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# Synthesis, Pairing, and Cellular Uptake Properties of C(6′)-Functionalized Tricyclo-DNA

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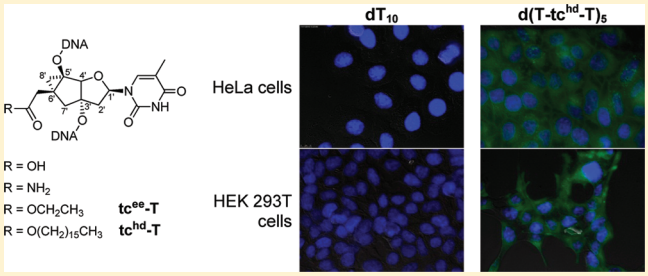
Supporting Information

**ABSTRACT:** Tricyclo-DNA (tc-DNA) is a promising candidate for oligonucleotide-based therapeutic applications exhibiting increased affinity to RNA and increased resistance to nucleases. However, as many other oligonucleotide analogs, tc-DNA does not readily cross cell membranes. We wished to address this issue by preparing a prodrug of tc-DNA containing a metabolically labile group at C(6′) that promotes cellular uptake. Two monomeric nucleoside building blocks bearing an ester function at C(6′) (tc$^{ee}$-T and tc$^{hd}$-T) were synthesized starting from a known C(6′) functionalized bicyclic sugar unit to which the cyclopropane ring was introduced via carbene addition. NIS-mediated nucleosidation of the corresponding glycal with in situ persilylated thymine afforded the $\beta$-iodonucleoside exclusively that was dehalogenated via radical reduction. Diversity in the ester function was obtained by hydrolysis and reesterification. The two nucleosides were subsequently incorporated into DNA or tc-DNA by standard phosphoramidite chemistry. The reactivity of the ester function during oligonucleotide deprotection was explored and the corresponding C(6′) amide, carboxylic acid, or unchanged ester functions were obtained, depending on the deprotection conditions. Compared to unmodified DNA, these tc-DNA derivatives increased the stability of duplexes investigated with $\Delta T_m$/mod of +0.4 to +2.0 °C. The only destabilizing residue was tc$^{hd}$-T, most likely due to self-aggregation of the lipophilic side chains in the single stranded oligonucleotide. A decamer containing five tc$^{hd}$-T residues was readily taken up by HeLa and HEK 293T cells without the use of a transfection agent.




## INTRODUCTION

Control of translation by oligonucleotides was first described more than three decades ago[1] and has since evolved into powerful therapeutic strategies[2,3] among which the antisense, the siRNA, and the anti micro-RNA approaches are most promising.[4,5] However, the barriers to the development of nucleic acids as therapeutics are manifold and delivery into cells is often recognized as being the most challenging one.[6] Other important parameters include resistance toward nuclease degradation and affinity for the target RNA sequence. Chemical modification has emerged as a powerful tool to tackle these issues.[2] In particular, nucleoside analogues with restricted backbone flexibility have been shown to increase the affinity toward complementary RNA and improve the stability of the corresponding oligonucleotides.[7] Such conformationally constrained nucleic acid structures include hexitol nucleic acids (HNA),[8] locked nucleic acids (LNA),[9,10] and bicyclo- and tricyclo-DNA (Figure 1).[11−13]

Tricyclo-DNA (tc-DNA) is an RNA structural mimic which binds to complementary RNA with high affinity and high selectivity.[14] Furthermore, it is stable in serum, does not elicit RNaseH cleavage and has recently been evaluated as antisense and siRNA agent with promising results.[15−17] However, like most natural or modified oligonucleotides, tc-DNA also suffers from poor cellular uptake and thus relies on the use of transfection agents to help crossing of the cellular membrane.

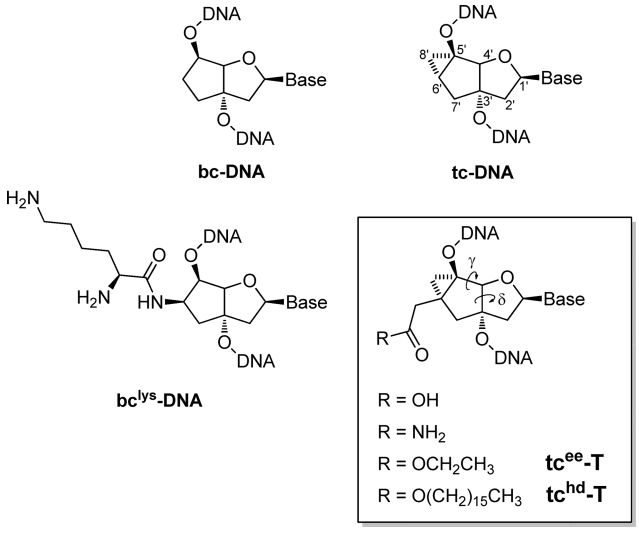



**Figure 1.** Chemical structures of selected derivatives of the bicyclo- and tricyclo-DNA family.

Transfection agents such as cationic lipids or polymers are perhaps the most known carriers for oligonucleotide delivery,

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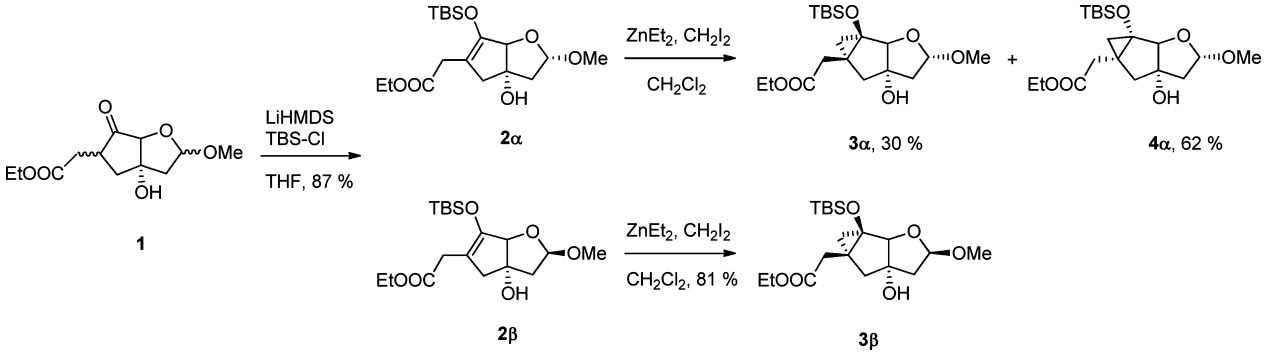
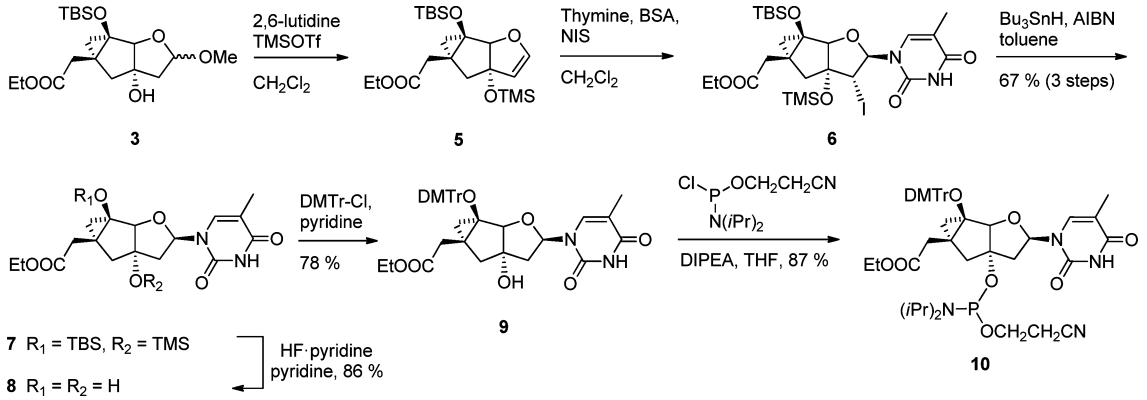
**Scheme 1. Synthesis of Tricyclo Compounds 3 and 4**




**Scheme 2. Synthesis of tc$^{ee}$-T Phosphoramidite 10**




but their inherent toxicity has prevented their use *in vivo*.[18] However, they represent only a fraction of a much broader arsenal of strategies for improving the cellular uptake of oligonucleotides. Such strategies include viral delivery methods,[19] physical and mechanical techniques,[20] and conjugation with gold nanoparticles[21] or with arginine-rich or cell-penetrating peptides.[22] Although they have been shown to improve the uptake of nucleic acids, limitations such as toxicity and immunogenicity have challenged their potential as universal delivery systems. Therefore, important research efforts were devoted to bypass the use of delivery agents.

Cationic or zwitterionic oligonucleotides have been shown to promote cellular uptake in selected cases.[23,24] In this context, we have recently reported on bicyclo-DNA carrying a lysine moiety able to cross the cell membrane without aid of transfection agents.[25] Bioconjugation to cholesterol or long aliphatic chains has also proven successful in carrier-free approaches.[26,27] Recently, LNA gapmers with phosphorothioates linkages have been evaluated in free cellular uptake through a process called gymnosis which requires higher oligonucleotide concentrations in the growth medium.[28]

Another promising concept consists in temporarily masking the negative charge of the phosphates and has been coined the "pro-oligonucleotide approach" in analogy to the prodrug approach.[29,30] The masked, charge neutral oligonucleotide can more easily penetrate into the intracellular compartment where endogenous enzymes convert the pro-oligonucleotide into the biologically active nucleic acid. Variations of this theme include modification of the O(2′) position of ribonucleotides to attach biolabile groups in order to improve the cellular uptake of RNA.[31,32]

In this work, we set out to synthesize "pro tricyclo-oligonucleotides" bearing an ester function at position C(6′). This position was selected since it can easily be synthetically manipulated by virtue of the neighboring C(5′)-oxo function. Moreover, this position is expected to be sufficiently distant from the nucleobase to exclude interference with base pairing.

## RESULTS AND DISCUSSION

**Synthesis of tc$^{ee}$-T and tc$^{hd}$-T Building Blocks 10 and 15.** The preparation of tc$^{ee}$-T and tc$^{hd}$-T phosphoramidites **10** and **15** started from the already known ketone **1** (mixture of four diastereoisomers), which was previously used in the synthesis of C(6′)-alkylated bicyclo nucleosides.[33] Abstraction of the $\alpha$-hydrogen of the ketone with LiHMDS followed by subsequent trapping of the enolate with TBS-Cl yielded silylenol ethers **2** which were separated into the pure anomeric forms (Scheme 1). The introduction of the cyclopropane ring into **2** was first envisaged via the Simmons−Smith procedure ($CH_2I_2$, Ag/Zn couple) but resulted in very low yields and several undesired side products. When the Ag/Zn couple was exchanged for $ZnEt_2$ (Furukawa's protocol[34]), the reaction proceeded smoothly and in good yields. However, while cyclopropanation of **2*β*** afforded tricyclo compound **3*β*** as the only isomer, the same conditions applied to **2*α*** led to a mixture of two diastereoisomers that could easily be separated. The major isomer **4*α*** (62%) resulted from reaction at the concave (*endo*) face of the bicyclic structure, while the desired isomer **3*α*** with *exo* configuration of the cyclopropane ring was only obtained in 30% yield. The stereochemical outcome of the reaction with **2*β*** can be explained by the directing effect of the homoallylic hydroxy group and steric hindrance at the alternative *endo* face.[35] In **2*α***, the methoxy substituent might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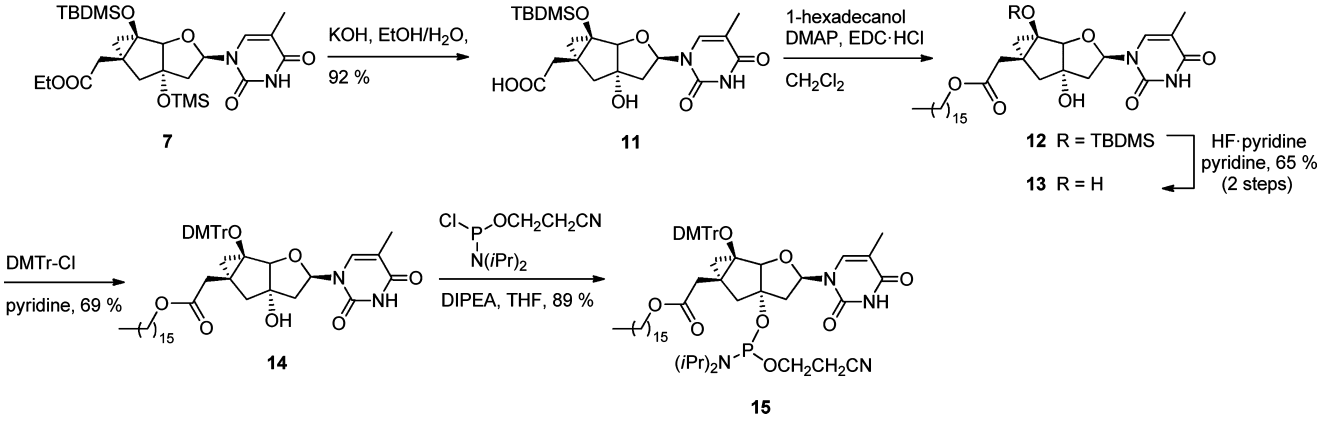
**Scheme 3. Synthesis of tc$^{hd}$-T Phosphoramidite 15**



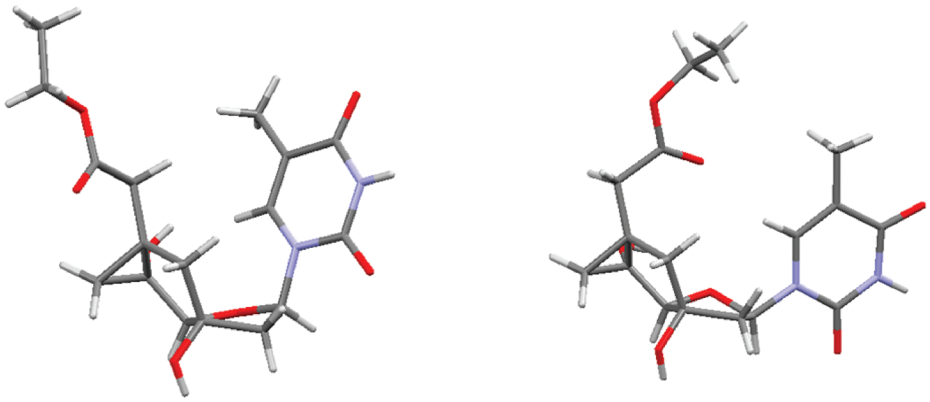



**Figure 2.** X-ray structures of nucleoside: **8A** (left), and **8B** (right).

engage in hydrogen bonding to the tertiary hydroxy group preventing it from coordinating to the reactive metal species, thus giving rise to a mixture of isomers. Furthermore, the ester function at C(6′) might also play a directing role in the cyclopropanation reaction.[36] The configuration at the cyclopropane ring was assigned by $^1$H NMR NOE, $^1$H–$^1$H COSY, and 2D NOESY experiments (see the Supporting Information).

For the nucleosidation step, we considered the *N*-iodosuccinimide induced addition of persilylated thymine to the corresponding glycal **5** (Scheme 2). Indeed, this strategy has been successfully applied to unmodified tricyclo pyrimidine nucleosides, yielding only *β*-nucleosides.[37] Therefore, the combined anomers of **3** were converted into glycal **5** by treatment with TMSOTf, affording only one product and thereby verifying the cyclopropyl ring configuration (Scheme 2). The crude, 3′-silylated glycal **5** was then subjected to a NIS-mediated nucleosidation with persilylated thymine which pleasingly yielded only the *β*-nucleoside **6**. Without further purification, the C(2′)-iodonucleoside **6** was subjected to radical reduction with $Bu_3SnH$ and AIBN, providing nucleoside **7** in 67% yield over three steps. Removal of the O(5′)- and O(3′)-silyl protecting groups with HF·pyridine gave compound **8**, the relative configuration at the anomeric center of which was assessed by $^1$H NMR difference NOE experiments. Irradiation of H(1′) led to strongly enhanced signals at the H(4′) (4%) and H(2′) (5.9%) (see the Supporting Information). These observations confirmed the *β*-configuration at C(1′). The structure of nucleoside **8** was also confirmed independently by X-ray crystallography.

With nucleoside **8** in hand, access to the phosphoramidite **10** was straightforward. Tritylation of the 5′-OH group with DMTr-Cl afforded intermediate **9** which was subsequently phosphitylated under standard conditions to give phosphoramidite **10**.

To obtain the long-chain alkyl ester derivative tc$^{hd}$-T, ester **7** was first saponified to afford the 3′-desilylated carboxylic acid **11** in good yield, which was used in the next step without further purification (Scheme 3). Condensation of **11** with 1-hexadecanol mediated by EDC·HCl and DMAP, followed by desilylation with HF·pyridine led to the free nucleoside **13** in 65% yield over two steps. Tritylation of O(5′) and phosphitylation of O(3′) to give **14** and **15**, respectively, proceeded under similar conditions as for building block **10** but required somewhat longer reaction times.

**X-ray Structure of Nucleoside 8.** To gain information on the conformational preferences of the new tricyclonucleoside derivatives, the solid-state structure of nucleoside **8** was solved by X-ray crystallography. The asymmetric unit contains two symmetry-unrelated molecules **8A** and **8B** (Figure 2). The main structural difference between **8A** and **8B** lies in the furanose substructure which belongs to the C(2′)-*exo* ($_2$E) conformation in **8A** and to the O(4′)-*endo* ($^0$E) conformation in **8B**, with calculated pseudorotation phase angles (*P*) of 342.8° (**8A**) and 85.2° (**8B**), respectively. Although the glycosidic torsion angles *χ* are slightly different (Table 1), both structures have the thymine moiety in the *anti* conformation. In the asymmetric unit, there exists a intricate pattern of intermolecular H-bonds as well as stacking contacts of the bases which probably accounts for the differences in *χ*


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**Table 1. Selected Torsion Angles and Furanose Puckers of Nucleoside 8 in Comparison to bc-, tc-, and Natural Deoxynucleosides**

| nucleoside | furanose pucker | $\gamma$ (deg) | $\delta$ (deg) | $\chi$ (deg) |
|---|---|---|---|---|
| **8A** | C(2′)-*exo* | 149.9 | 100.8 | −170 |
| **8B** | O(4′)-*endo* | 158.3 | 99.4 | −125.9 |
| **tc-dA**[a] | C(2′)-*exo* | 152 | 107 | −164 |
| **bc-T**[b] | C(1′)-*exo* | 149.3 | 133.5 | −107.6 |
| **dN**[c] | C(2′)-*endo* | 57 | 122 | −119 |

[a]Conformation of a tricyclodeoxyadenosine unit in a DNA duplex (ref [38]). [b]Data taken from ref 11. [c]Average deoxynucleotide conformation in B-DNA (ref [39]).

and *P* values. In both molecules **8A** and **8B** the torsion angle $\gamma$ falls into the antiperiplanar range, which is consistent with the values found for unmodified members of the bc-DNA and tc-DNA families.[11,38] In addition, the torsion angle $\delta$ is in a *+ac* orientation as observed in tc-DNA residues. Summarizing all the structural features, it appears that the addition of an alkyl group at position C(6′) does not significantly change the intrinsic conformational preferences of the tricyclic core unit.

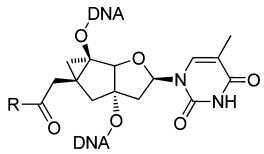
**Oligonucleotide Synthesis and Deprotection.** The previously prepared tc$^{ee}$-T and tc$^{hd}$-T phosphoramidites **10** and **15** were incorporated into a series of mixed-base decamer oligodeoxynucleotides as single or double substitutions by standard solid-phase synthesis on a 1.3 $\mu$mol scale (**ON 1**−**11**, Table 2). The modified units were introduced into either natural DNA (**ON 1**−**6**) or tc-DNA (**ON 7**−**11**). Additionally, decaoligothymidylates containing five alternating tc$^{ee}$-T, tc$^{hd}$-T, or tc-T substitutions and labeled with 6-carboxyfluorescein (FAM) at their 3′-end were prepared for cellular uptake experiments (**ON 12**−**14**). An increased concentration (0.15 M in $CH_3CN$) and an extended coupling time of 12 min were required in order to achieve coupling efficiencies of 94% for tc$^{ee}$-T **10** and 97% for tc$^{hd}$-T **15**, as judged from the trityl assay.

A set of conditions for deprotection and detachment from solid support has been elaborated that either allows to conserve the ester functions in the oligonucleotides or to transform them into amide or carboxy functions (Scheme 4).[33] Treatment with concd $NH_3$ (55 °C, 16 h) converted all ethyl ester groups into the corresponding amide functions yielding oligonucleotides **ON 1**, **3**, **5**, **7**−**9** (Table 2). Treatment with potassium hydroxide led to hydrolysis of the ester function (→ **ON 10**), while deprotection with 25% benzylamine left the ester groups untouched (→ **ON 11**).

For oligonucleotides containing tc$^{hd}$-T units we selected ethanolic ammonia (concd $NH_3$/EtOH 1:3) as a deprotection mixture. While after 24 h at 40 °C no trace of aminolysis of the ester function was observed by ESI$^-$-MS for singly modified sequences (**ON 2** and **4**), some aminolysis was observed with **ON 6** containing two hexadecyl ester moieties. Deprotection in concd $NH_3$/EtOH 1:3 at 40 °C for only 2 h was sufficient to produce the oligothymidylates containing five tc$^{ee}$-T or tc$^{hd}$-T units (**ON 12** and **13**). Indeed, no hydrolyzed ester in the crude material was detected by mass spectrometry.

All oligonucleotides were purified by ion-exchange (IE) HPLC using standard methods. Purification of **ON 13** bearing five $C_{16}$ chains, however, was not as straightforward and required the use of 1 M $NaClO_4$ as eluent and organic cosolvents. Under these conditions, **ON 13** eluted, somewhat surprisingly, as a broad peak with multiple shoulders, most likely arising from oligonucleotide aggregates. Reversed-phase HPLC on $C_4$ or $C_{18}$ columns was impossible due to very strong retention of the oligonucleotide on the solid phase. After purification and subsequent desalting, the integrity of all oligonucleotides was routinely verified either by ESI$^-$ or

**Table 2. Oligonucleotides Prepared with tc$^{ee}$-T and tc$^{hd}$-T Amidites 10 and 15**[a]

| Entry | Sequence (5′ to 3′) |  | Deprotection conditions |
|---|---|---|---|
| **ON 1** | AACTGtCACG | R = $NH_2$ | Conc. $NH_3$, 55 °C, 16 h |
| **ON 2** | | R = $O(CH_2)_{15}CH_3$ | $NH_3$/EtOH 1:3, 40 °C, 24 h |
| **ON 3** | AACtGTCACG | R = $NH_2$ | Conc. $NH_3$, 55 °C, 16 h |
| **ON 4** | | R = $O(CH_2)_{15}CH_3$ | $NH_3$/EtOH 1:3, 40 °C, 24 h |
| **ON 5** | AACtGtCACG | R = $NH_2$ | Conc. $NH_3$, 55 °C, 16 h |
| **ON 6** | | R = $O(CH_2)_{15}CH_3$ | $NH_3$/EtOH 1:3, 40 °C, 24 h |
| **ON 7** | *AACTGtCACG* | R = $NH_2$ | Conc. $NH_3$, 55 °C, 16 h |
| **ON 8** | *AACtGTCACG* | R = $NH_2$ | Conc. $NH_3$, 55 °C, 16 h |
| **ON 9** | *AACtGtCACG* | R = $NH_2$ | Conc. $NH_3$, 55 °C, 16 h |
| **ON 10** | | R = OH | 0.1 M KOH, 55 °C, overnight |
| **ON 11** | | R = $OCH_2CH_3$ | 25% $BnNH_2$, EtOH/$H_2O$, 65 °C, 8 h |
| **ON 12** | TtTtTtTtTt-FAM | R = $OCH_2CH_3$ | $NH_3$/EtOH 1:3, 40 °C, 2 h |
| **ON 13** | | R = $O(CH_2)_{15}CH_3$ | $NH_3$/EtOH 1:3, 40 °C, 2 h |
| **ON 14** | T*T*T*T*T*T*T*T-FAM[b] | | Conc. $NH_3$, 55 °C, 1 h |

[a]Capital letters: natural DNA nucleotides. Capital italic letters: 6′-unfunctionalized tricyclodeoxynucleotides tc-A, tc-C, tc-G, tc-T (structure, see Figure 1, top right). Lowercase letter: 6′-functionalized tc-T units. [b]FAM = 6-carboxyfluorescein.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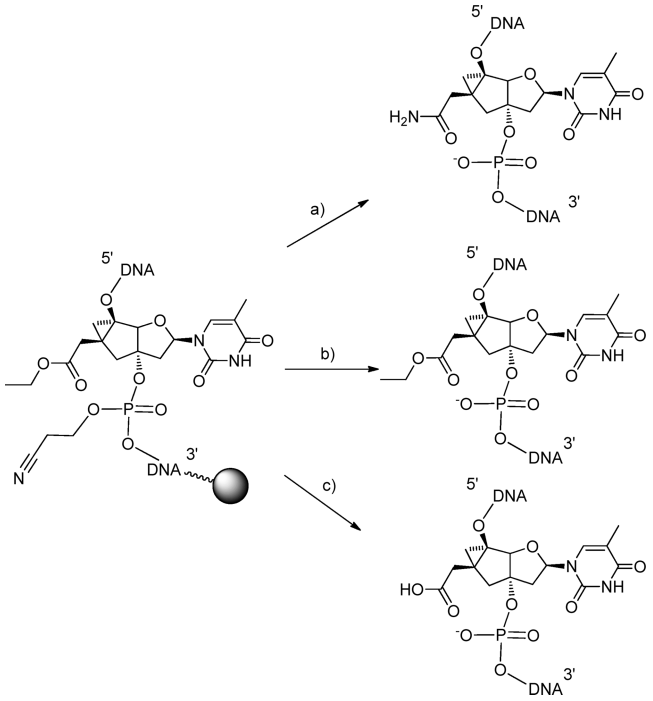
**Scheme 4**[a]




[a]Oligonucleotide deprotection conditions: (a) concd $NH_3$, 55 °C, 16 h; (b) 25% benzylamine in MeOH/$H_2O$ 1:2, 65 °C, 8 h; (c) 0.1 M KOH, 55 °C, overnight.

MALDI-TOF mass spectrometry (see the Supporting Information).

**Hybridization Properties of Substituted Oligodeoxynucleotides.** The affinities of the modified oligodeoxynucleotides to their DNA and RNA complements was assessed by UV melting curves at 260 nm in standard saline buffer (10 mM $Na_2HPO_4$, 150 mM NaCl, pH 7.0) (Table 3). Introduction of a single tc-T unit bearing an amide group at position C(6′) (**ON 1** and **3**) led to an increase in $T_m$ of +0.4 to +0.8 °C with a DNA complement and to +2.0 °C with an RNA complement. This clearly indicates that the amide substituent at C(6′) does not perturb duplex formation. Furthermore, the degree of stabilization is comparable to the single incorporation of an unmodified tc-T unit (−0.4 °C) in the same sequence.[14] Two modifications separated by a natural dG unit (**ON 5**) slightly decreased the $T_m$ of a DNA duplex when compared to the unmodified duplex (−1.0 °C/mod.). However, with RNA as a complement, **ON 5** exhibits again stabilization of the hybrid duplex with $T_m$'s comparable to those of single-modified oligonucleotides. This is also a feature found for unmodified tc-units in the same sequence context[14] and confirms again that the grafting of a substituent at C(6′) on the tricyclo scaffold does not affect the pairing efficiency.

**Table 3. $T_m$ data (°C) from UV−melting Curves (260 nm) of Modified Decamer Sequences with Complementary DNA and RNA**[a]

| entry | $T_m$ (°C) vs DNA ($\Delta T_m$/mod)[b] | $T_m$ (°C) vs RNA ($\Delta T_m$/mod)[c] |
|---|---|---|
| **ON 1** | 45.7 (+0.8) | 45.7 (+2.1) |
| **ON 2** | 37.0 (−9.0) | 38.4 (−5.2) |
| **ON 3** | 45.3 (+0.4) | 45.6 (+2.0) |
| **ON 4** | 38.0 (−8.0) | 38.9 (−4.7) |
| **ON 5** | 42.9 (−1.0) | 46.1 (+1.3) |
| **ON 6** | 20.1 (−13.0) | 23.1 (−10.2) |

[a]Conditions: 2 μM single strands in 150 mM NaCl and 10 mM $NaH_2PO_4$ at pH 7.0. [b]$T_m$ of the unmodified duplex: 44.9 °C; [c]$T_m$ of the unmodified duplex: 43.6 °C

On the other hand, upon replacement of an ethyl by a hexadecyl group, all duplexes were destabilized (Table 3). Moreover, this destabilization was particularly pronounced with DNA as a complement, where $\Delta T_m$s of −9.0 and −8.0 °C were measured with **ON 2** and **4**, respectively. When paired with complementary RNA, oligonucleotides containing one $tc^{hd}$-T unit were less destabilizing (−5.2 and −4.7 °C). Such a loss in affinity was cumulative, as a double-modified oligonucleotide (**ON 6**) gave $T_m$s of about half of that of the control (20.1 °C vs 44.9 °C; 23.1 °C vs 43.6 °C). Furthermore, this destabilization was associated with a lower hyperchromicity upon duplex melting, suggesting imperfect base-pairing (see the Supporting Information).

UV−melting experiments were also performed in the series of decathymidylates alternately substituted with either $tc^{ee}$-T, $tc^{hd}$-T, or tc-T units and carrying a 3′-FAM label (Table 4).

**Table 4. $T_m$ Data (°C) from UV−melting Curves (260 nm) of Modified Decathymidylates Sequences with Complementary DNA and RNA**[a]

| entry | $T_m$ (°C) vs DNA ($\Delta T_m$/mod)[b] | $T_m$ (°C) vs RNA ($\Delta T_m$/mod)[c] |
|---|---|---|
| **ON 12** | 18.3 (−1.1) | 20.6 (−0.2) |
| **ON 13** | n.d. | n.d. |
| **ON 14** | 19.2 (−1.0) | 19.9 (−0.3) |

[a]Conditions: 2 μM single strands in 150 mM NaCl and 10 mM $NaH_2PO_4$ at pH 7.0. [b]$T_m$ of the FAM-labeled unmodified duplex: 24.0 °C; [c]$T_m$ of the FAM-labeled unmodified duplex: 21.6 °C. n.d.: not detected.

Analysis of the $T_m$ data revealed that discontinuous tc-T incorporations (**ON 14**) were destabilizing when paired to $dA_{10}$ and $rA_{10}$ as a complement (−1.0 and −0.3 °C/mod, respectively), a result that was observed similarly also for **ON 12** containing five alternative $tc^{ee}$-T units. However, the presence of five $tc^{hd}$-T units (**ON 13**) made the determination of a $T_m$ value impossible. Indeed, no sigmoidal curves and thus no duplex formation was observed upon melting of **ON 13**·with cDNA or RNA (see the Supporting Information). We hypothesize that this is the result of a competition between base pairing and hydrophobic interactions of the C16 side chains in **ON 13**, organizing it in structures such as micelles or vesicles where the oligonucleotide is less accessible for hybridization.

**Hybridization Properties of Substituted Tricyclo-oligonucleotides.** The tc-T derivatives were also incorporated into tc-oligonucleotides to study the influence of a modified tc-unit on the pairing properties of tc-DNA (Table 5). UV−melting curve analysis showed that the strong stabilization brought about by the fully modified tc-DNA (+13.5 °C and +20.5 °C with cDNA and RNA, respectively) is conserved upon incorporation of the functionalized tc-units, with $\Delta T_m$s ranging from −1.6 to +2.0 °C overall. This indicates that short alkyl chains at position C(6′) do not interfere with the pairing properties of tc-DNA, suggesting again a smooth accommodation of all substituents in the duplexes. The amide and ethyl


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**Table 5. $T_m$ Data (°C) from UV−melting Curves (260 nm) of Modified Decamer Sequences in the Context of a tc-DNA Backbone with Complementary DNA and RNA[a]**

| entry | $T_m$ (°C) vs DNA ($\Delta T_m$/mod)[b] | $T_m$ (°C) vs RNA ($\Delta T_m$/mod)[c] |
|---|---|---|
| **ON 7** | 57.8 (−0.7) | 65.0 (+0.7) |
| **ON 8** | 57.1 (−1.4) | 64.4 (+0.1) |
| **ON 9** | 57.3 (−0.6) | 63.9 (−0.2) |
| **ON 10** | 56.9 (−0.8) | 66.3 (+1.0) |
| **ON 11** | 57.2 (−0.6) | 63.9 (−0.2) |

[a]Conditions: 2 $\mu$M single strands in 150 mM NaCl and 10 mM $NaH_2PO_4$ at pH 7.0. [b]$T_m$ of fully modified tricyclo-DNA/DNA duplex: 58.5 °C; [c]$T_m$ of fully modified tricyclo-DNA/RNA duplex: 64.3 °C.

ester groups were both found to have negligible effects on the thermal stability (**ON 9** and **11**).

Interestingly, additional carboxylate groups (as in **ON 10**) led to an increase in $T_m$ with RNA as a complement, implying that additional negative charges do not perturb the pairing affinity of tc-DNA. This is an important observation as according to the prodrug approach the carboxylic acid function is expected to be the functional group after enzymatic ester hydrolysis. It therefore appears that ester hydrolysis will be required to functionally activate the oligonucleotide.

**Fluorescence Emission Measurements.** The analysis of the UV−melting curves of **ON 13** led us to propose that self-association of the single strands via their hydrophobic hexadecyl ester functions might occur in water. Consequently, this should result in significant self-quenching of fluorescein fluorescence by proximity effects.[40] We measured the emission of fluorescence of all FAM-labeled oligonucleotides after excitation at 480 nm. In addition, we used the corresponding unmodified $dT_{10}$ with a fluorescein label as a control. The experiments were performed in pure water and in standard saline buffer (Figure 3). In water, low emission of fluorescence was obtained for all FAM-labeled oligonucleotides **ON 12−14**, suggesting a substantial degree of self-association under these conditions (Figure 3A). Upon addition of the detergent Triton x-100 above its critical micelle concentration, a large restoration of fluorescence emission was observed for **ON 12** and **14** and $dT_{10}$-FAM. However, the increase of fluorescence emission for **ON 13** was limited (Figure 3B). In standard saline, the intensity of fluorescence emission for **ON 12** and **14** and $dT_{10}$-FAM reached a maximum and did not vary upon addition of Triton x-100 (Figures 3C,D). On the other hand, under these conditions, a substantial quenching of fluorescence for **ON 13** was still detected and was only partially restored after addition of Triton x-100. Taken together, these results indicate that **ON 13** self-associates even in buffered saline. The limited restoration of fluorescence emission for **ON 13** after addition of a detergent implies that self-quenching is not entirely eliminated. It is therefore reasonable that this amphiphilic oligonucleotide still exists in an aggregated state even in the presence of large quantities of a detergent.

**Cellular Uptake.** The previously prepared fluorescein-labeled oligonucleotides were then evaluated for their ability to cross the cellular membrane in the absence of a transfection agent. Two human cell lines, HeLa and HEK 293T, were transfected with **ON 12**, **13** and **14** at 10 $\mu$M concentration each, and the unmodified $dT_{10}$-FAM was used as a control. After 2 days of incubation at 37 °C, the cells were fixed and analyzed by confocal fluorescence microscopy (Figure 4). No visible internalization of the control oligonucleotide $dT_{10}$-FAM was detected in any cell line. Similarly, the decathymidylates modified with either tc-T or $tc^{ee}$-T (**ON 12** and **14**) were not

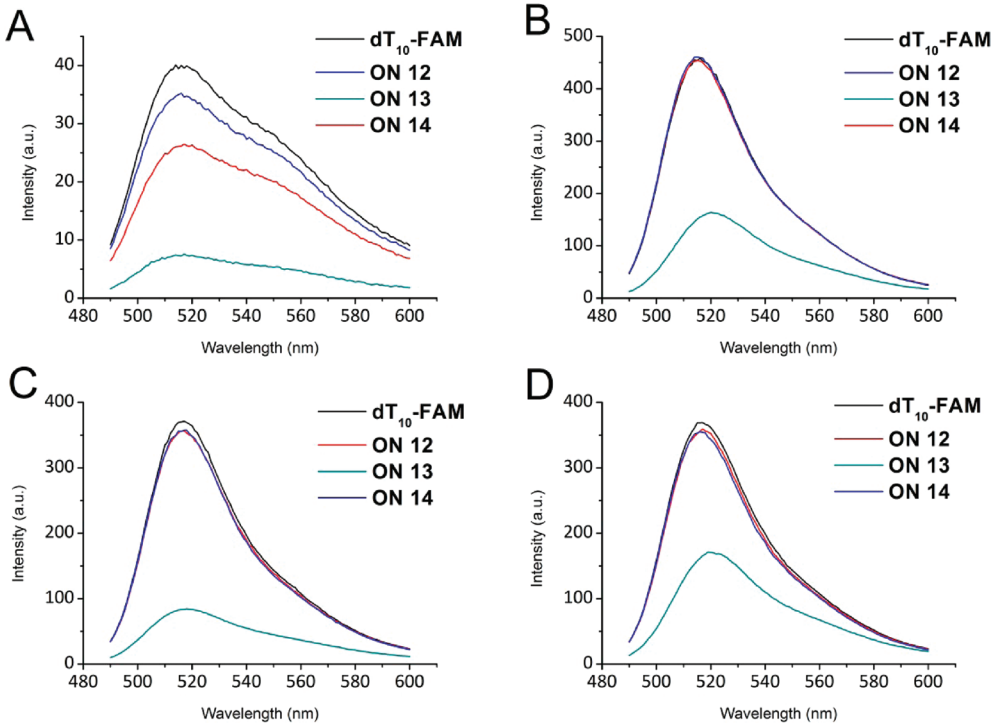


**Figure 3.** Fluorescence emission spectra of FAM-labeled oligonucleotides: (A) in $H_2O$; (B) in $H_2O$ after addition of 0.36 mM Triton x-100; (C) in 150 mM NaCl, 10 mM $NaH_2PO_4$, pH 7.0; (D) in 150 mM NaCl, 10 mM $NaH_2PO_4$, pH 7.0 after addition of 0.36 mM Triton x-100. Conditions: 2 $\mu$M single strand, excitation at 480 nm, 20 °C.


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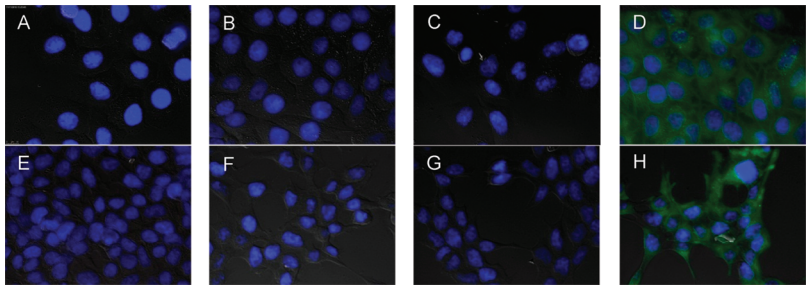


**Figure 4.** Fluorescence microscopy analysis of HeLa (top row) and HEK 293T cells (bottom row) treated with: (A, E) $dT_{10}$-FAM; (B, F) **ON 12**, (C, G) **ON 14**, (D, H) **ON 13**. Conditions: 10 $\mu$M ODN concentration, 2 days incubation at 37 °C, cells are then fixed with 3.7% paraformaldehyde and their nuclei stained with DAPI (blue channel).

able to penetrate through the cellular membrane. On the other hand, **ON 13** was readily taken up (Figure 4D,H) in both cell lines, thus highlighting the role of the side chains attached to the tricyclo skeleton. The distribution pattern was mainly cytoplasmic with a uniform rather than a granular localization. In HeLa cells, fluorescence was also visible on the contours of the cells, suggesting that some material remained associated with the cell membrane. In contrast, the contours of HEK cells were not fluorescent and **ON 13** was more homogeneously distributed throughout the cytosol. These observations raise questions about the mechanism of internalization and the subsequent trafficking. Indeed, the uniform cytosolic distribution implies that if endocytosis was the pathway for uptake, then most of the material has leaked out of the endosomal compartment. Furthermore, the presence of membrane-bound oligonucleotide suggests that endocytosis may not be the only route of internalization. As an alternative pathway, the fusion of **ON 13** with the cell surface can be assumed which has previously been shown to occur with cationic lipids as delivery agents.[41] Clearly, additional experiments will be required in order to identify the mechanism of uptake for **ON 13**.

## CONCLUSIONS

In this work, we have presented the synthesis of tc-T derivatives with functionalities connected to C(6′) of the tricyclic sugar structure with the aim of improving the cellular uptake of tc-DNA. Two phosphoramidites, $tc^{ee}$-T **10** and $tc^{hd}$-T **15**, were prepared in 8 and 10 steps, respectively, from ketone **1**. The key synthetic steps are the $\beta$-selective NIS-mediated nucleosidation and hydrolysis/re-esterification that allows for the preparation of a variety of tc-T esters without altering the major part of the synthetic scheme. Both phosphoramidites were successfully incorporated into oligonucleotides and the reactivity of the ester group was investigated under various deprotection conditions. Hydrolysis and aminolysis afforded the corresponding carboxylic acid and amide forms, whereas mild ethanolic ammonia treatment left the ethyl and hexadecyl esters untouched in most cases. Amide, acid or ethyl ester substituted oligonucleotides led to a significant stabilization when paired to cDNA, and this effect was even more expressed with RNA as a complement. With $tc^{hd}$-T however, all duplexes were destabilized and it appears that there exists a competition between base pairing and self-aggregation of the long aliphatic chains as evidenced by UV-melting experiments and fluorescence emission spectroscopy. Nevertheless, $tc^{hd}$-T maintains its potential as a nucleoside analogue for oligonucleotide-based therapies. Indeed, the hexadecyl ester is expected to be hydrolyzed after internalization into cells, thus releasing the corresponding carboxylic acid which was shown to increase DNA and RNA affinity. Furthermore, the cellular uptake of oligonucleotides containing either tc-T, $tc^{ee}$-T, or $tc^{hd}$-T units showed that a $C_{16}$ side chain is capable of helping to cross the cellular membrane of two different cell lines. Fluorescence microscopy revealed a rather uniform cytosolic distribution of **ON 13** together with surface-bound material. The exact mechanism by which **ON 13** enters cells remains elusive at this point. Taken together, these results represent an important step toward the preparation of a tc-DNA prodrug. Further studies on the enzymatic processing of the hexadecyl ester and preliminary tests on the antisense activities of tc-oligonucleotides containing $tc^{hd}$-T units are underway.

## EXPERIMENTAL SECTION

**General Methods.** All reactions were performed under Ar and in dried glassware. Anhydrous solvents for reactions were obtained by filtration through activated alumina or by storage over molecular sieves (4 Å). Column chromatography (CC) was performed on silica gel with an average particle size of 40 $\mu$m. All solvents for column chromatography were of technical grade and distilled prior to use. Thin-layer chromatography was performed on silica gel plates. Visualization was achieved either under UV light or by dipping in staining solution [$Cer^{IV}$-sulfate (10.5 g), phosphormolybdenic acid (21 g), conc. $H_2SO_4$ (60 mL), $H_2O$ (900 mL) or *p*-anisaldehyde (3.7 mL), concd $H_2SO_4$ (5 mL), glacial acetic acid (1.5 mL), ethanol (135 mL)] followed by heating with a heat gun. NMR spectra were recorded at 400 or 300 MHz field width ($^1$H) in either $CDCl_3$ or $CD_3OD$. $\delta$ in ppm relative to residual undeuterated solvent [$CHCl_3$: 7.26 ppm ($^1$H) and 77.0 ppm ($^{13}$C); $CHD_2OD$: 3.35 ppm ($^1$H) and 49.3 ppm ($^{13}$C)], *J* in Hz. Signal assignments are based on DEPT and on $^1$H−$^1$H and $^1$H−$^{13}$C correlation experiments (COSY/HSQC). High-resolution mass spectra were recorded on an ion-trap instrument in the $ESI^+$ mode.

**(1*S*,5*S*)-8-[(*tert*-Butyldimethylsilyl)oxy]-7-(ethoxycarbonyl)-methyl-5-hydroxy-3-methoxy-2-oxybicyclo[3.3.0]oct-7-ene (2).** To a solution of **1** (1.0 g, 3.87 mmol)[33] in dry THF (39 mL) was slowly added LiHMDS (842 mg, 5.03 mmol) 0.75 M THF at −78 °C. A solution of TBDMS-Cl (875 mg, 5.81 mmol) and $Et_3N$ (0.22 mL, 1.54 mmol) in dry THF (14 mL) was added dropwise after 20 min. The resulting clear yellow solution was allowed to warm to rt after 50 min and was stirred for an additional 2 h. The mixture was then diluted with EtOAc (150 mL) and washed with satd $NaHCO_3$ (2 × 125 mL) and brine (1 × 125 mL). The aqueous phase was extracted with EtOAc (2 × 400 mL), and the combined organic phases were dried over $MgSO_4$, filtered, and evaporated. The crude orange oil was purified by CC with EtOAc/hexane (1:5 → 1:1), affording **2*α*** (1.03 g, 72%) and **2*β*** (211 mg, 15%) as colorless oils.

*Data for* **2*α*:** $R_f$ = 0.51 (EtOAc/hexane 2:1); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 5.00 (*d*, *J* = 4.1 Hz, 1H, H−C(3)), 4.65 (*s*, 1H, H−C(1)), 4.09 (2*q*, *J* = 7.8, 4.0 Hz, 2H, $CH_3CH_2O$), 3.35 (*s*, 3H, *Me*O), 3.10 (*s*, 1H, O*H*), 3.02 (*s*, 2H, H−C(6)), 2.47 (*d*, *J* = 0.8 Hz, 2H, H−C(9)),


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2.19 (*d*, *J* = 13.4 Hz, 1H, H−C(4)), 2.01 (*dd*, *J* = 13.4, 4.1 Hz, 1H, H−C(4)), 1.22 (*t*, *J* = 7.1 Hz, 3H, $CH_3CH_2O$), 0.93 (*s*, 9H, $(CH_3)_3C$−Si), 0.18 (*s*, 3H, $(CH_3)_2Si$), 0.14 (*s*, 3H, $(CH_3)_2Si$); $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 171.1 ($CO_2Et$), 147.5 (C(8)), 110.73 (C(7)), 105.5 (C(3)), 92.0 (C(1)), 83.7 (C(5)), 60.8 ($CH_3CH_2O$), 54.7 (*Me*O), 47.2 (C(4)), 40.9 (C(6)), 32.4 (C(9)), 25.92 (($CH_3)_3C$−Si), 18.5 (($CH_3)_3C$-Si), 14.5 ($CH_3CH_2O$), −3.9, −4.2 (($CH_3)_2Si$); ESI$^+$-HRMS *m*/*z* calcd for $C_{18}H_{32}O_6NaSi$ ([M + Na]$^+$) 395.1860, found 395.1847.

*Data for* ***2β****:* $R_f$ = 0.29 (EtOAc/hexane 2:1); $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 5.15 (*dd*, *J* = 5.4, 2.7 Hz, 1H, H−C(3)), 4.53 (*s*, 1H, H−C(1)), 4.10 (*q*, *J* = 7.1 Hz, 2H, $CH_3CH_2O$), 3.30 (*s*, 3H, *Me*O), 3.16 (*d*, *J* = 15.8, 1H, H−C(6)), 2.89 (*dd*, *J* = 15.7, 0.8 Hz, 1H, H−C(6)), 2.74 (*d*, *J* = 15.6 Hz, 1H, H−C(9)), 2.48 (*d*, *J* = 15.6 Hz, 1H, H−C(9)), 2.32 (*dd*, *J* = 13.6, 5.4 Hz, 1H, H−C(4)), 2.16 (*dd*, *J* = 13.6, 2.7 Hz, 1H, H−C(4)), 1.99 (*s*, 1H, O*H*), 1.23 (*t*, *J* = 7.1 Hz, 3H, $CH_3CH_2O$), 0.94 (*s*, 9H, $(CH_3)_3C$−Si), 0.19, 0.18 (*2s*, 6H, $(CH_3)_2Si$). $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 171.2 ($CO_2Et$), 148.3 (C(8)), 110.4 (C(7)), 106.5 (C(3)), 92.2 (C(1)), 84.3 (C(5)), 60.8 ($CH_3CH_2O$), 55.3 (*Me*O), 48.8 (C(4)), 45.2 (C(9)), 32.6 (C(6)), 25.8 (($CH_3)_3C$−Si), 18.39 (($CH_3)_3C$-Si), 14.5 ($CH_3CH_2O$), −3.9, −4.1 (($CH_3)_2Si$); ESI$^+$-HRMS *m*/*z* calcd for $C_{18}H_{32}O_6NaSi$ ([M + Na]$^+$) 395.1860, found 395.1858.

**(1*S*,2*R*,4*R*,6*S*,8*R*)-2-[(*tert*-Butyldimethylsilyl)oxy]-4-(ethoxycarbonyl)methyl-6-hydroxy-8-methoxy-9-oxytricyclo-[4.3.0$^{1,6}$.0$^{2,4}$]nonane (3*β*).** To a solution of **2*β*** (0.35 mg, 0.95 mmol) in dry $CH_2Cl_2$ (10 mL) was added diethylzinc (1 M in hexane, 5.7 mL, 5.7 mmol) dropwise at 0 °C, followed by diiodomethane (0.76 mL, 9.5 mmol) after 15 min. A white precipitate formed, and the resulting suspension was allowed to warm to rt. After 5 h, the mixture was diluted with EtOAc (100 mL) and washed with satd $NH_4Cl$ (1 × 50 mL) and 10% $Na_2S_2O_3$ (1 × 50 mL). The aqueous phases were combined and extracted with EtOAc (2 × 100 mL). The organic phases were dried over $MgSO_4$, filtered, and evaporated. The crude dark yellow solid was purified by CC with EtOAc/hexane (1:5 → 1:3) to give the title compound **3*β*** (0.30 g, 81%) as a colorless oil.

*Data for* ***3β****:* $R_f$ = 0.43 (hexane/EtOAc 2:1); $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 5.03 (*d*, *J* = 4.8 Hz, 1H, H−C(8)), 4.18−4.09 (*m*, 3H, $CH_3CH_2O$, H−C(1)), 3.31 (*s*, 3H, *Me*O), 2.55 (*m*, 2H, H−C(10), H−C(5)), 2.23 (*m*, 2H, H−C(7), H−C(10)), 2.11 (*dd*, *J* = 12.4, 4.9 Hz, 1H, H−C(7)), 1.92 (*d*, *J* = 13.7 Hz, 1H, H−C(5)), 1.24 (*t*, *J* = 7.1 Hz, 3H, $CH_3CH_2O$), 0.88 (*s*, 9H, $(CH_3)_3C$−Si), 0.85−0.80 (*m*, 2H, H−C(3)), 0.18 (*s*, 3H, $(CH_3)_2Si$), 0.09 (*s*, 3H, $(CH_3)_2Si$); $^1H$ NMR difference NOE (400 MHz, $CDCl_3$) $\delta$ 5.03 (H−C(8)) → 3.31 (5.5%, O-*Me*), 2.11 (3.7%, H−C(7)), 0.85 (H−C(3)) → 4.12 (2.2%, H−C(1)), 2.23 (1.9%, H−C(10)), 1.92 (1.7%, H−C(5)); $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 173.3 ($CO_2Et$), 105.6 (C(8)), 91.9 (C(1)), 86.8 (C(6)), 68.2 (C(2)), 60.5 ($CH_3CH_2O$), 54.5 (*Me*O), 49.4, 49.2 (C(7), C(5)), 37.3 (C(10)), 30.7 (C(4)), 25.9 (($CH_3)_3C$−Si), 22.3 (C(3)), 18.1 (($CH_3)_3C$-Si), 14.2 ($CH_3CH_2O$), −3.57, −3.62 (($CH_3)_2Si$); ESI$^+$-HRMS *m*/*z* calcd for $C_{19}H_{35}O_6Si$ ([M + H]$^+$) 387.2197, found 387.2205.

**(1*S*,2*R*,4*R*,6*S*,8*S*)-2-[(*tert*-Butyldimethylsilyl)oxy]-4-[(ethoxycarbonyl)methyl]-6-hydroxy-8-methoxy-9-oxytricyclo[4.3.0$^{1,6}$.0$^{2,4}$]nonane (3*α*) and (1*S*,2*S*,4*S*,6*S*,8*S*)-2-[(*tert*-butyldimethylsilyl)oxy]-4-[(ethoxycarbonyl)methyl]-6-hydroxy-8-methoxy-9-oxytricyclo[4.3.0$^{1,6}$.0$^{2,4}$]nonane (4*α*).** To a heavily stirred solution of **2*α*** (1.01 g, 2.68 mmol) in dry $CH_2Cl_2$ (45 mL) was slowly added diethylzinc (1 M in hexane, 16.1 mL, 16.1 mmol) at 5−10 °C. After fuming had ceased, diiodomethane (2.15 mL, 2.68 mmol) was added dropwise and the mixture allowed to reach rt. After being stirred overnight, the reaction mixture was diluted with EtOAc (300 mL) and washed with satd $NH_4Cl$ (1 × 200 mL) and 10% $Na_2S_2O_3$ (1 × 200 mL). The aqueous phases were extracted with EtOAc (2 × 400 mL) and the combined organic phases dried over $MgSO_4$, filtered, and evaporated. Purification of the resulting yellow oil by CC (hexane/EtOAc 5:1 → 2:1) afforded **3*α*** (0.31 g, 30%) and **4*α*** (0.65 g, 62%) both as colorless oils.

*Data for* ***3α****:* $R_f$ = 0.58 (hexane/EtOAc 3:2); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 5.07 (*dd*, *J* = 5.3, 1.6 Hz, 1H, H−C(8)), 4.15−4.09 (*m*, 3H, $CH_3CH_2O$, H−C(1)), 3.36 (*s*, 3H, *Me*O), 2.52 (*d*, *J* = 15.8 Hz, 1H, H−C(10)), 2.43 (*dd*, *J* = 13.8, 5.3 Hz, 1H, H−C(7)), 2.32 (*d*, *J* = 15.8 Hz, 1H, H−C(10)), 2.14 (*dd*, *J* = 13.8, 1.2 Hz, 1H, H−C(5)), 2.00 (*dd*, *J* = 14.0, 1.6 Hz, 1H, H−C(7)), 1.96 (*d*, *J* = 13.9 Hz, 1H, H−C(5)), 1.88 (*s*, 1H, O-*H*), 1.24 (*t*, *J* = 7.1 Hz, 3H, $CH_3CH_2O$), 1.02 (*dd*, *J* = 6.1, 0.8 Hz, 1H, H−C(3)), 0.89 (*s*, 9H, $(CH_3)_3C$−Si), 0.79 (*dd*, *J* = 6.1, 1.6 Hz, 1H, H−C(3)), 0.19 (*s*, 3H, $(CH_3)_3C$−Si), 0.13 (*s*, 3H, $(CH_3)_3C$−Si); $^1H$ NMR difference NOE (400 MHz, $CDCl_3$) $\delta$ 5.07 (H−C(8)) → 3.36 (4.7%, O-*Me*), 2.43 (4.8%, H−C(7)); 0.79 ($H_\beta$-C(3)) → 4.10 (3.9%, H−C(1)), 1.96 (1.7%, H−C(5)), 0.85 (25%, $H_\alpha$-C(3)), 0.85 ($H_\alpha$-C(3)) → 2.32 (2.9%, H−C(10)), 0.79 (20.3%, $H_\alpha$-C(3)); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 173.0 ($CO_2Et$), 105.7 (C(8)), 89.5 (C(1)), 86.1 (C(6)), 66.7 (C(2)), 60.5 ($CH_3CH_2O$), 55.0 (*Me*O), 50.0 (C(7)), 46.9 (C(5)), 37.1 (C(10)), 29.9 (C(4)), 26.0 (($CH_3)_3C$−Si), 22.9 (C(3)), 18.2 (($CH_3)_3C$-Si), 14.5 ($CH_3CH_2O$), −3.4, −3.8 (($CH_3)_2Si$); ESI$^+$-HRMS *m*/*z* calcd for $C_{19}H_{34}O_6SiNa$ ([M + Na]$^+$) 409.2017, found 409.2008.

*Data for* ***4α****:* $R_f$ = 0.73 (hexane/EtOAc 3:2); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 5.10 (*d*, *J* = 4.2 Hz, 1H, H−C(8)), 4.70 (*d*, *J* = 1.5 Hz, 1H, H−C(1)), 4.12 (*qd*, *J* = 7.1, 1.6 Hz, 2H, $CH_3CH_2O$), 3.34 (*s*, 3H, *Me*O), 3.21 (*s*, 1H, O-*H*), 2.50 (*d*, *J* = 16.3 Hz, 1H, H−C(10)), 2.34 (*d*, *J* = 16.3 Hz, 1H, H−C(10)), 2.17−2.07 (*m*, 2H, H−C(5)), 1.95 (*d*, *J* = 13.9 Hz, 1H, H−C(7)), 1.79 (*dd*, *J* = 13.9, 4.3 Hz, 1H, H−C(7)), 1.24 (*t*, *J* = 7.1 Hz, 3H, $CH_3CH_2O$), 0.85 (*s*, 9H, $(CH_3)_3C$−Si), 0.85−0.82 (*m*, 1H, H−C(3)), 0.62 (*d*, *J* = 6.5 Hz, 1H, H−C(3)), 0.17 (*s*, 3H, $(CH_3)_3C$−Si), 0.08 (*s*, 3H, $(CH_3)_3C$−Si); $^1H$ NMR difference NOE (400 MHz, $CDCl_3$) $\delta$ 5.10 (H−C(8)) → 3.34 (5.7%, O-*Me*), 1.79 (3.7%, H−C(7)), 4.70 (H−C(1)) → 3.21 (2.7%, O-*H*); 0.62 ($H_\beta$-C(3)) → 2.32 (2.9%, H−C(5)), 0.83 (19.7%, $H_\alpha$-C(3)); $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 173.0 ($CO_2Et$), 106.9 (C(8)), 98.2 (C(1)), 87.1 (C(6)), 68.3 (C(2)), 60.6 ($CH_3CH_2O$), 54.5 (*Me*O), 46.6 (C(7)), 44.0 (C(10)), 38.0 (C(5)), 32.0 (C(4)), 25.9 (($CH_3)_3C$−Si), 22.3 (C(3)), 18.0 (($CH_3)_3C$-Si), 14.5 ($CH_3CH_2O$), −4.0, −4.3 (($CH_3)_2Si$); ESI$^+$-HRMS *m*/*z* calcd for $C_{19}H_{35}O_6Si$ ([M + H]$^+$) 387.2197, found 387.2198.

**(1*S*,2*R*,4*R*,6*S*)-2-[(*tert*-Butyldimethylsilyl)oxy]-4-[(ethoxycarbonyl)methyl]-6-hydroxy-9-oxytricyclo-[4.3.0$^{1,6}$.0$^{2,4}$]non-7-ene (5).** To a solution of **3** (323 mg, 0.84 mmol) in dry $CH_2Cl_2$ (6.1 mL) was added 2,6-lutidine (0.485 mL, 4.18 mmol) at 0 °C, followed by TMSOTf (0.43 mL, 2.51 mmol) after15 min. After an additional 30 min at 0 °C, the resulting yellow solution was warmed to rt and stirred for another 2 h. The reaction was then quenched by the dropwise addition of satd $NaHCO_3$ (10 mL) and diluted with EtOAc (30 mL). The organic phase was washed with satd $NaHCO_3$ (2 × 30 mL), and the aqueous phase was extracted with EtOAc (3 × 60 mL). The combined organic phases were then dried over $MgSO_4$, filtered, and concentrated to yield crude **5** (385 mg) that was used without further purification in the next step.

*Data for* ***5****:* $R_f$ = 0.88 (EtOAc/hexane 2:1); $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 6.30 (*d*, *J* = 2.7 Hz, 1H, H−C(8)), 5.05 (*d*, *J* = 2.7 Hz, 1H, H−C(7)), 4.45 (*s*, 1H, H−C(1)), 4.11 (*q*, *J* = 7.1 Hz, 3H, $CH_3CH_2O$), 2.47 (*d*, *J* = 15.8 Hz, 1H, H−C(5)), 2.31 (*d*, *J* = 15.8 Hz, 1H, H−C(5)), 2.17 (*dd*, *J* = 13.4, 1.7 Hz, 1H, H−C(10)), 2.02 (*d*, *J* = 13.3 Hz, 1H, H−C(10)), 1.23 (*t*, *J* = 7.1 Hz, 3H, $CH_3CH_2O$), 0.90 (*dd*, *J* = 5.9, 1.8 Hz, 1H, H−C(3)), 0.87 (*s*, 9H, $(CH_3)_3C$−Si), 0.81 (*d*, *J* = 5.9 Hz, 1H, H−C(3)), 0.17 (*s*, 3H, (($CH_3)_2Si$), 0.13 (*s*, 3H, (($CH_3)_2Si$), 0.07 (*s*, 9H, (($CH_3)_3Si$); $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 173.0 ($CO_2Et$), 146.4 (C(8)), 108.6 (C(7)), 95.0 (C(1)), 91.6 (C(6)), 67.3 (C(2)), 60.5 ($CH_3CH_2O$), 50.8 (C(5)), 37.1 (C(10)), 30.7 (C(4)), 26.0 (($CH_3)_3C$−Si), 23.9 (C(3)), 18.2 (($CH_3)_3C$-Si), 14.5 ($CH_3CH_2O$), 2.1 (($CH_3)_3Si$), −3.5, −3.6 (($CH_3)_2Si$); ESI$^+$-HRMS *m*/*z* calcd for $C_{21}H_{39}O_5Si_2$ ([M + H]$^+$) 427.2331, found 427.2331.

**1-[(3′*S*,5′*R*,6′*R*)-3′-*O*-Trimethylsilyl-5′-*O*-(*tert*-butyldimethylsilyl)-6′-[(ethoxycarbonyl)methyl]-2′-deoxy-2′-iodo-3′,5′-etha-no-5′,6′-methano-*β*-D-ribofuranosyl]thymine (6).** To a mixture of thymine (530 mg, 4.2 mmol) in dry $CH_2Cl_2$ (6 mL) was added BSA (1.53 mL, 6.3 mmol) at rt. It was left for 1 h until a fine suspension had formed. A solution of **5** (668 mg, 1.4 mmol) in dry $CH_2Cl_2$ (6 mL) was then slowly added. After being cooled to 0 °C, *N*-


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iodosuccinimide (504 mg, 2.24 mmol) was added portionwise over 1.5 h. The mixture was stirred for an additional 30 min at 0 °C and for 1.5 h at rt. The clear, brown solution was diluted with EtOAc (50 mL) and washed with satd $NaHCO_3$ (2 × 50 mL) and satd $Na_2CO_3$ (1 × 50 mL). The aqueous phase was extracted with EtOAc (3 × 200 mL), and the combined organic phases were dried over $MgSO_4$, filtered, and evaporated to give 1.2 g (>100%) of a viscous orange oil that was subjected to the next reaction without further purification.

*Data for* ***6****:* $R_f$ = 0.4 (EtOAc/hexane 2:1); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.48 (*br*, 1H, H−N(3)), 7.69 (*d*, *J* = 1.2 Hz, 1H, H−C(6)), 6.35 (*d*, *J* = 5.9 Hz, 1H, H−C(1′)), 4.95 (*d*, *J* = 5.9 Hz, 1H, H−C(2′)), 4.30 (*s*, 1H, H−C(4′)), 4.11 (*q*, 2H, $CH_3CH_2O$), 2.56 (*d*, *J* = 16.9 Hz, 1H, H−C(7′)), 2.22 (*d*, *J* = 16.9 Hz, 1H, H−C(7′)), 1.96 (*m*, 2H, H−C(9′)), 1.90 (*d*, *J* = 1.1 Hz, 3H, *Me*-C(5)), 1.24 (*t*, *J* = 7.1, 0.8 Hz, 3H, $CH_3CH_2O$), 0.94 (*d*, *J* = 6.3 Hz, 1H, H−C(8′)), 0.85 (*s*, 10H, $(CH_3)_3$C−Si, H−C(8′)), 0.19 (*s*, 9H, $(CH_3)_3$Si), 0.12 (*d*, *J* = 0.8 Hz, 6H, $(CH_3)_2$Si); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 172.4 ($CO_2$Et), 164.0 (C(4)), 150.6 (C(2)), 136.3 (C(6)), 110.7 (C(5)), 93.9 (C(1′)), 91.5 (C(4′)), 88.8 (C(3′)), 66.3 (C(5′)), 60.9 ($CH_3CH_2O$), 42.8 (C(9′)), 40.4 (C(2′)), 36.4 (C(7′)), 29.9 (C(6′)), 25.9 (($CH_3)_3$C−Si), 25.7 (C(8′)), 18.1 (($CH_3)_3$C-Si), 14.5 ($CH_3CH_2O$), 12.8 (*Me*-C(5)), 2.3 (($CH_3)_3$Si), −3.5, −3.9 (($CH_3)_2$Si); ESI$^+$-HRMS *m*/*z* calcd for $C_{26}H_{43}O_7N_2INaSi_2$ ([M + Na]$^+$) 701.1546, found 701.1557.

**1-[(3′*S*,5′*R*,6′*R*)-3′-*O*-Trimethylsilyl-5′-*O*-(*tert*-butyldimethylsilyl)-6′-[(ethoxycarbonyl)methyl]-2′-deoxy-3′,5′-ethano-5′,6′-methano-*β*-D-ribofuranosyl]thymine (7).** To an orange suspension of **6** (1.17 g, 1.4 mmol) and $Bu_3SnH$ (0.56 mL, 2.1 mmol) in dry toluene (12 mL) was added AIBN (115 mg, 0.7 mmol) at rt. The mixture was heated to 95 °C for 1 h. The yellow solution was then evaporated and the residue purified by CC with hexane/EtOAc (6:1 → 1:1) + 1% $Et_3N$. Nucleoside **7** (515 mg, 67% from **3**) was isolated as a white foam.

*Data for* ***7****:* $R_f$ = 0.65 (EtOAc/hexane 1:1); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.58 (*br*, 1H, H−N(3)), 7.79 (*d*, *J* = 1.1 Hz, 1H, H−C(6)), 5.97 (*dd*, *J* = 5.3, 3.4 Hz, 1H, H−C(1′)), 4.26 (*s*, 1H, H−C(4′)), 4.11 (*q*, *J* = 7.1 Hz, 2H, $CH_3CH_2O$), 2.56 (*m*, 2H, H−C(2′)), 2.23−2.20 (*m*, 2H, H−C(9′)), 2.15 (*d*, *J* = 14.2 Hz, 1H, H−C(7′)), 1.91 (*d*, *J* = 0.9 Hz, 3H, *Me*-C(5)), 1.75 (*d*, *J* = 14.1 Hz, 1H, H−C(7′)), 1.23 (*t*, *J* = 7.1 Hz, 3H, $CH_3CH_2O$), 1.01 (*d*, *J* = 6.2 Hz, 1H, H−C(8′)), 0.90 (*s*, 9H, $(CH_3)_3$C−Si), 0.88−0.81 (*m*, 2H, H−C(8′)), 0.19 (*s*, 3H, $(CH_3)_2$Si), 0.14 (*s*, 3H, $(CH_3)_2$Si), 0.10 (*s*, 9H, $(CH_3)_3$Si); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 172.4 ($CO_2$Et), 164.2 (C(4)), 150.2 (C(2)), 136.4 (C(6)), 109.6 (C(5)), 94.2 (C(4′)), 89.2 (C(1′)), 86.7 (C(3′)), 66.9 (C(5′)), 60.7 ($CH_3CH_2O$), 47.9 (C(2′)), 47.2 (C(7′)), 36.6 (C(9′)), 29.6 (C(6′)), 25.9 (($CH_3)_3$C−Si), 21.7 (C(8′)), 18.1 (($CH_3)_3$C-Si), 14.5 ($CH_3CH_2O$), 12.8 (*Me*-C(5)), 2.1 (($CH_3)_3$Si), −3.6, −3.7 (($CH_3)_2$Si); ESI$^+$-HRMS *m*/*z* calcd for $C_{26}H_{45}O_7N_2Si_2$ ([M + H]$^+$) 553.2760, found 553.2769.

**1-[(3′*S*,5′*R*,6′*R*)-6′-[(Ethoxycarbonyl)methyl]-2′-deoxy-3′,5′-ethano-5′,6′-methano-*β*-D-ribofuranosyl]thymine (8).** To a solution of **7** (510 mg, 0.92 mmol) in dry pyridine (6 mL) was carefully added HF (70% in pyridine, 0.34 mL, 18.4 mmol) at 0 °C. After 20 min of stirring, the reaction mixture was allowed to warm to rt. After a total of 32 h, the yellow reaction mixture was diluted with EtOAc (40 mL) and silica gel (4 g) was added. The mixture was concentrated, coevaporated with toluene (2 × 10 mL) and purified by CC ($CH_2Cl_2$/EtOH 95:5 to 9:1) to give **8** (290 mg, 86%) as white crystals.

*Data for* ***8****:* $R_f$ = 0.31 ($CH_2Cl_2$/EtOH 9:1); $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 7.94 (*d*, *J* = 1.2 Hz, 1H, H−C(6)), 6.16 (*dd*, *J* = 6.7, 5.7 Hz, 1H, H−C(1′)), 4.19−4.12 (*m*, 3H, $CH_3CH_2O$, H−C(4′)), 2.59 (*d*, *J* = 16.4 Hz, 1H, H−C(9′)), 2.49 (*dd*, *J* = 13.9, 6.8 Hz, 1H, H−C(2′)), 2.43−2.37 (*m*, 2H, H−C(2′), H−C(9′)), 2.15 (*dd*, *J* = 13.9, 1.4 Hz, 1H, H−C(7′)), 1.94 (*d*, *J* = 1.1 Hz, 3H, *Me*-C(5)), 1.92 (*d*, 1H, H−C(7′)), 1.26 (*t*, *J* = 7.1 Hz, 3H, $CH_3CH_2O$), 1.01 (*d*, *J* = 5.9 Hz, 1H, H−C(8′)), 0.82 (*dd*, *J* = 5.9, 1.6 Hz, 1H, H−C(8′)). $^1$H NMR difference NOE (400 MHz, $CD_3OD$) $\delta$ 7.95 (H−C(6)) →6.17 (2.5%, H−C(1′)), 2.41 (2.9%, H−C(2′), H−C(9′)), 2.16 (1.2%, H−C(7′)), 1.95 (5%, *Me*-C(5)); 6.17 (H−C(1′)) →7.94 (2.1%, H−C(6)), 4.14 (4%, H−C(4′)), 2.49 (5.9%, H−C(2′)); $^{13}$C NMR (100 MHz, $CD_3OD$) $\delta$ 174.4 ($CO_2$Et), 166.7 (C(4)), 152.3 (C(2)), 138.4 (C(6)), 111.2 (C(5)), 92.8 (C(4′)), 88.0 (C(1′)), 85.2 (C(3′)), 66.6 (C(5′)), 61.7 ($CH_3CH_2O$), 49.2 (C(2′)), 47.8 (C(7′)), 37.2 (C(9′)), 31.2 (C(6′)), 22.4 (C(8′)), 14.7 ($CH_3CH_2O$), 12.6 (*Me*-C(5)); ESI$^+$-HRMS *m*/*z* calcd for $C_{17}H_{23}O_7N_2$ ([M + H]$^+$) 367.1500, found 367.1507.

**1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-Dimethoxytriphenyl)methyl]-6′-[(ethoxycarbonyl)methyl]-2′-deoxy-3′,5′-ethano-5′,6′-methano-*β*-D-ribofuranosyl]thymine (9).** To a colorless solution of **8** (257 mg, 0.7 mmol) in dry pyridine (3.4 mL) was added DMTr-Cl (548 mg, 1.6 mmol) in four equal portions over a time range of 2.5 h at rt. The resulting orange solution was stirred for 8.5 h before addition of another 0.5 eq. of DMTr-Cl. After reaction overnight the mixture was diluted with EtOAc (30 mL) and subsequently washed with satd $NaHCO_3$ (2 × 25 mL). The aqueous phase was extracted with EtOAc (3 × 50 mL). The combined organic phases were dried over $MgSO_4$, filtered and evaporated. The resulting orange foam was purified by CC with EtOAc/hexane (1:1 → 4:1) containing 0.1% $Et_3N$ to give DMTr-protected nucleoside **9** (366 mg, 78%) as a white foam.

*Data for* ***9****:* $R_f$ = 0.58 (EtOAc/EtOH 95:5); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.42 (*br*, 1H, H−N(3)), 8.21 (*d*, *J* = 1.0 Hz, 1H, H−C(6)), 7.44−7.41 (*m*, 2H, H-arom), 7.33 (*dd*, *J* = 8.9, 6.8 Hz, 1H, H-arom), 7.22−7.19 (*m*, 3H, H-arom), 6.77 (*t*, *J* = 8.9 Hz, 1H, H-arom), 5.83 (*dd*, 1H, H−C(1′)), 4.16 (*m*, 2H, $CH_3CH_2O$), 3.76, 3.75 (2*s*, 6H, *Me*O), 2.86 (*d*, *J* = 16.6 Hz, 1H, H−C(9′)), 2.51 (*d*, *J* = 16.6 Hz, 1H, H−C(9′)), 2.30 (*dd*, *J* = 11.4, 5.9 Hz, 2H, H−C(2′)), 2.19 (*d*, *J* = 14.2 Hz, 1H, H−C(7′)), 2.11 (*s*, 3H, *Me*-C(5)), 1.90−1.87 (*m*, 2H, H−C(7′), O-*H*), 1.21 (*t*, *J* = 7.1 Hz, 1H, $CH_3CH_2O$), 0.98 (*d*, *J* = 5.8 Hz, 1H, H−C(8′)), 0.73 (*d*, *J* = 6.4 Hz, 1H, H−C(8′)); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 172.8 ($CO_2$Et), 164.0 (C(4)), 159.1 (MeO-*C*-arom), 150.3 (C(2)), 146.3, 136.9, 136.8 (C-arom), 136.7 (C(6)), 131.7, 131.6, 129.3, 127.7, 127.3, 113.0, 112.9 (*C*H-arom), 110.5 (C(5)), 91.3 (C(4′)), 86.4 (C(1′)), 88.1, 85.0 (C(3′)), *C*(Ph)$_3$), 68.7 (C(5′)), 60.7 ($CH_3CH_2O$), 55.5 (*Me*O−DMTr), 49.4 (C(2′)), 47.5 (C(7′)), 36.7 (C(9′)), 31.4 (C(6′)), 22.7 (C(8′)), 14.5 ($CH_3CH_2O$), 12.6 (*Me*-C(5)); ESI$^+$-HRMS *m*/*z* calcd for $C_{38}H_{40}O_9N_2Na$ ([M + Na]$^+$) 691.2626, found 691.2640.

**1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-Dimethoxytriphenyl)methyl]-6′-[(ethoxycarbonyl)methyl]-3′-*O*-(2-cyanoethoxy)-diisopropylaminophosphanyl-2′-deoxy-3′,5′-ethano-5′,6′-methano-*β*-d-ribofuranosyl]thymine (10).** To a solution of **9** (40 mg, 0.06 mmol) and *N*-ethyldiisopropylamine (0.04 mL, 0.24 mmol) in dry THF (0.4 mL) was carefully added 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.03 mL, 0.12 mmol) at rt. After the slightly yellow solution was stirred for 1 h, the mixture was diluted with EtOAc (20 mL) and washed with satd $NaHCO_3$ (2 × 10 mL). The aqueous phase was extracted with EtOAc (3 × 20 mL), and the combined organic phases were dried over $MgSO_4$. After filtration and evaporation, the residual oil was purified by CC (EtOAc/Hexane 1:1 → 4:1 + 0.1% $Et_3N$) to give the title compound **10** (45 mg, 87%) as a white foam.

*Data for* ***10****:* $R_f$ = 0.57, 0.63 (EtOAc/hexane 4:1); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.24 (*br*, 1H, H−N(3)), 8.17 (*d*, *J* = 1.2 Hz, 1H), 7.45−7.42 (*m*, 2H, H-arom), 7.33 (*dd*, *J* = 8.7, 5.8 Hz, 4H, H-arom), 7.23−7.21 (*m*, 3H, H-arom), 6.77 (*dd*, *J* = 8.9, 7.7 Hz, 4H, H-arom), 5.83 (*dd*, *J* = 6.8, 4.4 Hz, 1H, H−C(1′)), 4.18−4.09 (*m*, 2H, $CH_3CH_2O$), 3.78, 3.77, 3.76 (3*s*, 6H, *Me*O), 3.71−3.38 (*m*, 4H, $OCH_2CH_2CN$, $(Me_2CH)_2N$), 2.89−2.82 (2*d*, 1H, H−C(9′)), 2.72 (*dd*, *J* = 14.3, 6.9 Hz, 1H, H−C(2′)), 2.58−2.51 (*m*, 3H, $OCH_2CH_2CN$, H−C(9′)), 2.48−2.44 (*m*, 2H, H−C(7′), H−C(4′)), 2.32 (*dd*, *J* = 13.6, 4.9 Hz, 0.5H, H−C(2′)), 2.25 (*dd*, *J* = 14.1, 4.2 Hz, 0.5H, H−C(2′)), 2.10 (*br*, 3H, *Me*-C(5)), 2.05−1.99 (*m*, 1H,), 1.21, 1.20 (2*t*, *J* = 7.0 Hz, 3H, $CH_3CH_2O$), 1.10−1.05 (*m*, 12H, $(Me_2CH)_2N$), 0.95 (*m*, 1H, H−C(8′)), 0.85 (*d*, *J* = 6.5 Hz, 0.5H, H−C(8′)), 0.77 (*d*, *J* = 6.3 Hz, 0.5H, H−C(8′)); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 172.7, 172.5 ($CO_2$Et), 164.1 (C(4)), 159.0 (MeO-*C*-arom), 150.2 (C(2)), 146.3, 146.2, 136.9, 136.8 (C-arom), 136.74, 136.66 (C(6)), 131.7, 131.6, 129.3, 127.7, 127.3, 127.2 (CH-arom), 117.77, 117.7 (*C*N), 113.0, 112.9 (*C*H-arom), 110.32, 110.26 (C(5)), 91.8, 91.2 (C(4′)), 88.3, 88.2, 88.1 (C(3′)), *C*(Ph)$_3$), 87.2, 86.9 (C(1′)),


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68.5 (C(5′)), 60.7 ($CH_3CH_2O$), 58.1, 57.9 ($J_{C,P}$ = 15.5 Hz, $OCH_2CH_2CN$), 55.43, 55.40 (*Me*O−DMTr), 47.1, 47.0 (C(2′)), 45.2, 45.1 (C(7′)), 43.6, 43.5, 43.3 ($J_{C,P}$ = 12.7 Hz, $(Me_2CH)_2N$), 36.8, 36.7 (C(9′)), 31.33, 31.26, 24.7, 24.6, 24.5, 24.4 ($(Me_2CH)_2N$), 22.3 (C(8′)), 20.5, 20.4 ($J_{C,P}$ = 8.1 Hz, $OCH_2CH_2CN$), 14.5 ($CH_3CH_2O$), 12.7 (*Me*-C(5)). $^{31}$P NMR (122 MHz, $CDCl_3$) $\delta$ 144.0, 142.3; ESI$^+$-HRMS *m*/*z* calcd for $C_{47}H_{58}O_{10}N_4P$ ([M + H]$^+$) 869.3885, found 869.3896.

**1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-(*tert*-Butyldimethylsilyl)-6′-(carboxymethyl)-2′-deoxy-3′,5′-ethano-5′,6′-methano-β-D-ribofuranosyl]thymine (11).** To a solution of **7** (520 mg, 0.94 mmol) in EtOH (2 mL) was added 4 N KOH (1.06 mL, 4.23 mmol) at rt. The resulting yellow solution was stirred for 2.5 h and was then diluted with $H_2O$ (20 mL). Neutralization was performed by 1 M $KHSO_4$ (until pH 3 was reached, 12 mL). Extraction of the aqueous phase with $CH_2Cl_2$ (4 × 40 mL), followed by drying of the organic phase over $MgSO_4$, filtration, and evaporation yielded **11** (391 mg, 92%) as a yellow foam that was used without further purification in the next step.

*Data for* ***11****:* $R_f$ = 0.18 ($CH_2Cl_2$/EtOH 9:1); $^1$H NMR (300 MHz, MeOD) $\delta$ 7.91 (*d*, *J* = 1.0 Hz, 1H, H−C(6)), 6.06 (*dd*, *J* = 6.4, 3.4 Hz, 1H, H−C(1′)), 4.25 (*s*, 1H, H−C(4′)), 2.60 (*dd*, *J* = 13.9, 3.4 Hz, 1H, H−C(2′)), 2.49 (*dd*, *J* = 13.9, 6.5 Hz, 1H, H−C(2′)), 2.35 (*q*, *J* = 15.9 Hz, 2H, H−C(9′)), 1.96 (*s*, 2H, H−C(7′)), 1.90 (*d*, *J* = 0.9 Hz, 3H, *Me*-C(5)), 1.02 (*d*, *J* = 6.2 Hz, 1H, H−C(8′)), 0.94 (*s*, 9H, $(CH_3)_3$C−Si), 0.91 (*d*, *J* = 4.4 Hz, 1H, H−C(8′)), 0.23 (*s*, 3H, $(CH_3)_2$Si), 0.19 (*s*, 3H, $(CH_3)_2$Si); $^{13}$C NMR (75 MHz, MeOD) $\delta$ 176.0 ($CO_2H$), 166.8 (C(4)), 152.3 (C(2)), 138.2 (C(6)), 110.5 (C(5)), 94.1 (C(4′)), 89.8 (C(1′)), 86.3 (C(3′)), 68.6 (C(5′)), 48.8 (C(2′)), 47.7 (C(7′)), 37.5 (C(9′)), 31.1 (C(6′)), 26.4 ($(CH_3)_3$C−Si), 23.4 (C(8′)), 19.0 ($(CH_3)_3$C-Si), 13.0 (*Me*-C(5)), −3.4, −3.6 ($(CH_3)_2$Si); ESI$^+$-HRMS *m*/*z* calcd for $C_{21}H_{33}O_7N_2Si$ ([M + H]$^+$) 453.2052, found 453.2059.

**1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-(*tert*-Butyldimethylsilyl)-6′-[(hexadecoxycarbonyl)methyl]-2′-deoxy-3′,5′-ethano-5′,6′-methano-β-D-ribofuranosyl]thymine (12).** To a slightly turbid solution of **11** (50 mg, 0.11 mmol) in dry $CH_2Cl_2$ (1.5 mL) was added 1-hexadecanol (32 mg, 0.13 mmol) followed by DMAP (7 mg, 0.056 mmol) at rt. The resulting mixture was cooled in an ice bath, and a solution of EDC·HCl (32 mg, 0.17 mmol) in dry $CH_2Cl_2$ (1.5 mL) was added dropwise. The mixture was allowed to warm to rt and was stirred for another 1 h. The clear solution was then diluted with EtOAc (25 mL) and washed with satd $NaHCO_3$ (2 × 15 mL) and $H_2O$ (1 × 15 mL). The aqueous phase was extracted with EtOAc (3 × 45 mL), and the combined organic phases were dried over $MgSO_4$, filtered, and concentrated. The crude material was used without further purification in the next step. For characterization, a sample of the crude material was purified by column chromatography (5% EtOH in $CH_2Cl_2$).

*Data for* ***12****:* $R_f$ = 0.51 ($CH_2Cl_2$/EtOH 9:1); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.60 (*s*, 1H, H−N(3)), 7.82 (*d*, *J* = 1.1 Hz, 1H, H−C(6′)), 6.03 (*dd*, *J* = 6.1, 3.1 Hz, 1H, H−C(1′)), 4.27 (*s*, 1H, H−C(4′)), 4.03 (*t*, *J* = 6.8 Hz, 2H, $CH_2$O), 2.63 (*dd*, *J* = 14.0, 3.2 Hz, 1H, H−C(2′)), 2.56 (*dd*, *J* = 14.1, 6.2 Hz, 1H, H−C(2′)), 2.30 (*q*, *J* = 16.1 Hz, 2H, H−C(9′)), 2.13 (*s*, 1H, O*H*), 2.04−1.90 (*m*, 2H, H−C(7′)), 1.91 (*d*, *J* = 0.8 Hz, 3H, *Me*-C(5)), 1.63−1.52 (*m*, 2H, $CH_2CH_2$O), 1.28−1.23 (*m*, 29H, $CH_2$-alk), 1.00 (*d*, *J* = 6.4 Hz, 1H, H−C(8′)), 0.89 (*s*, 10H, $(CH_3)_3$C−Si, H−C(8′)), 0.88−0.83 (*m*, 3H, $CH_3$-alk), 0.18 (*s*, 3H, $(CH_3)_2$Si), 0.13 (*s*, 3H, $(CH_3)_2$Si); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 172.6 ($CO_2$R), 164.2 (C(4)), 150.3 (C(2)), 136.4 (C(6)), 109.9 (C(5)), 92.9 (C(4′)), 88.8 (C(1′)), 85.4 (C(3′)), 67.2 (C(5′)), 65.1 ($CH_2$O), 48.4 (C(2′)), 47.7 (C(7′)), 36.5 (C(9′)), 32.1 (C(6′)), 29.91, 29.87, 29.84, 29.81, 29.75, 29.6, 29.5 ($CH_2$-alk), 28.8 ($CH_2CH_2$O), 26.1 ($CH_2$-alk), 25.9 ($(CH_3)_3$C−Si), 22.9 ($CH_2$-alk), 22.3 (C(8′)), 18.1 ($(CH_3)$C-Si), 14.3 ($CH_3$-alk), 12.8 (*Me*-C(5)), −3.7, −3.6 ($(CH_3)_2$Si); ESI$^+$-HRMS *m*/*z* calcd for $C_{37}H_{65}O_7N_2Si$ ([M + H]$^+$) 677.4556, found 677.4556.

**1-[(3′*S*,5′*R*,6′*R*)-6′-[(Hexadecoxycarbonyl)methyl]-2′-deoxy-3′,5′-ethano-5′,6′-methano-β-D-ribofuranosyl]thymine (13).** HF·pyridine (0.19 mL, 10.4 mmol) was slowly added at 0 °C to a solution of **12** (374 mg, 0.52 mmol) in pyridine (4 mL), and the mixture was left for 20 min before it was allowed to warm to rt. After being stirred overnight, the mixture was heated to 45 °C for 4 h to complete conversion. The resulting turbid solution was diluted with EtOAc (20 mL) and the product adsorbed on silica gel (3 g). Evaporation followed by purification of the adsorbed material by CC ($CH_2Cl_2$/EtOH 9:1) afforded **13** (192 mg, 65% over two steps) as a white foam.

*Data for* ***13****:* $R_f$ = 0.60 ($CH_2Cl_2$/EtOH 9:1); $^1$H NMR (300 MHz, MeOD) $\delta$ 7.93 (*d*, *J* = 1.1 Hz, 1H, H−C(6)), 6.15 (*dd*, *J* = 6.6, 5.7 Hz, 1H, H−C(1′)), 4.14 (s, 1H, H−C(4′)), 4.11 (*td*, *J* = 6.6, 1.6 Hz, 2H, $CH_2$O), 2.59 (*d*, *J* = 16.3 Hz, 1H, H−C(9′)), 2.49 (*dd*, *J* = 13.9, 6.8 Hz, 1H, H−C(2′)), 2.44−2.35 (*m*, 2H, H−C(2′), H−C(9′)), 2.13 (*dd*, *J* = 13.9, 1.1 Hz, 1H, H−C(7′)), 1.94 (*d*, *J* = 0.9 Hz, 3H, *Me*-C(5)), 1.94−1.89 (*m*, 1H, H−C(7′)), 1.70−1.59 (*m*, 2H, $CH_2CH_2$O), 1.33−1.29 (m, 26H, $CH_2$-alk), 1.01 (*d*, *J* = 5.9 Hz, 1H, H−C(8′)), 0.90 (*t*, *J* = 6.7 Hz, 3H, $CH_3$-alk), 0.82 (*dd*, *J* = 5.9, 1.4 Hz, 1H, H−C(8′)); $^{13}$C NMR (101 MHz, MeOD) $\delta$ 174.5 ($CO_2$R), 166.8 (C(4)), 152.4 (C(2)), 138.4 (C(6)), 111.2 (C(5)), 92.8 (C(4′)), 88.0 (C(1′)), 85.1 (C(3′)), 66.7 (C(5′)), 65.9 ($CH_2$O), 49.8, 49.6, 49.4, 49.3, 49.2, 49.0, 48.7, 48.5 ($CHD_2$OD, C(2′)), 47.8 (C(7′)), 37.2 (C(9′)), 33.2 (C(6′)), 31.2, 31.0, 30.91, 30.89, 30.87, 30.81, 30.78, 30.6, 30.5 ($CH_2$-alk), 29.9 ($CH_2CH_2$O), 27.2, 23.9 ($CH_2$-alk), 22.4 (C(8′)), 14.6 ($CH_3$-alk), 12.7 (*Me*-C(5)); ESI$^+$-HRMS *m*/*z* calcd for $C_{31}H_{51}O_7N_2$ ([M + H]$^+$) 563.3691, found 563.3678.

**1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-Dimethoxytriphenyl)methyl]-6′-[(hexadecoxycarbonyl)methyl]-2′-deoxy-3′,5′-ethano-5′,6′-methano-β-D-ribofuranosyl]thymine (14).** To a colorless solution of **13** (190 mg, 0.34 mmol) in dry pyridine (2.5 mL) was added DMTr-Cl (343 mg, 1.01 mmol) in four equal portions over 1.5 h. The resulting orange solution was stirred for 22 h before being diluted with EtOAc (30 mL). The organic phase was washed with satd $NaHCO_3$ (2 × 20 mL) and the aqueous phase extracted with EtOAc (3 × 50 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated. The crude orange foam was purified by CC (hexane/EtOAc 1:1 + 0.1% $Et_3N$, then hexane/EtOAc 1:4) to yield **14** (200 mg, 69%) as a slightly yellow foam.

*Data for* ***14****:* $R_f$ = 0.48 (EtOAc/hexane 4:1); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.89 (*s*, 1H, H−N(3)), 8.24 (*d*, *J* = 0.8 Hz, 1H, H−C(6)), 7.42 (*d*, *J* = 9.1 Hz, 2H, H-arom), 7.32 (*t*, *J* = 8.3 Hz, 4H, H-arom), 7.23−7.12 (*m*, 3H, H-arom), 6.79−6.70 (*m*, 4H, H-arom), 5.84 (*t*, *J* = 6.1 Hz, 1H, H−C(1′)), 4.13−4.03 (*m*, 2H, $CH_2$O), 3.73 (*s*, 3H, *Me*O), 3.71 (*s*, 3H, *Me*O), 2.88 (*d*, *J* = 16.7 Hz, 1H, H−C(9′)), 2.52 (*d*, *J* = 16.7 Hz, 1H, H−C(9′)), 2.46 (*s*, 1H, O*H*), 2.33 (*dd*, *J* = 14.0, 6.7 Hz, 1H, H−C(2′)), 2.29−2.18 (*m*, 3H, H−C(7′), H−C(4′)), 2.12 (*s*, 3H, *Me*-C(5)), 1.90 (*d*, *J* = 14.0 Hz, 1H, H−C(7′)), 1.61−1.50 (*m*, 2H, $CH_2CH_2$O), 1.29−1.15 (*m*, 26H, $CH_2$-alk), 0.95 (*d*, *J* = 6.1 Hz, 1H, H−C(8′)), 0.86 (*t*, *J* = 6.7 Hz, 3H, $CH_3$-alk), 0.73 (*d*, *J* = 6.3 Hz, 1H, H−C(8′)); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 173.0 ($CO_2$R), 164.2 (C(4)), 159.1 (MeO-C-arom), 150.5 (C(2)), 146.4, 136.9 (C-arom), 136.7 (C(6)), 131.7, 131.6, 129.2, 127.6, 127.1, 112.9, 112.8 (CH-arom), 110.8 (C(5)), 91.0 (C(4′)), 87.9 (C(3′)), 86.0 (C(1′)), 85.2 ($C(Ph)_3$), 68.5 (C(5′)), 64.9 ($CH_2$O), 55.4 (*Me*O), 49.5 (C(2′)), 47.4 (C(7′)), 36.6 (C(9′)), 32.1 (C(6′)), 31.3, 29.91, 29.87, 29.77, 29.75, 29.6, 29.4 ($CH_2$-alk), 28.8 ($CH_2CH_2$O), 26.2, 22.9 ($CH_2$-alk), 22.8 (C(8′)), 14.3 ($CH_3$-alk), 12.6 (*Me*-C(5)); ESI$^+$-HRMS *m*/*z* calcd for $C_{52}H_{68}O_9N_2Na$ ([M + Na]$^+$) 887.4817, found 887.4814.

**1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-Dimethoxytriphenyl)methyl]-6′-[(hexadecoxycarbonyl)methyl]-3′-*O*-[(2-cyanoethoxy)-diisopropylaminophosphanyl]-2′-deoxy-3′,5′-ethano-5′,6′-methano-β-D-ribofuranosyl]thymine (15).** To a colorless solution of *N*-ethyldiisopropylamine (0.16 mL, 0.93 mmol, 4 equiv) and nucleoside **15** (200 mg, 0.23 mmol) in 2.5 mL of dry THF (2.5 mL) was added 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.1 mL, 0.45 mmol) at rt. The resulting yellow solution was stirred for 1 h and gradually turned into a suspension. The reaction mixture was then diluted with EtOAc (40 mL) and washed with satd $NaHCO_3$ (2 × 25 mL). The aqueous phase was extracted with EtOAc (3 × 50 mL), and the combined organic phases were dried over $MgSO_4$, filtered, and evaporated. The crude yellow oil was purified by CC (hexane/EtOAc 1:1 +0.1% $Et_3N$) to give **15** (220 mg, 89%, mixture of isomers) as a white foam.


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*Data for* ***15****:* $R_f$ = 0.77, 0.70 (EtOAc/hexane 7:3); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.31 (*br*, 1H, *H*-N(3)), 8.21−8.15 (*m*, 1H, H−C(6)), 7.47−7.38 (*m*, 2H, H-arom), 7.33 (*dd*, *J* = 8.8, 6.3 Hz, 4H, H-arom), 7.23−7.18 (*m*, 3H, H-arom), 6.76 (*dd*, *J* = 8.9, 7.8 Hz, 4H, H-arom), 5.83 (*dd*, *J* = 6.7, 4.4 Hz, 1H, H−C(1′)), 4.10−4.02 (*m*, 2H, $CH_2O$), 3.79−3.75 (*m*, 6H, *Me*O), 3.71−3.37 (*m*, 4H, $OCH_2CH_2CN$, $(Me_2CH)_2N$), 2.86 (*dd*, *J* = 16.6, 3.2 Hz, 1H, H−C(9′)), 2.76−2.67 (*m*, 1H, H−C(2′)), 2.59−2.41 (*m*, 4.5H, $OCH_2CH_2CN$, H−C(9′), H−C(4′), H−C(7′)), 2.36−2.20 (*m*, 1.4H, H−C(7′), H−C(2′)), 2.12−2.08 (*m*, 3H, *Me*-C(5)), 2.07−2.00 (*m*, 1H, H−C(7′), H−C(2′)), 1.58−1.49 (*m*, 2H, $CH_2CH_2O$)), 1.32−1.16 (*m*, 26H, $CH_2$-alk), 1.12−1.03 (*m*, 12H, $(Me_2CH)_2N$), 0.94 (*dd*, *J* = 10.0, 6.5 Hz, 1H, H−C(8′)), 0.85 (*t*, *J* = 6.7 Hz, 3H, $CH_3$-alk), 0.76 (*d*, *J* = 6.3 Hz, 1H, H−C(8′)); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 172.9, 172.7 ($CO_2R$), 164.1 (C(4)), 159.04, 159.01 (MeO-*C*-arom), 150.2 (C(2)), 146.3, 146.2 (C-arom), 136.9, 136.8, 136.7 (C-arom, C(6)), 131.7, 131.6, 129.3, 127.7, 127.24, 127.17 (*C*H-arom), 117.8, 117.7 (*C*N), 112.94, 112.87 (*C*H-arom), 110.4, 110.3 (C(5)), 91.71, 91.67, 91.2, 91.1 (C(4′)), 88.5, 88.4, 88.3, 88.2, 88.1 (C(3′), $C(Ph)_3$), 87.1, 86.8 (C(1′)), 68.4 (C(5′)), 65.0 ($CH_2O$), 58.1, 58.0, 57.9, 57.8 ($J_{C,P}$ = 18.8 Hz, 19.5 Hz, $OCH_2CH_2CN$), 55.41, 55.38 (*Me*O), 47.2, 47.1 (C(2′)), 45.3, 45.1 (C(7′)), 43.6, 43.47, 43.45, 43.3 ($J_{C,P}$ = 12.9 Hz, 12.7 Hz, $(Me_2CH)_2N$), 36.7, 36.6 (C(9′)), 32.1 (C(6′)), 31.4, 31.3, 30.0, 29.9, 29.8, 29.7, 29.6, 29.5 ($CH_2$-alk), 28.8 ($CH_2CH_2O$), 26.2 ($CH_2$-alk), 24.7, 24.6, 24.54, 24.47, 24.4 ($(Me_2CH)_2N$), 22.9 ($CH_2$-alk), 22.24, 22.20 (C(8′)), 20.51, 20.49, 20.44, 20.41 ($J_{C,P}$ = 8.0 Hz, 7.3 Hz, $OCH_2CH_2CN$), 14.33($CH_3$-alk), 12.7 (*Me*-C(5)). $^{31}$P NMR (162 MHz, $CDCl_3$) $\delta$ 143.7, 142.2; ESI$^+$-HRMS *m*/*z* calcd for $C_{61}H_{86}O_{10}N_4P$ ($[M + H]^+$) 1065.6076, found 1065.6113.

**Oligonucleotide Synthesis.** Syntheses of oligonucleotides were performed on the 1.3 $\mu$mol scale of a DNA synthesizer using standard solid-phase phosphoramidite chemistry. Oligomers were assembled using the manufacturer's protocols on nucleoside preloaded CPG or fluorescein-labeled (FAM) solid supports (Roche Diagnostics). Natural phosphoramidites (dT, $dC^{4Bz}$, $dA^{6Bz}$,$dG^{2dmf}$, Vivotide) were coupled as a 0.1 M solution in $CH_3CN$, tricyclophosphoramidites (tc-T, tc-$C^{4Bz}$, tc-$A^{6Bz}$, tc-$G^{2dmf}$) as 0.15 M solution in $CH_3CN$ (0.15 M in dichloroethane for tc-$A^{6Bz}$ phosphoramidite). The coupling step was 90 s for natural phosphoramidites. An extended coupling time of 12 min for tricyclonucleosides was necessary to achieve average coupling efficiencies of 94% for $tc^{ee}$-T **10** and 97% for $tc^{hd}$-T **15** (trityl assay). As a coupling reagent, 5-(ethylthio)-1*H*-tetrazole (0.25 M in $CH_3CN$) was used. Capping was performed with a solution of DMAP (0.5 M in $CH_3CN$, Cap A) and a solution of 25% $Ac_2O$ and 12.5% *sym*-collidine in $CH_3CN$ (Cap B). Oxidation was performed with a solution of 20 mM $I_2$ and 0.45 M *sym*-collidine in 2.1:1 $CH_3CN/H_2O$. Detritylation was carried out using a solution of 3% dichloroacetic acid in dichloroethane.

**Oligonucleotide Deprotection and Purification.** Deprotection of the oligonucleotides containing amide-tc-T units (**ON 1**, **3**, **5**, **7**−**9**), and detachment from the solid support was carried out using standard conditions (concd aq $NH_3$ for 16 h at 55 °C). To maintain the ethyl and hexadecyl ester functions, **ON 2**, **4**, **6**, **12**, and **13** were treated with a 1:3 $NH_3$/EtOH solution at 40 °C for 24 h. **ON 11** was deprotected in 25% benzylamine ($EtOH/H_2O$ 1:2) at 65 °C for 8 h. In all cases, the solutions were centrifuged after deprotection, the supernatants were removed and the remaining beads washed with 0.25 mL of $H_2O$. The combined supernatants were then concentrated to dryness. To perform hydrolysis of the ethyl ester (**ON 10**), the solid phase was treated with a 0.1 M KOH solution. After an overnight shaking at 55 °C, the solution was neutralized with 1 M HCl and desalted (see below). Crude oligomers were purified by ion-exchange HPLC. As mobile phases, the following buffers were prepared: (A) 25 mM Trizma (2-amino-2-hydroxymethyl-1,3-propanediol) in $H_2O$, pH 8.0; (B) 25 mM Trizma, 1.25 M NaCl in $H_2O$, pH 8.0. For oligos containing $tc^{hd}$-T building blocks, **ON 6**: (A) 25 mM Trizma in $H_2O$, pH 8.0; (B) 25 mM Trizma, 1 M $NaClO_4$ in $H_2O$, pH 8.0. **ON 13**: (A) 25 mM Trizma in $H_2O$/ACN 4:1, pH 8.0; (B) 25 mM Trizma, 1 M $NaClO_4$ in $H_2O$/ACN 4:1, pH 8.0. Linear gradients of B in A were used (typically 0 to 40% or 0 to 50% B in A over 30 min), with a 1 mL/min flow rate and detection at 260 nm. Purified oligonucleotides were desalted over Sep-Pak cartridges, quantified at 260 nm with a Nanodrop spectrophotometer, and analyzed by ESI$^-$ mass spectrometry. Oligonucleotides were then stored at −18 °C.

**UV−Melting Curves.** UV−melting curves were recorded on a Varian Cary Bio100 UV/vis spectrophotometer. Absorbances were monitored at 260 nm, and the heating rate was set to 0.5 °C/min. A cooling−heating−cooling cycle in the temperature range 15−80 °C was applied. $T_m$ values were obtained from the maximum of the first derivative curves and reported as the average of at least three ramps (±1 °C error). To avoid evaporation of the solution, the sample solutions were covered with a layer of dimethylpolysiloxane. All measurements were carried out in NaCl (150 mM), $Na_2HPO_4$ (10 mM) buffer at pH 7.0 with a duplex concentration of 2 $\mu$M.

**Fluorescence Spectroscopy.** The solutions were prepared in NaCl (150 mM), $Na_2HPO_4$ (10 mM) buffer at pH 7.0 with a duplex concentration of 2 $\mu$M. Measurements were performed on a Varian Cary Eclipse fluorescence spectrophotomer in quartz cuvettes with a path length of 1 cm. The photomultiplier voltage was set to 500 V, and the solutions were excited at 480 nm. The emission spectra were recorded at 20 °C from 490 to 600 nm.

**Cell Culture and Transfection.** HeLa and HEK 293T cells were grown at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/mL of penicillin and 100 $\mu$g/mL of streptomycin (P/S). Cells were split 1:10 every 2 or 3 days. For cellular uptake experiments, 1 × $10^5$ HeLa and 2 × $10^5$ HEK 293T cells were seeded in duplicate in six-well plates containing coverslips 24 h before treatment with oligonucleotides. Then, the medium was replaced by a 1 mL solution of fluorescein-labeled oligonucleotide (10 $\mu$M final concentration) in DMEM + /+ (FCS, P/S). The medium was removed after 48 h at 37 °C and the cells were washed with 2 × 1 mL PBS. They were finally suspended in 1 mL fresh DMEM +/+.

**Cell Imaging.** Fixation of the cells was carried out using a 1 mL solution of paraformaldehyde (3.7% PFA in PBS) for 10 min, after which the cells were washed with 2 × 1 mL PBS. After permeabilization of the cell membrane with 0.2% Triton x-100 for 10 min and washing with 2 × 1 mL PBS the cells were mounted on coverslips and treated with a few drops of polyvinyl alcohol (Mowiol) containing nuclear stain 4′,6′-diamidino-2-phenylindole (DAPI) and analyzed on a fluorescence microscope (Leica DMI6000 B, Leica Microsystems with Leica Application Suite software).

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

$^1$H and $^{13}$C NMR spectra of all compounds, $^{31}$P NMR spectra of phosphoramidites **10** and **15**. $^1$H−$^1$H COSY and 2D NOESY spectra of compounds **3*α*** and **4*α***, $^1$H NMR NOE spectrum and crystal data of compound **8**. Characterization of oligonucleotides. Representative melting curves of **ON 2**, **4**, **6**, and **12**−**14**. HPLC trace and mass spectrum of **ON12** as a representative example. This material is available free of charge via the Internet at http://pubs.acs.org.

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