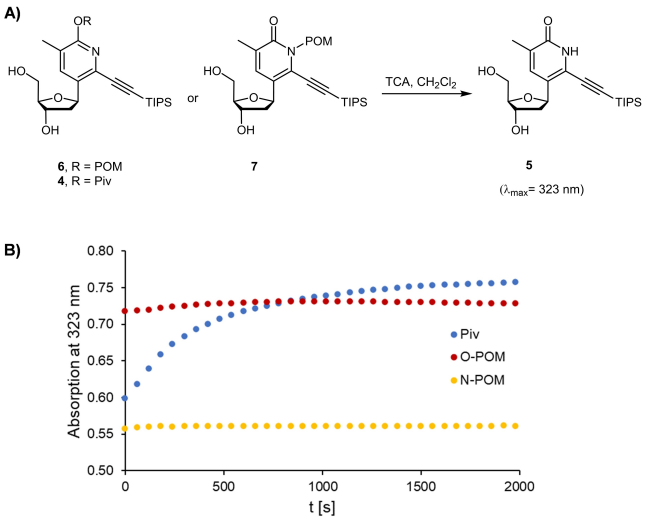

Figure 1. Stability of protected nucleosides to the Deblock solution of automated DNA synthesis (3% TCA in dichloromethane), as monitored by UV-absorbance at λ_{max} = 323 nm. A) reaction Scheme; B) Kinetics of formation of free pyridone **5**, starting from 82 μM solution of the respective nucleoside. TCA = trichloroacetic acid.

The second route to **3** introduces the POM group earlier, starting from **9**,[18] and proceeding via free pyridone **10**, which was *O*-alkylated to POM-protected **11** in 82% yield, with just 9% of the *N*-protected counterpart formed. This higher chemoselectivity may be due to the bromine at the 2-position and its steric and/or electronic effect. The POM-protected aglycone was used in the Heck reaction with glycal **12**, which was prepared as described previously.[10] Thus, protected **13** was obtained in a yield of 64% over 3 steps, including the cross coupling itself, desilylation and stereoselective reduction to the 2′-deoxyribonucleoside. Silyl enol ether **16** and ketone **17** were not purified, as they are unstable, as described for similar molecules in the literature.[10,18] The subsequent Sonogashira reaction gave 61% yield, and the 5′-DMT protection to **8** and phosphitylation to **3** proceeded in 67% and 93% yield, respectively.

Phosphoramidite **3** was then used for the synthesis of two different ODNs (Figure 2A). Oligonucleotide **14** is a sequence previously prepared by the industrial group that reported the branching problem.[20] The 5′-DMT protection was left in place and deprotection in aqueous ammonia for 2 h at room temperature was used to retain the TIPS groups, mirroring the conditions of the literature work. Reversed-phase HPLC yielded **14** in 32%, and no later eluting peaks for branched oligonucleotides were observed. This is in contrast to the corresponding HPLC trace obtained with the Piv protecting group, which is shown in ref. 20. The sequence of ODN **15** is that of a primer for PCR tests detecting the SARS-COV-2 virus.[37] Here, solid-phase chain assembly was followed by deprotection with ammonium hydroxide for 16 h at 55 °C. Although about half of the TIPS groups were removed during this step, no hydration of the ethynyl residue, as described for similar ethynyl-substituted bases,[38] was observed. After ammonia deprotection, the remaining TIPS groups were removed with TBAF. The HPLC trace of the crude showed no peaks for branching products (Figure 2B), and **15** was obtained in 48% yield, i.e. the highest yield published for an ODN containing an ethynylpyridone *C*-nucleoside to date.

Conclusions

A broad screen of protecting groups identified the pivaloyloxymethyl (POM) protecting group as an acid-stable replacement for the pivaloyl group of the commercial building block for the strongly pairing *C*-nucleoside W. The corresponding phosphoramidite building block (**3**) was successfully employed in the synthesis of oligonucleotides under standard DNA synthesis conditions. The high-yielding synthesis of W-containing strands opens the door to a routine use of the high-

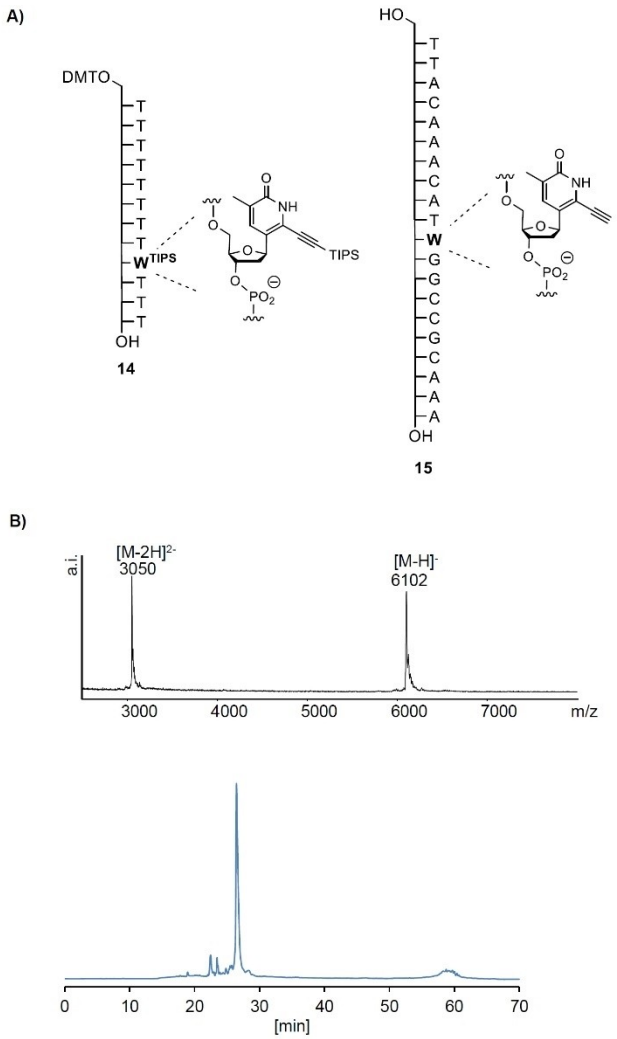

Figure 2. Synthesis of oligonucleotides containing W. A) Sequences prepared using **3**. B) HPLC trace of the crude **15** (C18 column, TEAA buffer/CH_3CN, 55 °C) (top), and MALDI-TOF mass spectrum of **15** after HPLC purification in linear negative mode (bottom).

affinity replacement for thymidine and thus oligodeoxynucleotides that can hybridize to their target sequences with high affinity and fidelity.

Experimental Section

Reagents and solvents were purchased from Acros Organics (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany), Carbolution Chemicals (Saarbrücken, Germany), Carbosynth (Compton, United Kingdom), Thermo Fisher (Karlsruhe, Germany), Sigma-Aldrich (Schnelldorf, Germany) or TCI Europe (Zwijndrecht, Belgium). All reactions were performed under argon atmosphere. For thin layer chromatography Macherey-Nagel ALUGRAM® Xtra SIL G/UV254 precoated silica gel sheets were used, and the analytes were visualized by ultraviolet light or staining with phosphomolybdatocerium(IV) sulfate solution (25 g phosphomolybdic acid hydrate, 10 g cerium(IV) sulfate tetrahydrate, 60 mL conc. sulfuric acid diluted with water to 1 L). Column chromatography was carried out on silica from Macherey-Nagel (Düren, Deutschland). The NMR spectra were measured on Bruker Ascend 400, Avance 500 or Ascend 700 spectrometers . ^{1}H-NMR spectra were measured at 400, 500 or 700 MHz, ^{13}C-NMR spectra at 100, 125 or 175 MHz, and ^{31}P-NMR-spectra were recorded at 162 MHz. Chemical shifts are reported as δ values, relatively to the chemical shifts of the solvent signal; coupling constants (*J*) are given in Hz. Multiplicity is described as: s (singlet), br s (broad singlet), d (doublet), t (triplet) or m (multiplet). High resolution mass spectra were measured on a Bruker Daltonics microTOF–Q mass spectrometer. MALDI-TOF mass spectra were measured in linear, negative mode on a Bruker microflex spectrometer with the matrix mixture 2,4,6-trihydroxyacetophenone (THAP, 0.3 M in ethanol) and triammonium citrate (CIT, 0.1 M in water) at a ratio of 2:1 (v/v). The m/z values are those of the unresolved isotope envelope of the pseudomolecular ions ($[M-H]^-$). Quantification of oligonucleotides was performed with a NanoDrop 1000 spectral photometer from NanoDrop Technologies (Wilmington, United States). The UV-VIS spectra were measured on a Perkin Elmer Lambda 25 spectrometer.

2-Bromo-3-iodo-5-methyl-6-pyridone (10). 2-Bromo-3-iodo-5-methyl-6-pivaloyloxypyridine 9 (500 mg, 1.26 mmol, 1 eq) was dissolved in acetonitrile (2 mL) and aqueous ammonia (25 %, 2 mL) was added. The reaction mixture was stirred for 3 h at room temperature. After TLC showed full conversion, water (10 mL) was added, ammonia was removed with a stream of nitrogen, and the suspension was lyophilized. Pyridone 10 (391 mg, 1.25 mmol, 99 %) was obtained as a colorless solid. TLC (petroleum ether/ethyl acetate, 5:1, *v/v*): $R_f = 0.63$; ^{1}H-NMR (700 MHz, DMSO-d_6): δ (ppm) = 11.81 (br s, 1H), 7.90 (s, 1H), 2.02-2.00 (m, 3H); ^{13}C-NMR (175 MHz, DMSO-d_6): δ (ppm) = 161.5, 149.6, 140.0, 120.9, 85.6, 14.5; HRESIMS (ESI-TOF): *m/z* calc. for: C_6H_5BrINO $[M+H]^+$ 313.867, found 313.867.

2-Bromo-3-iodo-5-methyl-6-(pivaloyloxymethoxy)-pyridine (11). Pyridone 10 (103 mg, 0.33 mmol, 1.0 eq) was dissolved in dry DMF (2 mL) and K_2CO_3 (0.14 g, 0.98 mmol, 3.0 eq) and chloromethyl pivalate (76.0 μL, 0.52 mmol, 1.6 eq) were added. After stirring at room temperature for 16 h, the reaction mixture was filtered through celite, and it was then washed with ethyl acetate (15 mL). The combined filtrates were washed with $NaHCO_3$ (sat. aq. solution, 10 mL). The aqueous phase was extracted with ethyl acetate (10 mL). The combined organic layers were washed with water (15 mL). After drying over Na_2SO_4, the solvent was removed *in vacuo*. The crude was purified by chromatography on silica (10 g) with a gradient of ethyl acetate (5–20 %) in petroleum ether. Title compound 11 was obtained as a colorless oil (115 mg, 0.27 mmol, 82 %). TLC (petroleum ether/ethyl acetate, 5:1, *v/v*): $R_f = 0.74$; ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.79-7.75 (m, 1H), 6.02 (s, 2H), 2.11-2.09 (m, 3H), 1.20 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 177.5, 159.1, 150.4, 141.1, 122.1, 89.4, 82.7, 39.0, 27.0, 14.8; HRESIMS: *m/z* calc. for: $C_{12}H_{15}BrINO_3$ $[M+H]^+$ 427.935, found 427.935. Reactions on larger scales gave similar yields.

2-Bromo-5-methyl-6-(pivaloyloxymethoxy)-3-[3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxy-2′,3′-didehydro-β-D-ribofuranos-1′-yl]-pyridine (16). Samples of $Pd(OAc)_2$ (43.0 mg, 0.19 mmol, 0.2 eq) and $P(PhF_5)$ (203 mg, 0.38 mmol, 0.4 eq) were dissolved in dry acetonitrile (4 mL), and the brown solution was stirred for 30 min at room temperature. Subsequently, glycal 12 (217 mg, 0.94 mmol, 1 eq) and aglycone 11 (443 mg, 1.04 mmol, 1.1 eq), dissolved in dry acetonitrile (4 mL), were added, followed by addition of Ag_2CO_3 (260 mg, 0.94 mmol, 1 eq). The reaction mixture was stirred for 16 h at room temperature. After TLC showed full conversion, the grey suspension was filtered through celite and eluted with acetonitrile (15 mL). The solvent was removed *in vacuo*. The resulting crude 16 (907 mg) was obtained as a dark brown oil and was used in the subsequent step without purification. TLC (petroleum ether/diethyl ether, 1:1, *v/v*): $R_f = 0.66$.

2-Bromo-5-methyl-6-(pivaloyloxymethoxy)-3-(3′-dehydro-2′,3′-dideoxy-3′-oxo-β-D-ribofuranos-1′-yl)-pyridine (17). In a polypropylene tube, crude 16 (907 mg, 0.94 mmol), obtained as described in the preceding protocol, was dissolved in dry THF (18 mL). After addition of 3HF·NEt_3 (0.30 mL, 1.89 mmol, 2.0 eq) the reaction mixture was shaken for 30 min at room temperature. To quench remaining HF, methoxytrimethylsilane (2 mL) was added, and the mixture was shaken for 30 min at room temperature. The dark solution was filtered through celite and eluted with ethyl acetate (20 mL). After removing the solvent *in vacuo*, crude 17 (851 mg) was obtained, which was used without further purification. TLC (petroleum ether/diethyl ether, 1:1, *v/v*): $R_f = 0.51$.

2-Bromo-5-methyl-6-(pivaloyloxymethoxy)-3-(2′-deoxy-β-D-ribofuranos-1′-yl)-6-(tri*iso*propylsilylethynyl)-pyridine (13). Crude 17 (851 mg, 0.94 mmol) was dissolved in a mixture of dry acetonitrile and acetic acid (3:1, *v/v*,15 mL) and cooled to 0 °C. Then, $NaBH(OAc)_3$ (300 mg, 1.42 mmol, 1.5 eq) was added, and the reaction mixture was stirred for 2 h at 0 °C. After TLC showed full conversion, methanol (5 mL) was added, and the solvent was removed *in vacuo*. The crude product was purified by chromatography on silica (50 g) with a gradient of methanol (1-5 %) in dichloromethane, yielding 254 mg (0.60 mmol, 64 % over 3 steps) of the desired *C*-nucleoside 13 as an orange oil. TLC (dichloromethane/methanol, 9:1, *v/v*): $R_f = 0.49$; ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.53 (s, 1H), 6.06-6.00 (m, 2H), 5.32-5.26 (m, 1H), 4.41-4.35 (m, 1H), 4.02-3.95 (m, 1H), 3.85-3.75 (m, 2H), 2.51-2.42 (m, 1H), 2.12(s, 3H), 1.86-1.75 (m, 1H), 1.20-1.16 (m, 9H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) = 177.6, 158.6, 138.6, 133.9, 131.6, 120.5, 87.1, 82.8, 78.0, 73.7, 63.5, 42.9, 39.0, 27.04, 26.98, 15.2; HRESIMS: *m/z* calc. for: $C_{17}H_{24}NO_6Br$ $[M+Na]^+$ 440.068, found 440.069.

3-Methyl-2-(pivaloyloxymethoxy)-5-(2′-deoxy-β-D-ribofuranos-1′-yl)-6-(tri*iso*propylsilylethynyl)-pyridine (6). A sample of *C*-nucleoside 13 (139 mg, 0.33 mmol, 1 eq) was dissolved in dry DMF (5 mL) and transferred to a pressure-stable reaction vessel. Then, $Pd(PPh_3)_2Cl_2$ (23.0 mg, 0.02 mmol, 0.1 eq), CuI (13.0 mg, 0.07 mmol, 0.2 eq), NEt_3 (92.0 µL„ 0.66 mmol, 2 eq) and tri*iso*propylsilylacetylene (0.37 mL, 1.66 mmol, 5 eq) were added, the reaction vessel was provided with dry DMF (3 mL) and sealed. After stirring for 16 h at 80 °C, the reaction mixture was filtered through celite and eluted with ethyl acetate (20 mL). The solvent was removed *in vacuo* and the crude product was purified by chromatography on silica (15 g) with a gradient of methanol (1-5 %) in dichloromethane, yielding 105 mg (0.20 mmol, 61 %) of title compound 6 as a light brown oil. TLC (dichloromethane/methanol, 9:1, *v/v*): $R_f = 0.60$; ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.53 (s, 1H), 6.10 (d, $J = 5.3$ Hz, 1H), 6.05 (d, $J =$ 5.4 Hz, 1H), 5.56 (dd, $J = 9.6$ Hz, $J = 6.3$ Hz, 1H), 4.39-4.35 (m, 1H), 3.96-3.93 (m, 1H), 3.84-3.75 (m, 2H), 2.40-2.36 (m, 1H), 2.18 (s, 3H), 1.87-1.82 (m, 1H), 1.20-1.06 (m, 30H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 177.7, 158.9, 136.4, 134.73, 134.69, 122.1, 103.5,95.2, 87.1, 83.0, 76.8, 73.5, 63.4, 43.2, 38.9, 27.7, 27.0, 18.809, 18.802, 15.9, 11.4; HRESIMS: *m/z* calc. for: $C_{28}H_{45}NO_6Si$ $[M+H]^+$ 520.309, found 520.309.

3-Methyl-5-(2′-deoxy-β-D-ribofuranos-1′-yl)-6-(tri*iso*propylsilylethynyl)-2-pyridone (5). The *C*-nucleoside starting material 4 (504 mg, 1.02 mmol, 1 eq) was dissolved in acetonitrile (5 mL), and aqueous ammonia (25 %, 5 mL) was added. The reaction mixture was stirred for 2 h at room temperature. After TLC showed full conversion, water (10 mL) was added, remaining ammonia was removed with a stream of nitrogen, and the suspension was lyophilized to dryness. Title compound 5 was obtained as a colorless solid in a yield of 360 mg (0.89 mmol, 87 %). TLC (dichloromethane /methanol, 9:1, *v/v*): $R_f = 0.34$; ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 7.69–7.64 (m, 1H), 5.42 (dd, $J = 10.3$ Hz, $J = 5.5$ Hz, 1H), 4.37-4.32 (m, 1H), 3.91–3.86 (m, 1H), 3.75-3.65 (m, 2H), 2.16-2.09 (m, 4H), 1.97-1.84 (m, 1H), 1.22–1.10 (m, 21 H); ^{13}C-NMR (100 MHz, CD_3OD): δ (ppm) = 164.9, 138.4, 132.6, 126.6, 124.6, 102.7, 97.7, 89.0, 77.4, 74.1, 63.7, 43.5, 19.1, 16.8, 12.4; HRESIMS: *m/z* calc. for: $C_{22}H_{35}NO_4Si$ $[M+Na]^+$ 428.223, found 428.223.

3-Methyl-2-(pivaloyloxymethoxy)-5-(2′-deoxy-β-D-ribofuranos-1′-yl)-6-(tri*iso*propylsilylethynyl)-pyridine (6). Nucleosidic pyridone 5 (360 mg, 0.89 mmol, 1.0 eq) was dissolved in dry DMF (10 mL), and K_2CO_3 (184 mg, 1.33 mmol, 1.5 eq) as well as chloromethyl pivalate (192 µL, 1.33 mmol, 1.5 eq) were added. After stirring at room temperature for 16 h, the reaction mixture was filtered through celite and was washed with ethyl acetate (10 mL). The combined filtrates were washed with water (10 mL). The aqueous phase was back-extracted with ethyl acetate (3 x 10 mL), and the combined organic layers were washed with water (3×30 mL). After drying over Na_2SO_4, the solvent was removed *in vacuo*. The crude was purified by chromatography on silica (30 g) with a gradient of methanol (1-5 %) in dichloromethane. Besides 205 mg (0.39 mmol, 44 %) *O*-alkylated *C*-nucleoside 6 as a colorless oil, 63.0 mg (0.12 mmol, 14 %) *N*-alkylated *C*-nucleoside 7 was obtained as a colorless solid. Analytical data for 6: TLC (dichloromethane/methanol, 9:1, *v/v*): $R_f = 0.60$; ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.53 (s, 1H), 6.10 (d, $J = 5.3$ Hz, 1H), 6.05 (d, $J = 5.4$ Hz, 1H), 5.56 (dd, $J =$ 9.6 Hz, $J = 6.3$ Hz, 1H), 4.39-4.35 (m, 1H), 3.96-3.93 (m, 1H), 3.84-3.75 (m, 2H), 2.40-2.36 (m, 1H), 2.18 (s, 3H), 1.87-1.82 (m, 1H), 1.20-1.06 (m, 30H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 177.7, 158.9, 136.4, 134.73, 134.69, 122.1, 103.5,95.2, 87.1, 83.0, 76.8, 73.5, 63.4, 43.2, 38.9, 27.7, 27.0, 18.809, 18.802, 15.9, 11.4; HRESIMS: *m/z* calc. for: $C_{28}H_{45}NO_6Si$ $[M+H]^+$ 520.309, found 520.309. Analytic data for 3-methyl-*N*-(pivaloyloxymethyl)-5-(2′-deoxy-β-D-ribofuranos-1′-yl)-6-(tri*iso*propylsilylethynyl)-2-pyridone (7): TLC (dichloromethane/methanol, 9:1, *v/v*): $R_f = 0.46$; ^{1}H-NMR (700 MHz, $CDCl_3$): δ (ppm) = 7.34 (s, 1H), 6.28–6.04 (m, 2H), 5.46 (dd, $J = 10.1$ Hz, $J = 5.8$ Hz, 1H), 4.46-4.42 (m, 1H), 3.96–3.93 (m, 1H), 3.86–3.77 (m, 2H), 2.21–2.12 (m, 4H), 1.97–1.91 (m, 1H), 1.19–1.09 (m, 30H); ^{13}C-NMR (175 MHz, $CDCl_3$): δ (ppm) = 177.6, 162.3, 135.0, 132.4, 126.0, 124.7, 106.2, 95.4, 87.2, 76.8, 73.6, 70.4, 63.4, 42.5, 39.0, 27.2, 18.779, 18.773, 17.7, 11.3; HRESIMS: *m/z* calc. for: $C_{28}H_{45}NO_6Si$ $[M+Na]^+$ 542.291, found 542.291.

3-Methyl-2-(pivaloyloxymethoxy)-5-(2′-deoxy-5′-*O*-(dimethoxytrityl)-β-D-ribofuranos-1′-yl)-6-(tri*iso*propylsilylethynyl)-pyridine (8). Protected *C*-nucleoside 6 (194 mg, 0.37 mmol, 1.0 eq) was coevaporated with dry pyridine (2×5 mL) and dissolved in dry pyridine (5 mL). Dimethoxytrityl chloride (91.0 mg, 0.27 mmol, 1.1 eq), previously coevaporated with dry pyridine (3 mL), was dissolved in dry pyridine (2 mL) and was added. After stirring for 16 h at room temperature, TLC showed full conversion. The solvent was removed *in vacuo* and the crude product was purified by chromatography on silica [20 g, deactivated with NEt_3 (0.5 % in dichloromethane) prior to use] with a gradient of methanol (0-1 %) in dichloromethane. Title compound 8 was obtained as a colorless glass (206 mg, 0.25 mmol, 68 %). TLC (dichloromethane/methanol, 40:1, *v/v*): $R_f = 0.75$; ^{1}H-NMR (500 MHz, CD_3CN): δ (ppm) = 7.76 (s, 1H), 7.51-7.18 (m, 9H), 6.89–6.82 (m, 4H), 6.05–6.01 (m, 2H), 5.46 (dd, $J =$ 9.7 Hz, $J = 5.8$ Hz, 1H), 4.36–4.27 (m, 1H), 3.98–3.93 (m, 1H), 3.74 (s, 6H), 3.28–3.20 (m, 2H), 3.17(d, $J = 3.8$ Hz, 1H), 2.33-2.27 (m, 1H), 2.02 (s, 3H), 1.93-1.84 (m, 1H), 1.17–1.13 (m, 30H); ^{13}C-NMR (125 MHz, CD_3CN): δ (ppm) = 178.0, 159.7, 146.3, 138.3, 137.3, 137.11, 137.07, 134.9, 131.0, 129.0, 128.8, 127.8, 123.3, 114.0, 104.6, 95.8, 87.4, 87.3, 87.0, 83.3, 77.4, 74.3, 74.2, 65.4, 55.9, 44.0, 39.4, 27.2, 19.1, 12.1; HRESIMS: *m/z* calc. for: $C_{49}H_{63}NO_8Si$ $[M+H]^+$ 822.440, found 822.440.

3-Methyl-2-(pivaloyloxymethoxy)-5-[2′-deoxy-3′-*O*-(2-cyanoethyl-*N*,*N*-di*iso*propylamino)-phosphino-5′-*O*-(dimethoxytrityl)-β-D-ribofuranos-1′-yl]-6-(tri*iso*propylsilylethynyl)-pyridine (3). The C-nucleoside 8 (85.0 mg, 103 µmol, 1.0 eq) was coevaporated with acetonitrile (3×2 mL) and briefly vacuum-dried dried together with

di*iso*propylammonium tetrazolide (12.5 mg, 0.07 mmol, 0.7 eq). Subsequently, dry acetonitrile (1 mL) and 2-cyanoethyl-*N,N,N′,N′*-tetra*iso*propyl phosphorodiamidite (43.0 μL, 0.07 mmol, 1.3 eq) were added, and the mixture was stirred for 4 h at room temperature. After TLC showed full conversion, the reaction mixture was chromatographed on silica (7 g), eluting with a mixture of methyl *tert*-butyl ether and petroleum ether (3:1, *v/v*, with 1% NEt_3). Phosphoramidite 3 was obtained as a colorless glass (98.6 mg, 96.4 μmol, 93%). TLC (petroleum ether/ethyl acetate, 5:1, *v/v*, with 1% NEt_3): $R_f = 0.70$; ^{31}P-NMR (121.5 MHz, CD_3CN): δ (ppm) = 147.73, 146.96; HRESIMS: *m/z* calc. for: $C_{58}H_{80}N_3O_9PSi$ $[M+H]^+$ 1022.547, found 1022.548.

DNA synthesis and deprotection. The desired oligodeoxynucleotide sequences were synthesized on LCCA controlled pore glass (cpg), up to the position where the W residue was to be incorporated, by automated DNA synthesis on a H-2 synthesizer (K&A Laborgeräte, Schaafheim, Germany) on a 1 μmol scale, using the protocol recommended by the manufacturer and commercial phosphoramidites. The cpg (40 mg, 1 μmol oligonucleotide loading) was dried in a pear-shaped flask *in vacuo*. Then, dry phosphoramidite 3 (28.5 mg, 28 μmol) was dissolved in "activator solution" for automated DNA synthesis (350 μL, 0.25 M 5-(ethylthio)tetrazole in dry acetonitrile). The solution was immediately added to the cpg, and the mixture was shaken in a horizontal shaker for 1 h at room temperature. The supernatant was removed, and the solid support was washed with dry acetonitrile (800 μL). Subsequently, "cap-A" (200 μL, 9% acetic anhydride in THF) and "cap-B" (200 μL THF/pyridine/1-methylimidazole, 80:10:10) solutions were added, and the mixture was shaken for 5 min. The supernatant was removed and the cpg was washed with dry acetonitrile (800 μL). Then, "oxidizer solution" (750 μL, 3% iodine in THF /water/pyridine) was added and the slurry was shaken for 10 min. The supernatant was again removed, and the solid support was washed with dry acetonitrile (5×800 μL), followed by drying *in vacuo*, and back-transfer to a column for DNA synthesis. The remaining synthesis cycles were performed by automated DNA synthesis as described for the first phase of chain assembly, above. Oligonucleotide 14 was thus synthesized with its 5′-terminal DMT group in place. The cpg was placed in a polypropylene tube and aqueous ammonia (25%, 1 mL) was added. After 2 h at room temperature, the supernatant was aspirated, and the cpg was washed with water (4× 300 μL). The ammonia in the combined solutions was removed with a gentle stream of nitrogen, and the sample was lyophilized. The resulting crude was purified by HPLC. The DMT group of the support bound form of oligonucleotide 15 was removed at the end of the chain assembly. The cpg was then placed in a polypropylene tube, and aqueous ammonia (25%, 1 mL) was added. After incubating for 16 h at 55 °C, the supernatant was collected, and the cpg was washed with water (4 x 300 μL). The ammonia of the combined aqueous solutions was removed with a stream of nitrogen directed onto the surface of the solution, and the sample was lyophilized. Then, TBAF (1 M in THF, 500 μL) was added and subsequently water (20 μL) was added, in order to fully dissolve the oligonucleotide. The mixture was shaken for 5 h at room temperature on a horizontal shaker. Then, triethylammonium acetate buffer (1 M in water, 500 μL) and water (500 μL) were added, and the THF was removed with a stream of nitrogen. The sample was lyophilized and purified by HPLC, as described below.

HPLC purification. Reversed-phase HPLC was performed on a DIONEX ULTIMATE 300 HPLC system (Thermo Fischer Scientific, Waltham, USA) using an Nucleosil C18 column (250×4.6 mm, Macherey-Nagel, Düren, Germany). The lyophilized oligonucleotides were dissolved in water (1 mL) and the solution was filtered (pore size: 0.45 μm) prior to injection. A gradient of acetonitrile in triethylammonium acetate buffer (0.1 M, pH 7) was used, at 55 °C and a flow of 0.6 mL/min.

5′-DMT-TTTTTTTW^{TIPS}TTT-3′ (14) HPLC: gradient of acetonitrile (1-50% in 40 min) in triethylammonium acetate buffer (0.1 M, pH 7), $t_r = 35.7$ min. yield: 324 nmol, 32%; MALDI-TOF-MS: m/z: calc. for $C_{153}H_{196}N_{22}O_{83}P_{11}Si$ $[M-H]^-$ 4054, found 4053.

5′-TTACAAACATWGGCCGCAAA-3′ (15) HPLC: gradient of acetonitrile (1-35% in 40 min) in triethylammonium acetate buffer (0.1 M, pH 7), $t_r = 26.1$ min. yield: 479 nmol, 48%; MALDI-TOF-MS: m/z: calc. for $C_{198}H_{245}N_{77}O_{113}P_{19}$ $[M-H]^-$ 6100, found 6102.

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Conflict of Interest

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.

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