The First Postsynthetic 5′-5′ Intercalators in Triplex DNA – Solid-Phase Postsynthetic *Sonogashira* Reaction and Homocouplings on Arylacetylenes

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We report herein a new technique for postsynthetic modifications of on-column oligonucleotides. CPG-Supported nonamer polypyrimidine oligodeoxynucleotides comprising 2-(3-iodobenzyloxy)ethyl phosphate at the terminal 5′-ends were successfully capped with 1,3-diethynylbenzene under *Sonogashira* reaction conditions, resulting in novel intercalating 5′-5′ linker in alternate strand *Hoogsteen* triplex-forming oligonucleotidej (TFO) **X**. In addition, novel 5′-5′-linked alternate strand TFOs, **Y** and **Z**, were isolated as a result of on-column *Sonogashira* coupling with 1,3-diethynylbenzene, followed by Cu-catalyzed oxidative arylacetylenic homocoupling(s) between neighboring terminal arylacetylenes. Linkers **X**, **Y**, and **Z** represent the first postsynthesis 5′-5′ intercalating linkers for alternate strand TFOs. Reproducibility and verification by independent syntheses using conventional phosphoramidite chemistry and DNA synthesis, as well as thermal stability and molecular modelling studies are included.

Introduction. – With the decoding of the human genome and with an increased knowledge of the gene functions, the necessity for sequence-specific recognition and targeting of double-stranded DNA (dsDNA) for diagnostic applications became of high interest. The formation of triple DNA helices *via* the binding of DNA probes to DNA duplexes through H-bonding is the key to sequence-selective dsDNA targeting [1][2]. Triplex-forming oligonucleotides (TFOs) can bind to dsDNA in either a parallel or antiparallel motif with respect to the homopurine strand of the duplex [3]. The main disadvantage, employing TFOs as a diagnostic tool, is the instability of the corresponding triplex under physiological conditions, since cytosine requires protonation at N(3) to form the $C^+ \cdot G-C$ *Hoogsteen* base triplet. In addition, G-rich TFOs are unlikely to form antiparallel triplexes under physiological ion concentrations, because, under these conditions, they form more stable aggregates, *e.g.*, quadruplexes, promoted by K^+ ions [4][5]; however, insertion of TINA (twisted intercalating nucleic acid) favors triplex formation [6][7]. Another major limitation for the triplex technology is the requirement of a homopurine tract of more than 15 purines in order to achieve high target-binding affinity [8].

Alternate-strand TFOs that target homopurine tracts located on each strand of the dsDNA have been designed to increase the number of targets in dsDNA (*Fig. 1*). Alternate-strand TFO consists of minimum two independent oligonucleotides covalently linked at their 3′- or the 5′-ends [9][10]. Since each oligonucleotide of the probe

should bind simultaneously to the target, in combination with specific point of strand alternation, a better specificity to dsDNA can be assumed when compared to targeting a homopurine sequence.

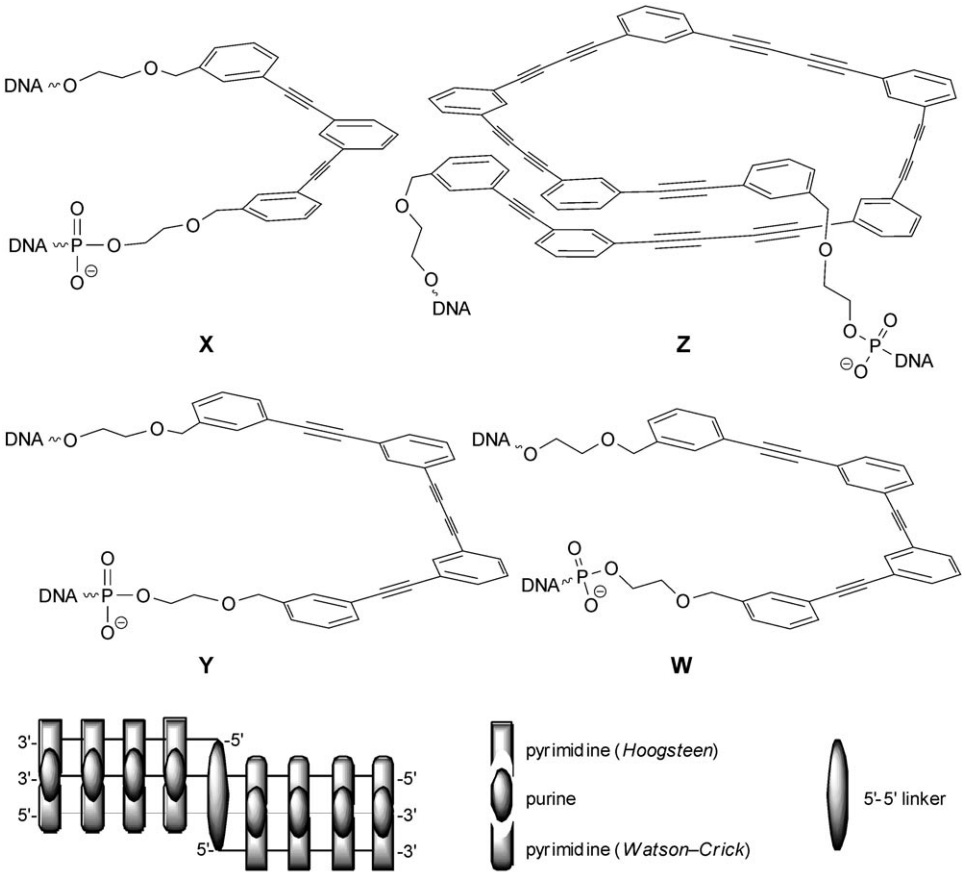


Fig. 1. *5′-5′ Intercalating linkers for alternate-strand TFOs* (**X**, **Y**, **Z**, and **W**) *and a sketch of the 5′-5′ alternate-strand triplex concept*

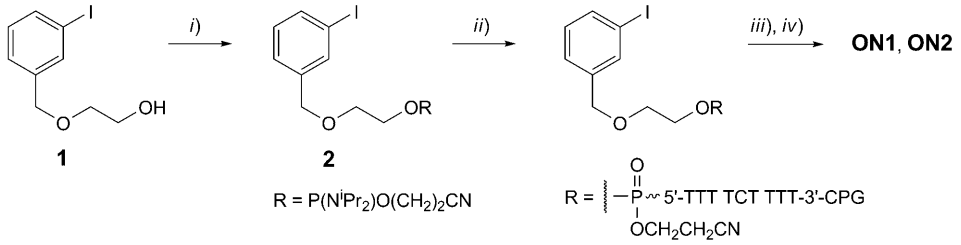
Herein, we report the synthesis of novel 5′-5′ linkers obtained *via* on-column postsynthetic derivatization of DNA. The postsynthetically Pd^0-catalyzed reaction between 2-(3-iodobenzyloxy)ethyl phosphate linked to the 5′-end of DNA and 1,3-diethynylbenzene resulted in different types of on-column oligonucleotide cross-linking (*Fig. 1*). The linker **X** was formed by *Sonogashira* reactions between two separate terminal 2-(3-iodobenzyloxy)ethyl phosphates and 1,3-diethynylbenzene. In addition, the linkers **Y** and **Z** were presumably formed by *Sonogashira* reactions between two separate terminal 2-(3-iodobenzyloxy)ethyl phosphates and two or six 1,3-diethynylbenzenes *via* oxidative arylacetylenic homocoupling(s). To our knowledge, this is the first observation of one-pot postsynthetic *Sonogashira* coupling and

acetylenic homocoupling between neighboring on-column oligonucleotides, representing a new technique in postsynthetic modifications of on-column oligonucleotides. In continuation of this work, the linker **W** was anticipated as an interesting target, and it was synthesized by usual amidite chemistry.

Results and Discussion. – *Postsynthetic 5′-5′ Intercalators.* Recently, we have shown the possibility of achieving high thermal stability of a matched 5′-5′ linked alternate-strand triplex under physiological conditions [11]. Therefore, we decided to investigate the possibility of synthesizing a novel 5′-5′ linker in an alternate-strand TFO, by capping the modified 5′-ends of a nonamer oligonucleotide together through two separate solid-phase postsynthetic *Sonogashira* reactions. Postsynthetic modification of CPG-supported oligonucleotides has shown to be an effective and time-saving alternative to standard phosphoramidite synthesis, and several reports on postsynthetically Pd^0-catalyzed reactions have been reported [6][12–15].

A nonamer oligodeoxynucleotide was synthesized by standard DNA automated phosphoramidite approach in a 1.0-μmol scale on 500-Å CPG-support and subsequent coupled at the 5′-end with the phosphoramidite of 2-(3-iodobenzyloxy)ethanol (*Scheme 1*). In the first postsynthesis, the modified oligonucleotide was treated afterwards with a reaction mixture containing 1,3-diethynylbenzene (38 μmol), $Pd(PPh_3)_4$ (22 μmol), and CuI (26 μmol) in dry DMF/Et_3N (350 μl/150 μl) under Ar and at room temperature over 3 h. After washing the CPG-support with DMF (2 × 2 ml) and MeCN (2 × 2 ml), the oligonucleotide derivative was cleaved from the support and deprotected by 32% aqueous NH_3 (55°, overnight), followed by removal of excess reagents and impurities from the postsynthesis by *NAP™ 10* column. Major fractions from the RP-HPLC purification were collected and analyzed by MALDI-TOF-MS (*Table 1*).

Scheme 1. *Synthesis of Phosphoramidite and Oligonucleotides* (ONs) **ON1** *and* **ON2**



i) $NC(CH_2)_2OP(N^iPr_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, 19 h; 25%. *ii*) Incorporation of **2** at the 5′-end of nonamer oligodeoxynucleotide synthesized by standard DNA synthesis on a 500-Å 1.0-μmol CPG-support. *iii*) Postsynthesis: 1,3-diethynylbenzene (38 μmol), $Pd(PPh_3)_4$ (22 μmol), and CuI (26 μmol) in dry DMF/Et_3N (350 μl/150 μl) under Ar. *iv*) 32% aq. NH_3, 55°, overnight.

Surprisingly, from MALDI-TOF analysis, two different 5′-5′ linked oligonucleotides (ONs) were isolated, **ON1** and **ON2**. **ON1** was isolated as the major product and identified from the molecular mass as the possible product of *Sonogashira* couplings between 1,3-diethynylbenzene and two neighboring oligonucleotides possessing a

Table 1. *Reproducibility and Verification Study: Calculated and Found Masses of Synthesized ONs*

Entry	Synthesis[a])	Sequence	Mass [*m*/*z*]	
			Found[b])	Calc.
ON1	1st Postsynth.	3′-TTTTCTTTT-5′-**X**-5′-TTTTCTTTT-3′	5868.7	5871.1
ON3	2nd Postsynth.		5872.5	–
ON7	Amidite chem.		5867.5	–
ON2	1st Postsynth.	3′-TTTTCTTTT-5′-**Y**-5′-TTTTCTTTT-3′	5995.0	5996.3
ON4	2nd Postsynth.		5991.1	–
ON5	3rd Postsynth.		5995.8	–
ON8	Amidite chem.		5996.5	–
ON6	4th Postsynth.	3′-TTTTCTTTT-5′-**Z**-5′-TTTTCTTTT-3′	6487.1	6491.8

[a]) Reaction conditions are discussed in the text. [b]) Determined by MALDI-TOF-MS.

terminal 2-(3-iodobenzyloxy)ethyl phosphate resulting in the formation of the 5′-5′ linker **X**. **ON2** was isolated as the minor product, and from the molecular mass, we assumed that it contained a larger 5′-5′ linker corresponding to the structure **Y**. Traces of atmospheric O_2 during postsynthesis presumably catalyzed an arylacetylenic homocoupling either before or after *Sonogashira* coupling with the CPG-supported oligonucleotide [16–18]. *Minakawa et al.* [19] showed in 2003 the possibility of a postsynthetically Cu-catalyzed oxidative acetylenic homocoupling between the 5′-ends of on-column hexamer ONs performed on a 500-Å CPG-support. But to our knowledge, this is the first observation of one-pot postsynthetic *Sonogashira* and homocoupling between neighboring on-column ONs. The possibility of synthesizing novel 5′-5′ linkers through two different carbon–carbon reactions performed postsynthetically encouraged us to investigate reproducibility of **X** and **Y**, and to verify their structures by conventional chemistry.

Reproducibility and Verification Study. Reproducibility of **ON1** and **ON2** were performed *via* a second postsynthesis using equal amounts of reactant and reagents, but under atmospheric O_2 in order to ensure sufficient amounts of O_2 for the oxidative acetylenic homocoupling of **ON2**. As seen from *Table 1*, oligonucleotides with masses equal to **ON1** and **ON2** were isolated, *i.e.*, **ON3** and **ON4**, respectively, still with the same distribution in yield. To investigate the possibility of increasing the yields of the oligonucleotide comprising the linkage **Y**, we performed two additional postsyntheses (*third postsynthesis:* the CPG-supported oligonucleotide was treated twice with equal *Sonogashira* mixtures both performed under Ar; *fourth postsynsthesis:* the CPG-supported oligonucleotide was treated twice with two different *Sonogashira* mixtures, first with both reactant and reagents under Ar, followed with only a reagent mixture under atmospheric O_2, as to ensure sufficient O_2 for the oxidative acetylenic homocoupling). In the case of the *third postsynthesis*, only an ON with a mass equal to **ON2** was isolated, *i.e.*, **ON5**. It is plausible that by saturating the CPG-supported oligonucleotide with 1,3-diethynylbenzene, a full conversion of 2-(3-iodobenzyloxy)-ethyl into 2-{3-[(3-ethynylphenyl)ethynyl]benzyloxy}ethyl is achieved before the on-column cross-linking takes place. This results in the exclusive formation of the linkage **Y**. In the case of the *fourth postsynthesis*, an oligonucleotide, **ON6**, with surprisingly

high molecular weight of 6487.1 g/mol was isolated. From the MALDI-TOF analyses, a 5′-5′ linker **Z**, which contains five homocoupled aryldiacetylenes with a calculated mass of 6491.8 g/mol, was proposed. All novel 5′-5′ linked alternate-strand TFOs, *i.e.*, **ON1**–**ON6**, were used in thermal-stability studies to evaluate their ability to stabilize matched and mismatched alternate-strand triplexes (*Table 2*). In addition, we wanted to use these T_m values along with MALDI-TOF data for comparison with the values obtained using the same 5′-5′-linked alternate-strand TFOs synthesized *via* conventional phosphoramidite chemistry as a way to verify the structures of linkers **X** and **Y**.

Table 2. *Reproducibility and Verification Study:* T_m [°] *Data for Third-Strand Melting, Taken from UV Melting Curves* ($\lambda = 260$ nm)[a])

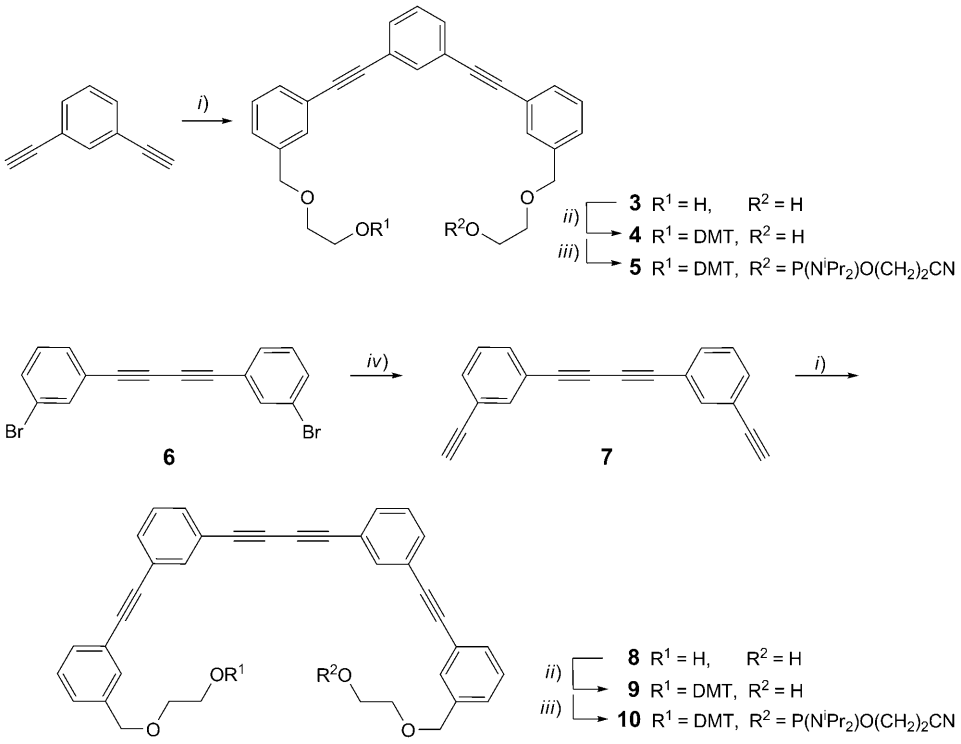
Entry	Synthesis[b])	Sequence	**D1** pH 6.0	**D1** pH 7.2	**D2** pH 6.0	**D3** pH 6.0
ON1	1st Postsynth.	3′-TTTTCTTTT-5′-**X**-5′-TTTTCTTTT-3′	55.5	46.5	28.5	25.5
ON3	2nd Postsynth.		55.5	46.0	28.5	25.5
ON7	Amidite chem.		54.5	45.5	27.5	25.0
ON2	1st Postsynth.	3′-TTTTCTTTT-5′-**Y**-5′-TTTTCTTTT-3′	51.5	43.5	23.0	21.0
ON4	2nd Postsynth.		52.0	43.5	22.5	20.0
ON5	3rd Postsynth.		53.0	44.0	24.5	21.5
ON8	Amidite chem.		51.5	43.0	24.0	21.5
ON6	4th Postsynth.	3′-TTTTCTTTT-5′-**Z**-5′-TTTTCTTTT-3′	54.5	46.0	30.0	24.5
D1		3′-GGTCAGGGAAAAGAAAATTTTCTTTTCACC-5′ 5′-CCAGTCCCTTTTCTTTTAAAAGAAAAGTGG-3′				
D2		3′-GGTCAGGGAA*C*A*C*AAAATTTTCTTTTCACC-5′ 5′-CCAGTCCCTT*G*T*G*TTTTAAAAGAAAAGTGG-3′				
D3		3′-GGTCAGGGAAAAGAAAATT*GG*CTTTTCACC-5′ 5′-CCAGTCCCTTTTCTTTTAA*CC*GAAAAGTGG-3′				

[a]) $c = 1.5$ μM of **ON1**–**8** and 1.0 μM of each strand of dsDNA (**D1**–**D3**) in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0 and 7.2; duplex T_m 67.5° (**D1**; pH 6.0), 70.5° (**D2**; pH 6.0), 71.0° (**D3**; pH 6.0), and 68.0° (**D1**; pH 7.2); target regions are underlined, and bases in italics are mismatch for TFO hybridization. [b]) Reaction conditions are discussed in the text.

Therefore, the phosphoramidites **5** and **10** of **X** and **Y**, respectively, were synthesized (*Scheme 2*). Phosphoramidite **5** was synthesized starting from the central 1,3-diethynylbenzene, which was coupled with 2-(3-iodobenzyloxy)ethanol (**1**) under *Sonogashira* conditions to produce the diol **3** of linker **X**. Phosphoramidite **10** was synthesized starting from the central 1,4-bis(3-bromophenyl)buta-1,3-diyne (**6**) [20], which was synthesized from 1-bromo-3-iodobenzene *via* one-pot synthesis involving a *Sonogashira* reaction with (trimethylsilyl)acetylene, followed by a cleavage of the silyl protection group to achieve an oxidative homocoupling between the arylacetylenes.

The phosphoramidites were used in standard DNA syntheses to obtain **ON7** and **ON8**, corresponding to **ON1** and **ON2**, respectively. As can be seen from *Table 1*, equal masses for **ON1**, **ON3**, and **ON7**, and **ON2**, **ON4**, **ON5**, and **ON8**, respectively, were determined. Final verification of the linker structures **X** and **Y** was achieved *via* identical melting curves of thermal denaturation experiments of triplexes, shown as first derivative plots in *Fig. 2*. Obtained T_m values (within the uncertainty of the first

Scheme 2. *Synthesis of the phosphoramidite of* **X** *and* **Y**



i) 2-(3-Iodobenzyloxy)ethanol (**1**), $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, overnight. *ii*) Dimethoxytrityl chloride (= bis(4-methoxyphenyl)(phenyl)methyl; DMT-Cl), Et_3N, CH_2Cl_2, overnight. *iii*) $NC(CH_2)_2OP(N^iPr_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, overnight. *iv*) $Pd(PPh_3)_2Cl_2$, CuI, PPh_3, (trimethylsilyl)acetylene (TMSA), Et_3N, 17 h; followed by $Et_3N \cdot 3$ HF, 2 h.

derivative method for determination of T_m [21]) for *Hoogsteen*-matched (*i.e.*, **D1**) and *Hoogsteen*-mismatched triplexes (*i.e.*, **D2** and **D3**) are shown in *Table 2*.

As it can be seen from *Table 2*, all TFOs formed highly stabile triplexes with the complementary duplex **D1** at pH 6.0 and 7.2, respectively. In addition, it was established that all TFOs formed 5′-5′-linked alternate-strand triplexes at pH 6.0, as a large drop in T_m was observed upon insertion of pyrimidine mismatches in the homopurine tracts, *e.g.*, in **D2** and **D3**, corresponding to loss of stabilizing *Hoogsteen* hybridization.

TFOs with the linker **X** were found to form more stabile triplexes than TFOs with the linker **Y** (T_m(**ON7**/**D1**) = 54.5° and T_m(**ON8**/**D1**) = 51.5°, resp., at pH 6.0. *Jessen* and *Pedersen* [10] showed in 2004 that the 1,3-bis(phenylethynyl)benzene moiety of linker **X** is able to form $\pi-\pi$ interaction with all four strands at the specific point of alternation. Therefore, the lower triplex stability observed for the use of **Y** could be the result of poorer $\pi-\pi$ interaction caused by the larger structure of the 1,4-bis(3-(phenylethynyl)phenyl)buta-1,3-diyne moiety in addition to its lower rigidity when

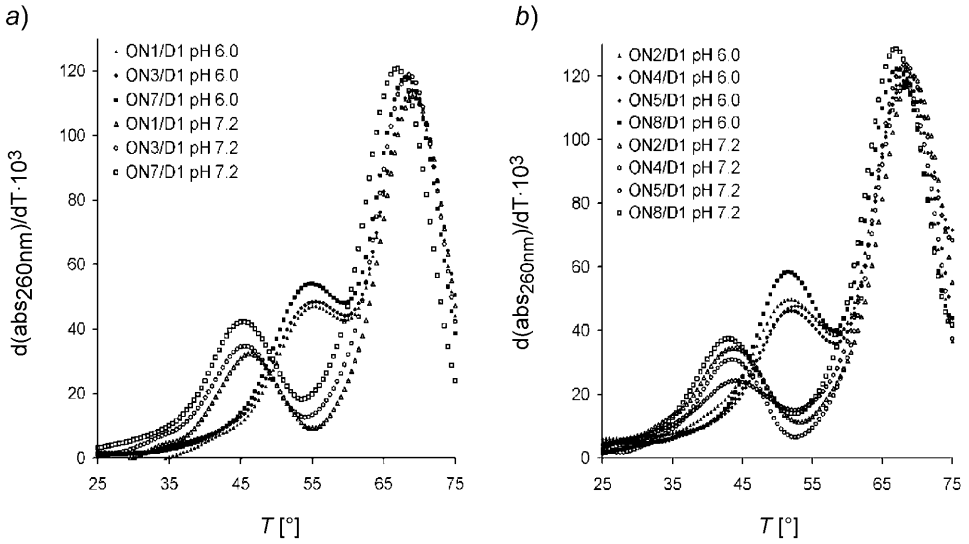


Fig. 2. *First-derivative plots of thermal denaturation experiments of triplexes:* a) **ON1**, **ON3**, *and* **ON7/D1**, b) **ON2**, **ON4**, **ON5**, *and* **ON8/D1** (recorded in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0 and 7.2, at 260 nm *vs.* temperature, with a heating of 1.0°/min)

compared with **X**. Surprisingly, the much larger linkage **Z** (in **ON6**) was able to stabilize the triplex better than **Y**, the T_m values being nearly identical to those found for **X**.

Molecular Modelling. To assess the novel linkers' ability to intercalate the triplex, as well as to evaluate the variations in T_m values for **Y** and the unexpected high T_m values observed for **Z**, we performed molecular-modelling studies using a modified AMBER* force field to generate representative low-energy structures with linkers **X**, **Y**, and **Z** (*Fig. 3*, *a*–*h*). As can be seen from *Fig. 3*, *a* and *e*, linker **X** intercalates the triplex, positioning the central 1,3-diethynylbenzene moiety and the peripheral aromatic rings so that they form π–π interaction with the *Watson*–*Crick* duplex and the adjacent nucleobases of the TFO, respectively. From molecular dynamics of linker **Y**, three low-energy structures were found (*Fig. 3*, *b*). Even though the intercalating part of **Y** was found to be similar to that of linker **X** (*Fig. 3*, *f*), the diacetylene moiety resulted in two benzene rings to be positioned outside the triplex where no interaction with the surrounding nucleobases could be formed. We believe that the unfixed part of **Y** will wobble increasingly with elevated temperatures during thermal denaturation measurements, resulting in a lowering of thermal stability (*Table 2*). To our surprise, the much larger structure of linker **Z** was able to form a helical macrocycle *via* intramolecular π–π interactions in addition to triplex intercalation (*Figs. 3*, *c* and *g*). Hence, the formation of intramolecular π–π interactions in the linker would prevent wobbling and thereby lowering of T_m during thermal denaturation measurements as asserted for the linker **Y**. As can be seen from *Fig. 3*, *g*, the intercalating part of **Z** is equal to that of **X**, which could explain why similar T_m values for **Z** and **X** were observed.

Optimized 5′-5′ Intercalator. These findings encouraged us to synthesize a novel linker, **W**, where the central diacetylene of **Y** was replaced with a single C≡C bond

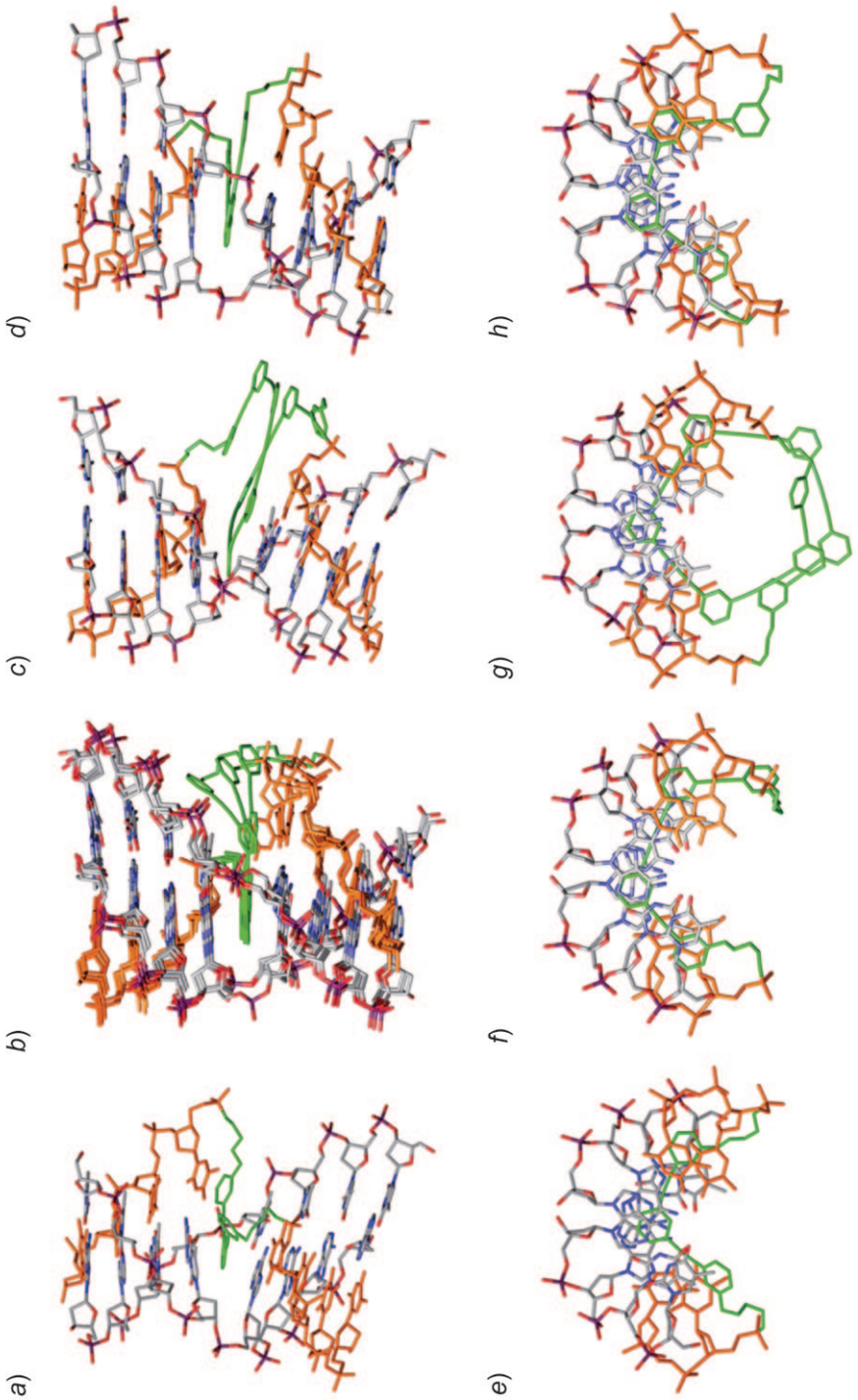
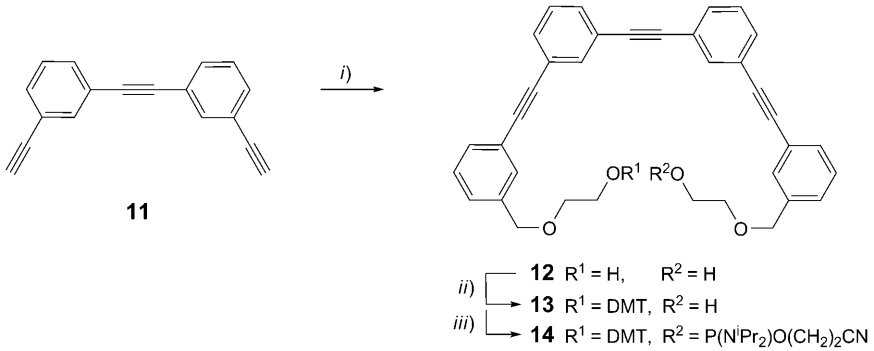


Fig. 3. *Representative low-energy structures of the truncated 5′-5′-linked alternate-strand triplexes obtained by molecular modelling.* The TFO is shown in orange and the intercalating linkers **X** (*Fig. 3*, *a* and *e*), **Y** (*Fig. 3*, *b* and *f*), **Z** (*Fig. 3*, *c* and *g*), and **W** (*Fig. 3*, *d* and *h*) in green. Top view of the structures is shown on *Fig. 3*, *a*–*d*; side view is shown on *Fig. 3*, *e*–*h*.

(*Fig. 3*, *d* and *e*). By shortening the diacetylene moiety of linker **Y**, a linker, which would fit better within the base triplets and thereby eliminate the possibility of wobbling, could be obtained. The phosphoramidites **14** and **10** (*Schemes 3* and *2*, resp.) of linkers **W** and **Y**, respectively, were used for the synthesis of TFOs complementary to the HIV-1 sequence **D4** (*Table 3*) [22][23].

Scheme 3. *Synthesis of the Phosphoramidite of* **W**



i) 2-(3-Iodobenzyloxy)ethanol (**1**), $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, 18 h. *ii*) DMT-Cl, Et_3N, CH_2Cl_2, 15 h. *iii*) $NC(CH_2)_2OP(N^iPr_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, 18 h.

Table 3. T_m [°] *Data for Third-Strand Melting, Taken from UV Melting Curves* ($\lambda = 260$ nm)[a])

Entry	Sequence	Mass [*m*/*z*]		**D4**	**D4**	**D5**	**D6**
		Found	Calc.	pH 6.0	pH 7.2	pH 6.0	pH 6.0
ON9	3′-TTTTCTTTT-5′-**Y**-5′-TTTTT-3′	4806.8	4808.4	39.5	35.0	16.0	19.5
ON10	3′-TTTTCTTTT-5′-**Y**-5′	3287.2	3287.5	23.5	15.5	< 5.0	–
ON11	3′-TTTTCTTTT-5′-**W**-5′-TTTTT-3′	4783.2	4784.4	46.0	42.0	22.0	26.0
ON12	3′-TTTTCTTTT-5′-**W**-5′	3263.1	3263.4	31.0	23.5	23.0	–
ON13[b])	3′-TTTTCTTTT-5′	–	–	16.0	< 5.0	n.d.[c])	–
D4	3′-GGTCAGGGGGGAAAAGAAAATTTTTCACC-5′						
	5′-CCAGTCCCCCCTTTTCTTTTAAAAAGTGG-3′						
D5	3′-GGTCAGGGGGGAAAAGAAA*G*TTTTTCACC-5′						
	5′-CCAGTCCCCCCTTTTCTTT*C*AAAAAGTGG-3′						
D6	3′-GGTCAGGGGGGAAAAGAAAATT*GG*TCACC-5′						
	5′-CCAGTCCCCCCTTTTCTTTTAA*CC*AGTGG-3′						

[a]) $c = 1.5$ μM of **ON9**–**13** and 1.0 μM of each strand of dsDNA (**D4**–**D6**) in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0 and 7.2; duplex T_m 68.0° (**D4**; pH 6.0), 70.0° (**D5**; pH 6.0), 70.0° (**D6**; pH 6.0), and 68.0° (**D4**, pH 7.2); target regions are underlined, and bases in italics are mismatch for TFO hybridization; C is 5-methylcytosine. [b]) Oligonucleotides and T_m data are from [11]. [c]) n.d. = Not determined.

This sequence is normally targeted at the 16-mer G-rich polypurine tract (PPT) which, for the parallel motif, is strongly pH-dependent. The dsDNA HIV-1 target allows us to use an asymmetric alternate-strand TFO consisting of a 14-mer pyrimidine sequence containing only one 5-methylcytosine to be protonated for triplex formation.

Indeed, only a moderate decrease in the triplex melting temperature was observed for **ON9** and **ON11** going from pH 6.0 to 7.2 ($\Delta T_m = -4.5$ and $-4.0°$, resp.). The 5-Me derivative was selected because, at physiological pH, it is easier to protonate than the native cytosine [4]. Mismatch studies using the dsDNAs **D5** and **D6** were used to confirm that both alternate strands hybridized to purine regions of dsDNA (*Table 3*). As it was expected from molecular-modelling studies, the use of linker **W** instead of **Y** led to increased thermal stability at pH 6.0 and 7.2: ΔT_m(**ON11/D4** – **ON9/D4**) = +6.5° and ΔT_m(**ON11/D4** – **ON9/D4**) = +7.0°, respectively (*Table 3*). Furthermore, improved intercalating properties of **W** in comparison with **Y** was observed as an increase in T_m value when **W** and **Y** were used as terminal 5′-intercalators: ΔT_m(**ON12/D4** – **ON13/D4**) = +15.0° compared to ΔT_m(**ON10/D4** – **ON13/D4**) = +7.5°. Upon insertion of mismatch in either side of the purine tract (*i.e.*, **D5** and **D6**), thermal stability dropped significantly for **ON9** and **ON11** at pH 6.0. In addition, thermal stability dropped considerably for the two non-alternating triplexes **ON10/D5** and **ON12/D5**. These observations are a clear indication of alternate-strand triplex formation for **ON9** and **ON11**. Besides, it is worth mentioning that the mismatch studies also show that **ON9** and **ON11** are sequence-specific with a good mismatch discrimination (ΔT_m(**ON9/D5** – **ON9/D4**) = –23.5° and ΔT_m(**ON11/D5** – **ON11/D4**) = –24.0°).

Conclusions. – We have successfully demonstrated a new technique in postsynthetical modifications of on-column oligonucleotides. *Via* Pd^0-catalyzed postsynthetic reaction with CPG-supported oligodeoxynucleotides possessing 2-(3-iodobenzyloxy)-ethyl phosphate at the terminal 5′-ends and 1,3-diethynylbenzene, three novel intercalating 5′-5′ linkers, **X**, **Y**, and **Z**, in alternate-strand TFOs were produced. Reproducibility, and verification of **X** and **Y** were achieved through independent postsyntheses and conventional phosphoramidite chemistry in DNA synthesis, respectively. All novel 5′-5′ linkers were shown to form stabile alternate-strand *Hoogsteen*-type triplexes at physiological pH. Molecular modelling was used to study the $\pi-\pi$ interaction between linker and surrounding nucleobases leading to the structurally optimized linker **W**. An interesting feature of using the alternate-strand triplexes is the shift from a G-rich target sequence used in the HIV-1 polypurine tract to an alternate sequence with only one G which makes the TFO less pH-dependent in the parallel motif. A 14-mer oligonucleotide with the linker **W** showed in the latter case low pH dependence (ΔT_m(pH 7.2 – pH 6.0) = –4.0°) and high thermal stability at physiological pH (T_m(pH 7.2) = 42.0°).

Experimental Part

General. M.p.: *Büchi* melting-point apparatus; uncorrected. TLC: Plates *60* F_{254} (*Merck*); visualization with UV light (254 nm). Column chromatography (CC): silica gel (SiO_2; 0.040–0.063 mm, *Merck*). Solvents for CC and reagents were used as purchased without further purification. NMR Spectra: *Varian Gemini 2000* spectrometer in $CDCl_3$ at 300 (^1H), 75 (^{13}C), and 121.5 MHz (^{31}P), resp.; δ in ppm rel. to Me_4Si (δ 0.00; ^1H) and $CDCl_3$ (δ 77.00; ^{13}C) as internal standards, and H_3PO_4 (δ 0.00, ^{31}P) as external standard; *J* in Hz. Accurate ion mass determinations: *4.7 Tesla Ultima Fourier Transform* (FT) mass spectrometer (*Ion Spec*, Irvine, CA). The $[M+Na]^+$ ions were peaks matched

using ions derived from the 2,5-dihydroxybenzoic acid matrix. EI-MS: *Finnigan SSQ 710*; in *m/z* (rel. %). MALDI-TOF-MS: *MicroFlex LT* from *Bruker Daltronics*.

1. *2-(3-Iodobenzyloxy)ethanol* (**1**). Compound **1** was synthesized according to the procedure in [10] using 3-iodobenzyl bromide as reactant instead of 4-iodobenzyl bromide. Yield 86%. Yellow oil. ^{1}H-NMR: 2.44 (br. *s*, OH); 3.58–3.61 (*m*, CH_2OH); 3.75 (*t*, *J* = 4.5, CH_2CH$_2$OH); 4.48 (*s*, $C_6H_4CH_2O$); 7.07 (*t*, *J* = 7.8, 1 arom. H); 7.29 (*d*, *J* = 7.5, 1 arom. H); 7.61 (*d*, *J* = 7.8, 1 arom. H); 7.69 (*s*, 1 arom. H). ^{13}C-NMR: 61.7 (*C*H$_2$OH); 71.6 (O*C*H$_2$CH$_2$OH); 72.2 ($C_6H_4CH_2O$); 94.3, 126.7, 130.1, 136.5, 136.7, 140.3 (C_6H_4). HR-MALDI-MS: 300.9700 ([*M* + Na]$^+$, $C_9H_{11}INaO_2^+$; calc. 300.9701).

2. *2-({[3-(3-{3-[(2-Hydroxyethoxy)methyl]phenyl}ethynyl)phenyl]ethynyl}benzyloxy)ethanol* (**3**). 1,3-Diethynylbenzene (360 mg, 2.86 mmol) was dissolved in dry Et$_3$N (50 ml) and flushed with Ar before CuI (56 mg, 0.29 mmol) and Pd(PPh$_3$)$_2$Cl$_2$ (130 mg, 0.19 mmol) were added. Compound **1** (1.77 g, 6.37 mmol) was added *via* a syringe, and the mixture was stirred at r.t. under Ar for 22 h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (100 ml) which was washed with 0.3M aq. EDTA (2 × 100 ml). After back-extraction with CH_2Cl_2 (100 ml), the combined org. phases were washed with brine (100 ml), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The residue was purified by CC (SiO_2; 0–85% AcOEt in cyclohexane (*v*/*v*)) to afford **3** (1.16 g, 43%). Yellow oil. ^{1}H-NMR: 3.62 (*t*, *J* = 4.6, 4 H, 2 OCH_2CH$_2$OH); 3.78 (*t*, *J* = 4.6, 4 H, 2 OCH$_2$CH_2OH); 4.57 (*s*, 4 H, 2 $C_6H_4CH_2O$); 7.28–7.71 (*m*, 12 arom. H). ^{13}C-NMR: 61.8 (*C*H$_2$OH); 71.6 (O*C*H$_2$CH$_2$OH); 72.7 ($C_6H_4CH_2O$); 88.6, 89.8 (*C*≡*C*); 123.1, 123.5, 127.7, 128.5, 130.8, 130.9, 131.3, 134.6, 138.4 (3 C_6H_4). HR-MALDI-MS: 449.1707 ([*M* + Na]$^+$, $C_{28}H_{26}NaO_4^+$; calc. 449.1729).

3. *1,4-Bis(3-bromophenyl)buta-1,3-diyne* (**6**). 1-Bromo-3-iodobenzene (2.40 g, 8.5 mmol) was dissolved in dry THF (30 ml) and dry Et$_3$N (2 ml), and flushed with Ar, before Pd(PPh$_3$)$_2$Cl$_2$ (122 mg, 0.17 mmol) and CuI (89 mg, 0.47 mmol) were added. (Trimethylsilyl)acetylene (1.00 g, 10.5 mmol) was added *via* a syringe, and the mixture was stirred at r.t. under Ar for 21 h, before Et$_3$N·3 HF (1.20 g, 7.7 mmol) was added. After 30 min, additional CuI (80 mg, 0.42 mmol) was added, and the mixture was flushed with O_2, closed with a septum, and stirred at r.t. under O_2 for 22 h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (50 ml), which was washed with 0.3M aq. EDTA (100 ml), H_2O (100 ml), brine (100 ml), and 10% HCl (100 ml). The org. phase was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The residue was purified by dry column vacuum chromatography (CVC; SiO_2; 0–50% AcOEt in cyclohexane (*v*/*v*)) to afford **6** (1.09 g, 71%). Pale yellow solid. M.p. 97–98°. ^{1}H- and ^{13}C-NMR: identical to those reported in [20]. EI-MS: 362 (50, [*M* + 2]$^+$), 360 (100, [$C_{16}H_8Br_2$]$^+$), 358 (50, [*M* − 2]$^+$), 200 (70, [*M* − 2 Br]$^+$).

4. *1,4-Bis(3-ethynylphenyl)buta-1,3-diyne* (**7**). Compound **6** (1.99 g, 5.5 mmol), Pd(PPh$_3$)$_2$Cl$_2$ (287 mg, 0.41 mmol), CuI (130 mg, 0.68 mmol), and powdered PPh$_3$ (331 mg, 1.13 mmol) were dissolved in dry Et$_3$N (75 ml). The mixture was flushed with Ar before closing with a septum. After addition of (trimethylsilyl)acetylene (3.30 g, 33.6 mmol), the mixture was stirred and refluxed under Ar for 17 h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (100 ml), which was washed with H_2O (100 ml) and 0.3M aq. EDTA (2 × 100 ml). After back-extraction of aq. layers with CH_2Cl_2 (100 ml), the combined org. phases were washed with brine (100 ml), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The residue was dissolved in dry CH_2Cl_2 (50 ml), and Et$_3$N·3 HF (948 mg, 5.88 mmol) was added. After 2 h, the mixture was concentrated under reduced pressure and purified by dry CVC (SiO_2; cyclohexane) to afford **7** (902 mg, 65%). Pale yellow solid. M.p. 122–126°. ^{1}H-NMR: 3.10 (*s*, 2 C≡C*H*); 7.27–7.32 (*m*, 2 arom. H); 7.46–7.51 (*m*, 4 arom. H); 7.64 (*t*, *J* = 1.5, 2 arom. H). ^{13}C-NMR: 74.4 (C≡*C*–*C*≡C); 78.2 (C≡*C*H); 80.7 (*C*≡CH); 82.4 (*C*≡C–C≡*C*); 122.0, 122.7, 128.6, 132.7, 132.8, 135.9 (2 C_6H_4). EI-MS: 250 (100, M^+), 248 (30, [*M* − 2 H]$^+$).

5. *2-{3-[(3-{4-[3-({3-(2-Hydroxyethoxy)methyl]phenyl}ethynyl)phenyl]buta-1,3-diynyl}phenyl)ethynyl]benzyloxy}ethanol* (**8**). Compound **7** (550 mg, 2.20 mmol) was dissolved in dry Et$_3$N (50 ml) and flushed with Ar, before CuI (32 mg, 0.17 mmol) and Pd(PPh$_3$)$_2$Cl$_2$ (84 mg, 0.12 mmol) were added. Compound **1** (1.44 g, 5.2 mmol) was added *via* a syringe, and the mixture was stirred at r.t. under Ar for 17 h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (100 ml) which was washed with 0.3M aq. EDTA (2 × 100 ml). After back-extraction with CH_2Cl_2 (100 ml), the combined org. phases were washed with brine (100 ml), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Crystallization from MeCN gave **8** (1.04 g, 86%). Yellow solid. M.p. 130–135°.

^{1}H-NMR: 2.15 (br. *s*, 2 CH_2O*H*); 3.60–3.63 (*m*, 2 C*H*$_2$OH); 3.78 (br. *s*, 2 C*H*$_2$CH$_2$OH); 4.56 (*s*, $C_6H_4CH_2O$); 7.30–7.37 (*m*, 6 arom. H); 7.45–7.69 (*m*, 8 arom. H); 7.69 (*s*, 2 arom. H). ^{13}C-NMR: 61.9 (*C*H$_2$OH); 71.5 (*C*H$_2$CH$_2$OH); 72.7 ($C_6H_4CH_2O$); 74.3 (C≡*C*–*C*≡C); 80.9, 88.3 (C_6H_4–*C*≡*C*–C_6H_4); 90.1 (*C*≡C–C≡*C*); 122.0, 122.9, 123.7, 127.8, 128.6, 128.6, 130.8, 131.0, 132.2, 132.3, 135.4, 138.4 (4 C_6H_4). EI-MS: 550 (100, M^+), 440 (60, $[M-C_4H_{10}O_3]^+$).

6. *2-{3-[(3-{[3-({3-[(2-Hydroxyethoxy)methyl]phenyl}ethynyl)phenyl]ethynyl}phenyl)ethynyl]benzyloxy}ethanol* (**12**). *1,2-Bis(3-ethynyl-phenyl)ethyne* (**11** [24]; 455 mg, 2.01 mmol) was dissolved in dry Et_3N (50 ml) and flushed with Ar, before CuI (34 mg, 0.18 mmol) and $Pd(PPh_3)_2Cl_2$ (72 mg, 0.10 mmol) were added. Compound **1** (1.34 g, 4.8 mmol) was added *via* a syringe, and the mixture was stirred at r.t. under Ar for 18 h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (100 ml) which was washed with 0.3M aq. EDTA (2 × 100 ml). After back-extraction with CH_2Cl_2 (100 ml), the combined org. phase was washed with brine (100 ml), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The residue was purified by dry CVC (SiO_2; 0–50% AcOEt in cyclohexane (*v*/*v*)) to afford **12** (674 mg, 64%). Yellow solid. M.p. 124–130°. ^{1}H-NMR: 3.60–3.63 (*m*, 2 C*H*$_2$OH); 3.76–3.81 (*m*, 2 C*H*$_2$CH$_2$OH); 4.56 (*s*, 2 $C_6H_4CH_2O$); 7.31–7.37 (*m*, 6 arom. H); 7.45–7.71 (*m*, 8 arom. H); 7.71 (*s*, 2 arom. H). ^{13}C-NMR: 61.9 (*C*H$_2$OH); 71.5 (*C*H$_2$CH$_2$OH); 72.8 ($C_6H_4CH_2O$); 88.7, 89.1, 89.9 (*C*≡*C*); 123.2, 123.4, 123.6, 127.8, 128.5, 128.6, 130.9, 131.0, 131.4, 131.5, 134.7, 138.4 (4 C_6H_4). EI-MS: 526 (30, M^+), 420 (35, $[M-C_4H_{10}O_3]^+$), 44 (100, $[CH_2OCH_2]^+$).

7. *General Procedure for DMT Protection of the Diol.* The diol was dissolved in dry CH_2Cl_2 and dry Et_3N (2.5 equiv.), and the mixture was flushed with Ar. DMT-Cl (1.2 equiv.), dissolved in dry CH_2Cl_2 and dry Et_3N (0.5 equiv. in relation to DMT-Cl), was added dropwise under vigorous stirring. The mixture was stirred at r.t. under Ar overnight, before the reaction was quenched with H_2O. The org. phase was dried ($MgSO_4$), filtered, and concentrated under reduced pressure, before the residue was purified by dry CVC (SiO_2; 1% Et_3N *v*/*v*, 0–50% AcOEt in cyclohexane) to afford the desired DMT-protected alcohol.

8. *2-{3-[(3-{[3-({2-[Bis(4-methoxyphenyl)phenylmethoxy]ethoxy}methyl)phenyl]ethynyl}phenyl)ethynyl]benzyloxy}ethanol* (**4**). Yield: 38%. Yellow oil. ^{1}H-NMR: 3.28 (*t*, *J* = 5.1, C*H*$_2$O-DMT); 3.61 (*t*, *J* = 4.5, OC*H*$_2$CH$_2$OH); 3.69 (*t*, *J* = 5.1, OC*H*$_2$CH$_2$O-DMT); 3.76 (*s*, 2 MeO); 3.76–3.80 (*m*, C*H*$_2$OH); 4.57 (*s*, $C_6H_4CH_2O$); 4.61 (*s*, $C_6H_4CH_2O$); 6.80, 6.83, 7.19–7.70 (*m*, 21 arom. H). ^{13}C-NMR: 55.30, 55.31 (2 *C*H$_2$O); 62.1 (*C*H$_2$OH); 63.5 (*C*H$_2$O-DMT); 70.1 (*C*H$_2$CH$_2$O-DMT); 71.6 (*C*H$_2$CH$_2$OH); 72.7 ($C_6H_4CH_2O$); 72.9 ($C_6H_4CH_2O$); 86.1 (Ar_3C); 88.7, 88.9, 89.9, 90.2 (2 *C*≡*C*); 113.2, 123.2, 123.3, 123.7, 123.8, 126.8, 127.7, 127.9, 128.4, 128.6, 128.6, 128.7, 130.2, 130.7, 130.8, 131.0, 131.1, 131.4, 131.5, 134.8, 136.5, 138.5, 139.2, 145.2, 158.5 (36 arom. C). HR-MALDI-MS: 751.3012 ($[M+Na]^+$, $C_{49}H_{44}NaO_6^+$; calc. 751.3036).

9. *2-[3-({3-[4-(3-{[3-({2-[Bis(4-methoxyphenyl)phenylmethoxy]ethoxy}methyl)phenyl]ethynyl}phenyl)buta-1,3-diynyl]phenyl}ethynyl)benzyloxy]ethanol* (**9**). Yield: 43%. Yellow oil. ^{1}H-NMR: 3.29–3.30 (*m*, C*H*$_2$O-DMT); 3.60–3.63 (*m*, C*H*$_2$OH); 3.67–3.71 (*m*, C*H*$_2$CH$_2$OH); 3.76 (*s*, 2 MeO); 3.76–3.80 (*m*, C*H*$_2$CH$_2$O-DMT); 4.57, 4.61 (*s*, 2 $C_6H_4CH_2O$); 6.82 (*d*, *J* = 7.1, 4 H, DMT); 7.25–7.69 (*m*, 25 arom. H). ^{13}C-NMR: 55.1 (2 MeO); 61.9 (*C*H$_2$OH); 63.3 (*C*H$_2$O-DMT); 70.0 (*C*H$_2$CH$_2$O-DMT); 71.5 (*C*H$_2$CH$_2$OH); 72.5, 72.7 (2 $C_6H_4CH_2O$); 74.3 (C≡*C*–*C*≡C); 80.8, 80.9, 88.1, 88.3 (2 C_6H_4–*C*≡*C*–C_6H_4); 86.0 (Ar_3C); 90.1, 90.4 (*C*≡C–C≡*C*); 113.0, 126.7, 127.6, 127.7, 127.8, 130.1, 136.3, 144.1, 158.4 (DMT); 122.0, 122.1, 123.8, 123.8, 128.2, 128.4, 128.6, 128.6, 130.6, 130.7, 130.8, 131.0, 132.1, 132.2, 132.3, 135.4, 138.4, 139.1 (4 C_6H_4). HR-MALDI-MS: 875.3323 ($[M+Na]^+$, $C_{59}H_{48}NaO_6^+$; calc. 875.3349).

10. *2-[3-({3-[(3-{[3-({2-[Bis-(4-methoxyphenyl)phenylmethoxy]ethoxy}methyl)phenyl]ethynyl}phenyl)ethynyl]phenyl}ethynyl)benzyloxy]ethanol* (**13**). Yield: 46%. Yellow oil. ^{1}H-NMR: 3.28–3.31 (*m*, C*H*$_2$O-DMT); 3.61–3.78 (*m*, C*H*$_2$OH, C*H*$_2$CH$_2$OH, C*H*$_2$CH$_2$O-DMT); 3.76 (*s*, 2 MeO); 4.57, 4.61 (*s*, 2 $C_6H_4CH_2O$); 6.82 (*d*, *J* = 6.8, 4 H, DMT); 7.29–7.71 (*m*, 25 arom. H). ^{13}C-NMR: 55.2 (2 MeO); 61.9 (*C*H$_2$OH); 63.3 (*C*H$_2$O-DMT); 70.0 (*C*H$_2$CH$_2$O-DMT); 71.5 (*C*H$_2$CH$_2$OH); 72.6, 72.8 (2 $C_6H_4CH_2O$); 86.0 (Ar_3C); 88.6, 89.2, 90.1 (*C*≡*C*); 113.1, 126.7, 127.5, 130.1, 136.3, 145.1, 158.4 (DMT); 123.0, 123.2, 123.3, 123.4, 123.6, 123.7, 127.8, 128.3, 128.4, 128.5, 128.5, 130.5, 130.7, 130.9, 131.0, 131.3, 131.4, 131.4, 131.5, 132.0, 132.2, 134.6, 138.4, 139.0 (C_6H_4). HR-MALDI-MS: 851.3369 ($[M+Na]^+$, $C_{57}H_{48}NaO_6^+$; calc. 851.3349).

11. *General Procedure for the Formation of Phosphoramidites.* The alcohol and diisopropylammonium tetrazolide (1.5 equiv.) were dissolved under Ar in dry CH_2Cl_2 or dry MeCN, followed by dropwise addition of 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphordiamidite (2.1 equiv.) *via* a syringe at 0°. The mixture was stirred under Ar at r.t. overnight, before the reaction was quenched with H_2O, and the org. phase was washed with H_2O. After back-extraction with CH_2Cl_2, the combined org. phases were dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The residue was purified by dry CVC (SiO_2; Et_3N 0.05% (*v/v*), 0–100% AcOEt in cyclohexane) to afford the desired phosphoramidite.

12. *1-{2-[(2-cyanoethoxy)(diisopropylamino)phosphinoxy]ethoxy}-3-iodobenzene* (**2**). Yield: 25%. Clear oil. ^{13}C-NMR: 20.3, 20.4 (*C*H_2CN); 24.5, 24.6, 24.6, 24.7 (2 *Me*$_2$CH); 43.0, 43.1 (2 Me$_2$*C*H); 58.3, 58.6 (O*C*H_2CH$_2$CN); 62.5, 62.8 (O*C*H_2CH$_2$OP); 70.4, 70.5 (CH$_2$*C*H_2OP); 72.2, (C_6H_4*C*H_2O); 117.6 (*C*N); 94.3, 126.6, 130.1, 136.4, 136.6, 104.7 (C_6H_4). ^{31}P-NMR: 149.0. HR-MALDI-MS: 501.0761 ([*M* + Na]$^+$, $C_{18}H_{28}IN_2NaO_3P^+$; calc. 501.0780).

13. *1-[(3-{2-[(2-Cyanoethyl)(diisopropyl)phosphinoxy]ethoxy}benzyloxy)ethynyl]-3-[(3-{2-[bis(4-methoxyphenyl)phenylmethoxy]ethoxy}benzyloxy)ethynyl]benzene* (**5**). Yield: 45%. Yellow oil. ^{13}C-NMR: 20.3, 20.4 (*C*H_2CN); 24.5, 24.6, 24.6, 24.7 (2 *Me*$_2$CH); 43.0, 43.2 (2 Me$_2$*C*H); 55.1 (2 MeO); 58.3, 58.6 (O*C*H_2CH$_2$CN); 62.6, 62.8 (O*C*H_2CH$_2$OP); 63.3 (*C*H_2O-DMT); 70.0 (*C*H_2CH$_2$O-DMT); 70.3, 70.4 (CH$_2$*C*H_2OP); 72.57, 72.63 (2 C_6H_4*C*H_2O); 86.0 (Ar_3*C*); 88.5, 88.6, 89.9, 90.0 (2 C_6H_4–*C*≡*C*–C_6H_4); 113.0, 126.6, 127.5, 127.7, 127.7, 130.1, 136.3, 145.1, 158.4 (DMT); 123.0, 123.5, 123.6, 128.2, 128.4, 128.4, 130.5, 130.7, 130.7, 130.8, 131.2, 131.3, 134.6, 138.7, 139.0 (3 C_6H_4). ^{31}P-NMR: 149.6.

14. *1-{3-[(3-{2-[Bis(4-methoxyphenyl)phenylmethoxy]ethoxy}benzyloxy)ethynyl]phenyl}-4-{3-[(3-{2-[(2-cyanoethoxy)(diisopropyl)phosphinoxy]ethoxy}benzyloxy)ethynyl]phenyl}buta-1,3-diyne* (**10**). Yield: 51%. Yellow oil. ^{13}C-NMR: 20.2, 20.4 (*C*H_2CN); 24.5, 24.6, 24.7 (2 *Me*$_2$CH); 43.0, 43.2 (2 Me$_2$*C*H); 55.2 (2 MeO); 58.3, 58.7 (O*C*H_2CH$_2$CN); 62.6, 62.9 (O*C*H_2CH$_2$OP); 63.3 (*C*H_2O-DMT); 70.0 (*C*H_2CH$_2$O-DMT); 70.3, 70.4 (CH$_2$*C*H_2OP); 72.6, 72.6 (2 C_6H_4*C*H_2O); 74.4 (C≡*C*–*C*≡C); 81.0, 88.2 (C_6H_4–*C*≡*C*–C_6H_4); 86.0 (Ar_3*C*); 90.3 (*C*≡C–C≡*C*); 117.6 (CN); 113.1, 126.7, 127.6, 127.7, 130.1, 136.3, 145.1, 158.4 (DMT); 122.0, 122.1, 122.9, 123.8, 123.9, 128.2, 128.4, 128.6, 128.6, 130.6, 130.7, 130.8, 130.8, 132.1, 132.3, 132.3, 135.4, 138.7, 139.1 (4 C_6H_4). ^{31}P-NMR: 149.7.

15. *1-{3-[(3-{2-[Bis(4-methoxyphenyl)phenylmethoxy]ethoxy}benzyloxy)ethynyl]phenyl}-2-{3-[(3-{2-[(2-cyanoethoxy)(diisopropyl)phosphinoxy]ethoxy}benzyloxy)ethynyl]phenyl}ethyne* (**14**). Yield: 50%. Yellow oil. ^{13}C-NMR: 20.3, 20.4 (*C*H_2CN); 24.5, 24.6, 24.6, 24.7 (2 *Me*$_2$CH); 43.0, 43.2 (2 Me$_2$*C*H); 55.1 (2 MeO); 58.3, 58.6 (O*C*H_2CH$_2$CN); 62.6, 62.9 (O*C*H_2CH$_2$OP); 63.3 (*C*H_2O-DMT); 70.0 (*C*H_2CH$_2$O-DMT); 70.3, 70.4 (CH$_2$*C*H_2OP); 72.6, 72.6 (2 C_6H_4*C*H_2O); 86.0 (Ar_3*C*); 88.5, 88.5, 89.1, 89.2, 90.0, 90.1 (*C*≡*C*); 113.0, 126.7, 127.5, 127.7, 127.8, 130.1, 136.3, 145.1, 158.4 (DMT); 123.0, 123.3, 123.4, 123.6, 123.7, 128.2, 128.4, 128.5, 128.5, 128.5, 130.6, 130.7, 130.8, 130.8, 131.3, 131.3, 131.4, 131.5, 134.6, 138.7, 139.0 (4 C_6H_4). ^{31}P-NMR: 149.6.

16. *Molecular Modelling.* Molecular modelling was performed with Macro Model v9.1 from *Schrödinger*. All calculations were conducted with AMBER* force field and the GB/SA water model. A parameter for the single bond in the diacetylene was added to the AMBER* force field using the same binding constants as for the C≡C bond but with a bond length of 1.3837 Å [25][26]. The dynamic simulations were performed with stochastic dynamics, a SHAKE algorithm to constrain bonds to H-atoms, time step of 1.5 fs and simulation temp. of 300 K. Simulation for 0.5 ns with an equilibration time of 150 ps generated 250 structures, which were minimized using the PRCG method with convergence threshold of 0.05 kJ/mol. The minimized structures were examined with Xcluster from *Schrödinger*, and representative low-energy structures were selected. The starting structures were generated with Insight II v97.2 from MSI, followed by incorporation of the modified nucleotide.

17. *Synthesis and Purification of Modified Oligonucleotides.* Modified oligonucleotides were synthesized in a 0.2-μmol or 1-μmol scale for postsynthesis on 500-Å CPG supports using *Expedite Nucleic Acid Synthesis System* model *8909* (*Applied Biosystems*). Standard procedures were used for the coupling of commercial phosphoramidites, whereas modified phosphoramidites, **2**, **5**, **10**, and **14**, were coupled with 1*H*-tetrazole as an activator and an extended coupling time (10 min). ONs were cleaved from the CPG support with 32% aq. NH_3 (1.2 ml) and deprotected at 55° overnight. **ON1**–**ON5** were purified with *NAP™ 10* columns from *GE Healthcare Bio-Sciences AB* before RP-HPLC. Purification of ONs was carried out on semiprep. RP-HPLC on a *Waters Xterra MS C18* column (10 μm, 7.8 × 150 mm).

ON6 and **ON7** were desalted with *NAP™ 10* columns from *GE Healthcare Bio-Sciences AB*. **ON8**–**ON13** were submitted to DMT-deprotection with 80% aq. AcOH (100 µl) for 20 min, followed by addition of H_2O (100 µl) and 3M aq. AcONa (50 µl), before precipitation from 99.9% EtOH (550 µl) at $-18°$. The purity of the obtained ONs was checked by ion-exchange chromatography on a *LaChrom* system (*Merck Hitachi*) using a *GenPak-Fax* column (*Waters*). Verification was achieved by MALDI-TOF analysis on a *Voyager Elite Bio* spectrometry research station (*Perspective Biosystems*).

18. *Postsynthesis.* In the case of **ON1**–**ON3**, a fresh mixture of $Pd(PPh_3)_4$ (22 µmol), CuI (26 µmol), and 1,3-diethynylbenzene (38 µmol) in dry DMF/Et_3N (350 µl/150 µl) was prepared in a 1-ml syringe, which was flushed with Ar prior use. The CPG support was flushed with Ar before the syringe containing the *Sonogashira* reagent mixture was attached to one end, and an empty 1-ml syringe was attached to the other end. The CPG support was treated several times with the reagent mixture every 45 min. After a coupling time of 3 h, the CPG support was washed with dry DMF (2×2 ml) and dry MeCN (2×2 ml), before it was dried with a flow of Ar. In the case of **ON4** and **ON5**, the CPG support was treated with an equal mixture as used for **ON1**–**ON3** but without the use of Ar. In the case of **ON6** and **ON7**, the postsynthesis were carried out in a similar way as for **ON1**–**ON3**, using a fresh mixture of $Pd(PPh_3)_4$ (22 µmol), CuI (26 µmol), and 1,3-diethynylbenzene (38 µmol) in dry DMF/Et_3N (350 µl/150 µl). The CPG supports were treated again under the same conditions and with equiv. reagent mixtures with the exception of 1,3-diethynylbenzene and the use of Ar in the case of **ON7**.

19. *Melting-Temperature Measurements.* T_m Measurements were performed on a *Perkin-Elmer Lambda 35* UV/VIS spectrometer with a *PTP 6* thermostat and *Perkin-Elmer* Templab 2.00 Software. Triplexes were formed by mixing 1.0 µM of each ssDNA and 1.5 µM of the TFO in the corresponding buffer soln. The solns. were heated to 80° for 5 min, and afterward cooled to 5° and kept at this temp. for 30 min. The absorbance of triplexes was measured at 260 nm from 5 to 80° with a heating rate of 1.0°/min or 0.5°/min. The T_m [°] values were determined as the maximum of the first-derivative plots of the melting curves. All T_m values are within the uncertainty $\pm 0.5°$ as determined by repetitive experiments.

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