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Design of a Ribosyltriazole-Annulated Cyclooctyne for Oligonucleotide Labeling by Strain-Promoted Alkyne–Azide Cycloaddition

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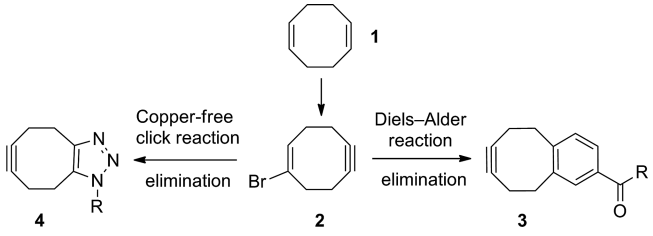
Keywords: Solid-phase synthesis / Oligonucleotides / Click chemistry / Conjugation / Dyes/Pigments

A ribosyltriazole ring-fused cyclooctyne was prepared and converted into the corresponding phosphoramidite, which was applied in the automated synthesis of DNA and RNA oligomers. Ensuing strain-promoted alkyne–azide cycloaddition of the obtained oligonucleotides to fluorescent azides yielded the corresponding fluorescent oligonucleotide conjugates.

Introduction

Thanks to the introduction[1] of strain-promoted[2] variants of the alkyne to azide Huisgen [2+3] dipolar cycloaddition, commonly referred to as the copper-free click reaction, (bio)molecules of large structural diversity and complexity can be connected without additives and under aqueous conditions. Following their initial discovery, several groups have focused their efforts on the development of cyclooctyne reagents of increased reactivity.[7–9] An important step forward comprised the recent discovery by van Delft and co-workers[10] of a readily available, relatively polar and highly reactive bicyclononyne click reagent.[3] In another recent instalment to the field, Varga et al.[4] reported copper-free click ligations with monobenzocyclooctyne **3** (Scheme 1), which was obtained starting from cyclooctadiene through a Diels–Alder reaction. Both of these recent approaches start from cyclooctadiene, a cheap and readily available material that is easily transformed into useful cyclooctyne derivatives for effective bioconjugation processes. Altogether, the merits of the strain-promoted alkyne–azide cycloaddition (SPAAC) have furthered the field of chemical biology[5] and in particular have made a major impact on the synthesis of complex bioconjugates.[6–8] For example, phosphoramidite derivatives have been developed to install a dibenzocyclooctyne or bicyclononyne moiety at either the 5′ or 3′ terminus of oligonucleotides and within the backbone of DNA and RNA fragments.[9–13] These cyclooctyne-derivatized oligonucleotides were subsequently conjugated to a wide variety of azide-containing (bio)-molecules such as oligosaccharides, oligopeptides, proteins, and fluorophores. Additionally, the copper-free click approach was applied in oligonucleotide crosslinking[14] and ligation[15] reactions, in which the two oligonucleotides were derivatized with an azide and a dibenzocyclooctyne, respectively. The versatility of cyclooctadiene as a starting compound en route to cyclooctynes inspired us and led to the design of triazole-fused cyclooctyne **4**. We reasoned that the annulated triazole moiety would increase both the polarity and the strain of the cyclooctyne ring compared to **3**, which would contribute to its solubility and reactivity, respectively.



Scheme 1. Literature[11] synthesis of monobenzocyclooctyne **3** and synthesis of triazole-annulated cyclooctyne **4** that is subject of this study.

Results and Discussion

We aimed to access cyclooctyne **4** in a two-step procedure by means of SPAAC followed by elimination (Scheme 1). A strategy employing cycloocta-1,5-diyne seemed unattractive in our case, as it was recently reported that monosubstitution of cyclooctadiynes through SPAAC is difficult to achieve for small molecules, and it has been only successful if applied to the fluorescent labeling of proteins under dilute conditions.[16] We herein describe the synthesis of ribosyl triazole annulated cyclooctyne **14**

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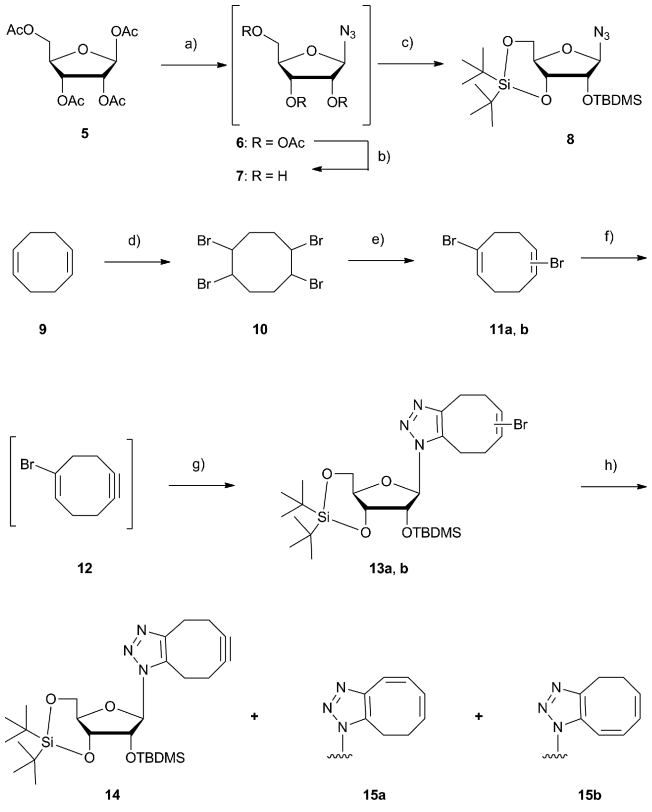
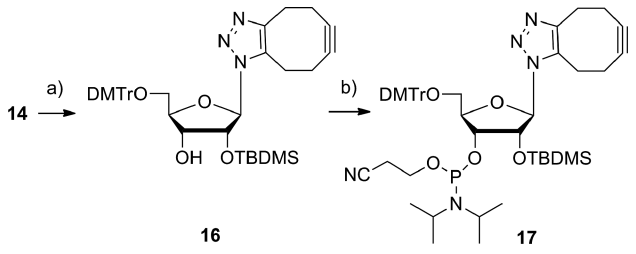
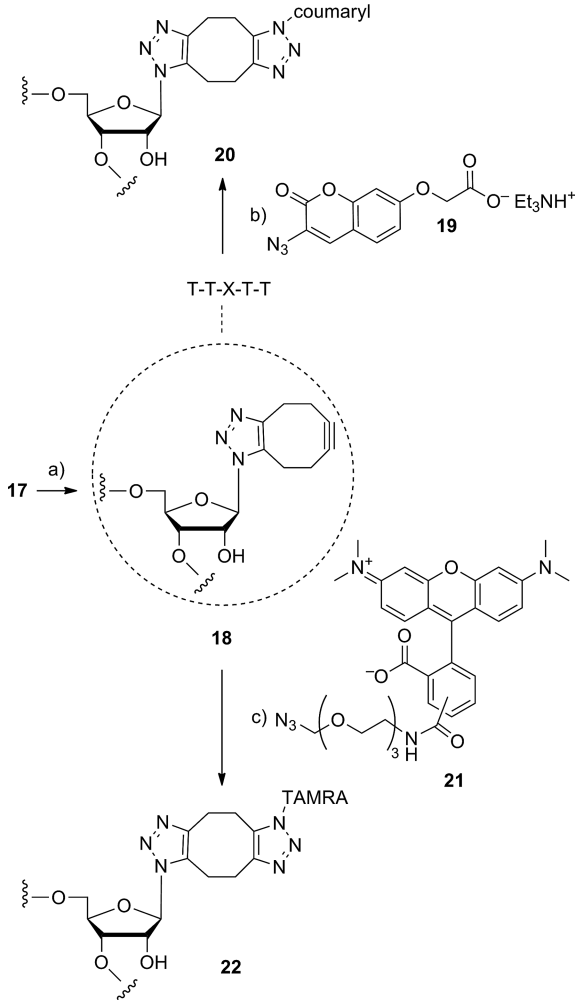
(Scheme 2), corresponding phosphoramidite **17** (Scheme 3), and incorporation of the latter into oligonucleotides as a nucleoside substitute. Phosphoramidite **17** was applied in the fully automated synthesis of a 13-mer RNA hetero-oligomer. This RNA fragment, as single-strand oligomer and as RNA–DNA duplex, was subsequently rendered fluorescent through strain-promoted click reaction with an azide-modified coumarin. The synthesis of ribosyl triazole annulated cyclooctyne **14** started with the conversion of commercially available β-1,2,3,5-tetra-*O*-acetyl-D-ribose (**5**) into fully protected azide **8** through a three-step reaction sequence (Scheme 2).[17] Treatment of **5** with trimethylsilyl azide and $SnCl_4$ stereoselectively yielded β-ribosyl azide **6**. Subsequent saponification of the acyl protective groups provided unprotected ribosyl azide **7**. Next, in a one-pot procedure, crude **7** was reprotected with two different silylating reagents to give protected ribose derivative **8**. According to a reported procedure,[18] bis(vinyl) bromides **11a** and **11b** were synthesized by bromination of cycloocta-1,4-diene (**9**) to yield tetrabromides **10**. Subsequent elimination by using KO*t*Bu yielded the mixture of regioisomeric dibromides **11a** and **11b**. Treatment of this mixture with potassium *tert*-butoxide at room temperature in hexanes yielded labile bromocyclooctyne **12**, which was found to deteriorate upon evaporation of the solvents. However, crude **12** proved to be sufficiently stable in solution to allow the [2+3] dipolar cycloaddition with anomeric azide **8** to proceed. With an estimated fourfold excess amount of crude cyclooctyne **12**, azide **8** was fully converted into the triazole-fused bromooctane as a mixture of two regioisomers, **13a** and **13b**, which were separable by silica gel column chromatography. Initially, the mixture of regioisomeric vinyl



Scheme 2. Reagents and conditions: (a) $TMSN_3$, $SnCl_4$, CH_2Cl_2. (b) NaOMe, MeOH. (c) $tBu_2Si(CF_3SO_3)_2$, DMF, then imidazole, TBDMSCl, 60 °C, 61% (3 steps, a, b and c). (d) Br_2, CH_2Cl_2, –78 °C, 66%. (e) KO*t*Bu, Et_2O, –78 °C to r.t., 72%. (f) KO*t*Bu, 18-crown-6, hexane. (g) **8**, hexane/MeCN/EtOAc, 96%. (h) KO*t*Bu, 18-crown-6, hexane, 23% (45% based on recovered **13**); TBDMS = *tert*-butyldimethylsilyl.



Scheme 3. Reagents and conditions: (a) HF·pyridine, pyridine/CH_2Cl_2, 0 °C, then DMTrCl, pyridine, 0 °C, 93% over two steps. (b) 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, 50%; DMTr = 4,4′-dimethoxytrityl, DIPEA = *N*,*N*-diisopropylethylamine.



Scheme 4. Reagents and conditions: (a) Solid-phase oligonucleotide synthesis. (b) 3-Azidocoumarin carboxylic acid **19**, H_2O. (c) TAMRA-N_3 (**21**), H_2O.

bromides **13a** and **13b** was used in the elimination reaction with KO*t*Bu.

TLC/MS analysis showed formation of the alkyne product co-eluting with the higher running regioisomer of the starting bromides (i.e., **13b**). Additionally, TLC analysis revealed formation of two less polar products as well as putative degradation products observed as baseline material. The two high-running side products could be isolated and were tentatively assigned by ^{1}H NMR spectroscopy as dienes **15a** and **15b**. To minimize the formation of side products, qualitative optimization experiments were performed, and they showed that optimal results were obtained by using short reaction times (<30 min) at room temperature. Although under these conditions both degradation and diene formation were suppressed, the reaction at this point did not proceed to completion. To avoid the isolation of an inseparable mixture, the most polar regioisomer (i.e., **13a**) was used in the ensuing elimination reaction to yield target alkyne **14** in 23% yield (45% yield based on recovered starting material).

En route to phosphoramidite building block **17** (Scheme 3), the silylidene protecting group was selectively[19] removed by treatment with pyridine·HF in pyridine/CH_2Cl_2 to yield the corresponding 3′,5′-diol that was converted into cyclooctyl riboside **16** by selective introduction of the DMTr protecting group. Subsequent phosphitylation of the 3′-hydroxy group with 2-cyanoethyl *N*,*N*-diisopropylaminochlorophosphoramidite yielded target phosphoramidite **17**.

The compatibility of triazolylcylcooctyne building block **17** with the conditions normally used in automated solid-phase oligonucleotide synthesis, that is, iodine, concentrated aqueous NH_4OH, and neat Et_3N·HF, were evaluated by the synthesis of oligonucleotide thymidine pentamer **18** (Scheme 4). Successful isolation of oligomer **18** demonstrated that the triazole cyclooctyne was stable under such solid-phase synthesis conditions.

Next, to assess the application of the triazole-fused cyclooctyne in the copper-free click reaction, pentamer **18** was conjugated with fluorescent label tetramethylrhodamine (TAMRA-N_3) **21** and 3-azidocoumarin **19**, which becomes fluorescent upon cycloaddition. As observed by HPLC analysis, both cycloadditions were approximately 95% complete after 1 h by using a small excess amount of the azide (Figure 1).

In the same vein, RNA 13-mer (ON-23) containing the cyclooctyne modification in the middle of the sequence was successfully synthesized (Scheme 5). Also, the copper-free click reaction proceeded uneventfully by subjection of oligomer ON-23 to an excess amount of azidocoumarin **19**, which resulted in fast conversion into labeled oligomer ON-26. The propensity of the triazole-annulated cyclooctyne moiety to function as a modified nucleobase was an incentive to explore whether the copper-free click reaction would also proceed in an oligonucleotide duplex. To this end, the solid-phase synthesis of DNA fragment ON-25 complementary to the cyclooctyne-containing RNA 13-mer (i.e., ON-23) was undertaken.

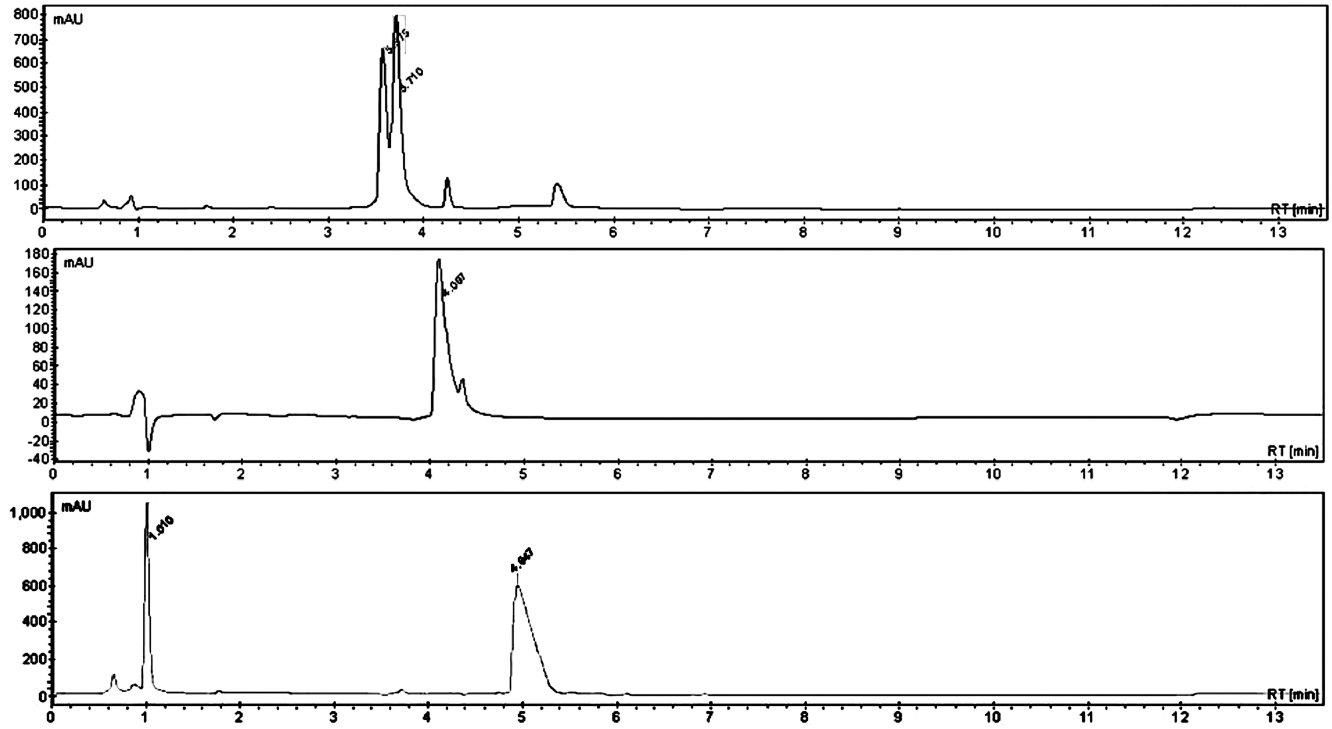
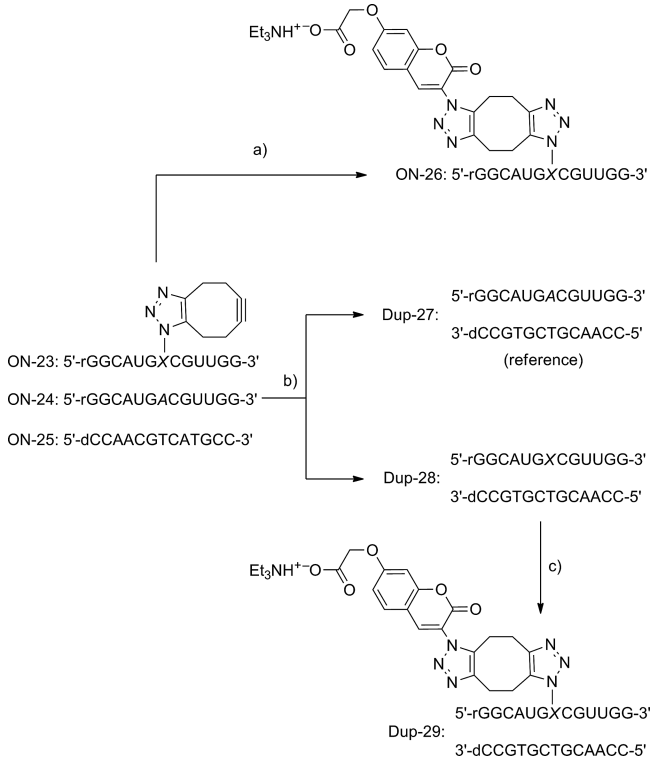


Figure 1. LC traces of the copper-free click reaction of pentamer **18** and azidocoumarin **20**. From top to bottom: reaction mixture after 1 h (**20**), oligomer **18**, azidocoumarin **19**.



Scheme 5. Reagents and conditions (a) 2-[(3-Azido-coumaryl-7-yl)-oxy] acetic acid (**19**), phosphate-buffered saline pH 7.3 (0.1 M/0.2 M). (b) Phosphate-buffered saline pH 7.3 (0.1 M/0.2 M). (c) 2-[(3-Azido-coumaryl-7-yl)oxy]acetic acid (X represents the ribosyl triazole cyclooctyne modification or the corresponding clicked adduct).

After mixing equal quantities of the RNA 13-mer (i.e., ON-23) and the complementary DNA 13-mer (i.e., ON-25) duplex Dup-28 was formed, and it was subjected to an excess amount of azidocoumarin **19** (Figure 2). LC–MS analysis of the reaction mixture revealed a rate of conversion similar to the one found with the single-stranded RNA (i.e., ON-23), and after 3 h, complete conversion of RNA–DNA duplex Dup-28 into labeled duplex Dup-29 was observed. LC analysis of the modified duplex was executed under denaturing HPLC (Figure 2) conditions, by which the signals for the DNA and modified RNA strand could be observed as partially resolved. This allowed the extraction of the corresponding MS information. Modified RNA ON-26 (m/z = 1491.0 $[M + 3H]^{3+}$) and DNA ON-25 (m/z = 1294.8 $[M + 3H]^{3+}$) oligomers were detected, whereas the absence of the signal for starting ON-23 (m/z = 1404 $[M + 3H]^{3+}$) indicated complete conjugation.

To assess the structural characteristics of our triazolyl-cyclooctyne building block in the RNA–DNA duplex, circular dichroism measurements were performed. In all three spectra, that is, those of the native (Dup-27), modified (Dup-28), and clicked duplex (Dup-29), the characteristic absorption profile for A helices of the DNA–RNA-type was observed.[20,21] To gauge the stability of the triazole cyclooctyne containing duplex (i.e., Dup-28) and the corresponding duplex containing the clicked adduct (i.e., Dup-29), UV-melting measurements were also performed. The melting temperature (T_m) values for Dup-28 (43 °C) and Dup-29 (40 °C) decreased by 15 and 18 °C, respectively, relative to that of unmodified duplex Dup-27 (58 °C). The similar T_m values of both modified duplexes before and after the addition of the coumaryl fluorophore suggests an outward rotation of the triazolo-cyclooctyne moiety, likely as a result of steric bulk, which thereby destabilizes the duplex. The bulge-out of the triazole cyclooctyne modification might explain its reactivity in the copper-free click reaction, as we observed no difference in the conversion rate of the duplex compared to that of the single-stranded RNA oligonucleotide.

Conclusions

In summary, we present a new conjugation handle based on a ribosyl triazole annulated cyclooctyne as a nucleoside substitute that can be incorporated through a standard so-

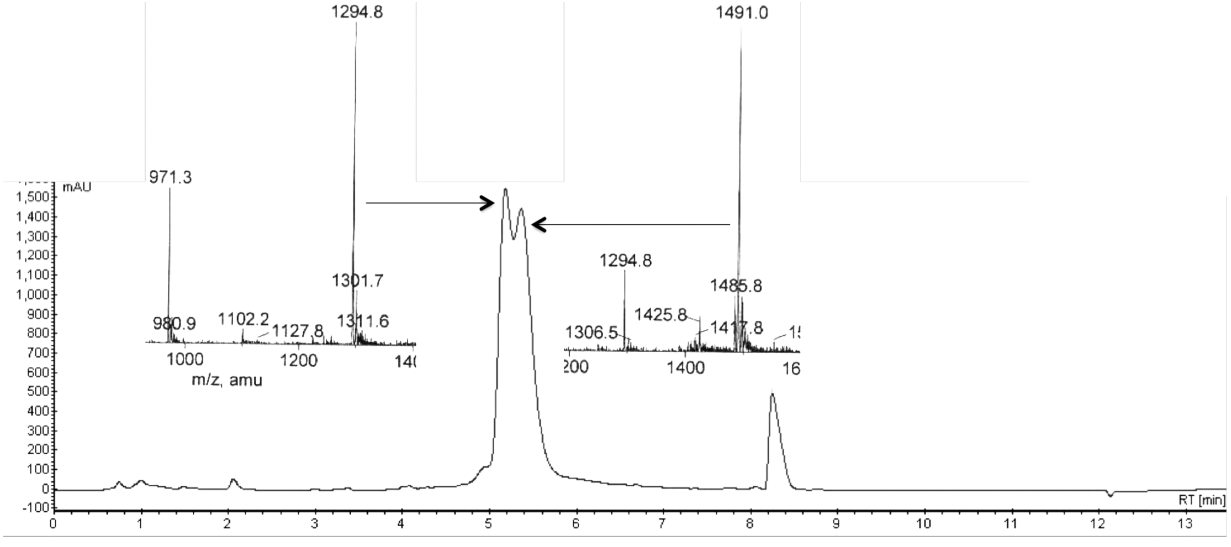


Figure 2. LC trace of Dup-29 and the extraction of the corresponding MS spectrum.

lid-phase synthesis approach at any position in oligonucleotides. The protected cyclooctyne-modified nucleoside and the corresponding phosphoramidite were prepared by a straightforward route of synthesis. A cyclooctyne-modified RNA 13-mer was assembled and fluorescently labeled in both the single-stranded and duplex forms by strain-promoted alkyne–azide cycloaddition with azidocoumarin. In the RNA–DNA hybrids, the stability before and after cycloaddition was essentially the same, but it was lower than that of the native duplex. The efficiency of the addition reaction on the duplex was found to be similar to that of the labeling of the single-stranded RNA. Additionally, the ready availability of azides and the ease of preparation of the ribosyl triazole annulated cyclooctyne described herein will be an incentive for broader application of other triazole-annulated cyclooctynes.

Experimental Section

1-[1,2-*O*-(*tert*-Butyldimethylsilyl)-3,5-*O*-(di-*tert*-butylsilanediyl)-β-D-ribofuranosyl]-7-bromo-6,7-didehydro-4,5,8,9-tetrahydro-1*H*-cycloocta[*d*][1,2,3]triazole and 1-[1,2-*O*-(*tert*-Butyldimethylsilyl)-3,5-*O*-(di-*tert*-butylsilanediyl)-β-D-ribofuranosyl]-6-bromo-6,7-didehydro-4,5,8,9-tetrahydro-1*H*-cycloocta[*d*][1,2,3]triazole (13a + 13b): 18-Crown-6 (661 mg, 5 mmol, 0.5 equiv.) and KO*t*Bu (4.49 g, 40 mmol, 4 equiv.) were added to a mixture of compounds **11a** and **11b** (2.66 g, 10 mmol) dissolved in dry *n*-hexane. The solution turned into a brown suspension upon the addition of KO*t*Bu. The mixture was stirred for 3.25 h. The reaction was quenched by the addition of saturated aqueous NH_4Cl (130 mL) and H_2O (100 mL). The layers were separated, and the organic layer was washed (2×) with saturated aqueous NH_4Cl (100 mL) and H_2O (100 mL). Compound **8** (1.05 g, 2.45 mmol, 0.25 equiv.) was added to the organic layer; acetonitrile (100 mL) and EtOAc (50 mL) were then added, and the mixture was stirred overnight. The mixture was concentrated in vacuo to a volume of 150 mL and was then washed with H_2O (50 mL). The organic layer was dried with Na_2SO_4, filtered, and concentrated on Celite in vacuo. The crude product was purified by automated silica gel column chromatography (EtOAc/pentane, 3:97–20:80), which yielded the two regioisomers as a thick oil and a crystalline solid [1.43 g, 2.34 mmol, 96% (2 steps)]. Data for **13a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.05 (t, J = 8.3 Hz, 1 H), 5.54 (s, 1 H), 5.04 (d, J = 4.6 Hz, 1 H), 4.48 (dd, J = 9.7, 4.6 Hz, 1 H), 4.35 (dd, J = 9.1, 5.1 Hz, 1 H), 4.18 (td, J = 10.1, 5.1 Hz, 1 H), 3.88 (dd, J = 10.3, 9.3 Hz, 1 H), 3.19–3.15 (m, 2 H), 3.01–2.87 (m, 3 H), 2.87–2.75 (m, 1 H), 2.65–2.55 (m, 2 H), 1.06 (s, 9 H), 1.02 (s, 9 H), 0.88 (s, 9 H), 0.13 (s, 3 H), 0.11 (s, 3 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 143.4, 132.6, 126.6, 128.8, 91.9, 76.3, 75.3, 74.9, 67.8, 39.2, 27.5, 27.0, 25.9, 25.0, 24.8, 22.8, 22.6, 20.3, 18.3, –4.3, –5.2 ppm. IR (neat): $\tilde{\nu}$ = 2933, 2858, 1472, 1249, 1124, 1081, 1052, 1002, 950, 900, 828 cm^{-1}. HRMS: calcd. for $C_{27}H_{49}BrN_3O_4Si_2$ [M + H]$^+$ 614.24450; found 614.24439. Data for **13b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.99 (t, J = 6.0 Hz, 1 H), 5.60 (s, 1 H), 5.00 (d, J = 4.6 Hz, 1 H), 4.50 (dd, J = 9.6, 4.6 Hz, 1 H), 4.36 (dd, J = 9.0, 5.1 Hz, 1 H), 4.19 (td, J = 10.1, 5.1 Hz, 1 H), 3.93–3.82 (m, 1 H), 3.22–3.07 (m, 3 H), 3.07–2.85 (m, 3 H), 2.52–2.42 (m, 2 H), 1.06 (s, 9 H), 1.02 (s, 9 H), 0.89 (s, 9 H), 0.13 (s, 3 H), 0.11 (s, 3 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 143.5, 132.6, 122.0, 131.5, 92.0, 76.3, 75.3, 75.0, 67.9, 35.0, 29.2, 27.5, 27.0, 25.9, 24.0, 22.6, 22.1, 20.3, 18.3, –4.3, –5.2 ppm. IR (neat): $\tilde{\nu}$ = 2933, 2859, 1472, 1254, 1171, 1130, 1054, 1002, 896, 828 cm^{-1}. HRMS: calcd. for $C_{27}H_{49}BrN_3O_4Si_2$ [M + H]$^+$ 614.24450; found 614.24445.

1-[1,2-*O*-(*tert*-Butyldimethylsilyl)-3,5-*O*-(di-*tert*-butylsilanediyl)-β-D-ribofuranosyl]-6,7-dehydro-4,5,8,9-tetrahydro-1*H*-cycloocta[*d*][1,2,3]triazole (14): The lower running isomer of compound **13** [TLC: EtOAc/petroleum ether, 10:90; R_f = 0.34 and 0.54] (615 mg, 1 mmol) was co-evaporated with 1,4-dioxane (3×), kept under an atmosphere of argon, and dissolved in *n*-hexane (30 mL). 18-Crown-6 (159 mg, 0.6 mmol, 0.6 equiv.) was co-evaporated with 1,4-dioxane (3×), kept under an atmosphere of argon, and dissolved in dry *n*-hexane (50 mL). KO*t*Bu (337 mg, 3 mmol, 3 equiv.) was added, and a solution of **13** was added to the resulting suspension. The mixture turned brown and was stirred for 25 min. The mixture was diluted with EtOAc (80 mL), washed with water (2 × 65 mL), washed with brine (65 mL), dried with $MgSO_4$, filtered, and concentrated on Celite. Silica gel column chromatography (EtOAc/petroleum ether, 4:96–4.75:95.25) afforded starting material compound **13** (272 mg, 0.44 mmol, 44%) and title compound **14** as a thick oil (125 mg, 0.23 mmol, 23%, 45% based on recovered **13**). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.63 (s, 1 H), 5.19 (d, J = 4.5 Hz, 1 H), 4.49 (dd, J = 9.6, 4.6 Hz, 1 H), 4.34 (dd, J = 9.0, 5.1 Hz, 1 H), 4.20 (dd, J = 10.1, 5.1 Hz, 1 H), 3.91–3.82 (m, 1 H), 3.27–3.04 (m, 4 H), 2.49–2.19 (m, 4 H), 1.07 (s, 9 H), 1.03 (s, 9 H), 0.89 (s, 9 H), 0.14 (s, 3 H), 0.11 (s, 3 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 145.5, 134.5, 98.1, 95.1, 92.1, 76.4, 75.4, 74.7, 67.8, 29.2, 27.5, 27.1, 26.4, 25.9, 22.6, 20.3, 19.7, 18.3, –4.3, –5.1 ppm. IR (neat): $\tilde{\nu}$ = 3444, 2028, 1472, 1053, 835, 827 cm^{-1}. HRMS: calcd. for $C_{27}H_{48}N_3O_4Si_2$ [M + H]$^+$ 534.31834; found 534.31732.

Supporting Information (see footnote on the first page of this article): Synthetic procedures, full compound characterization, and spectroscopic data for compounds **8**, **13**, **14**, **16**, and **17**; ^{1}H NMR spectroscopic data for **15**; solid-phase synthesis details, LC–MS, and HRMS analysis of all oligonucleotides; and circular dichroism and UV-melting measurements for duplexes Dup-27, Dup-28, and Dup-29.

Acknowledgments

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[21] See Figures S1 and S2 in the Supporting Information for the UV melting curves and the circular dichroism spectra of Dup-27, Dup-28, and Dup-29.

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