Universiteit
Leiden
The Netherlands

Synthetic Studies Towards Oligonucleotide Derivatives and Conjugates

Delft, P. van

Citation

Delft, P. van. (2013, September 3). *Synthetic Studies Towards Oligonucleotide Derivatives and Conjugates*. Retrieved from https://hdl.handle.net/1887/21632

Version:	Not Applicable (or Unknown)
License:	Leiden University Non-exclusive license
Downloaded from:	https://hdl.handle.net/1887/21632

Note: To cite this publication please use the final published version (if applicable).

Cover Page

The handle http://hdl.handle.net/1887/21632 holds various files of this Leiden University dissertation.

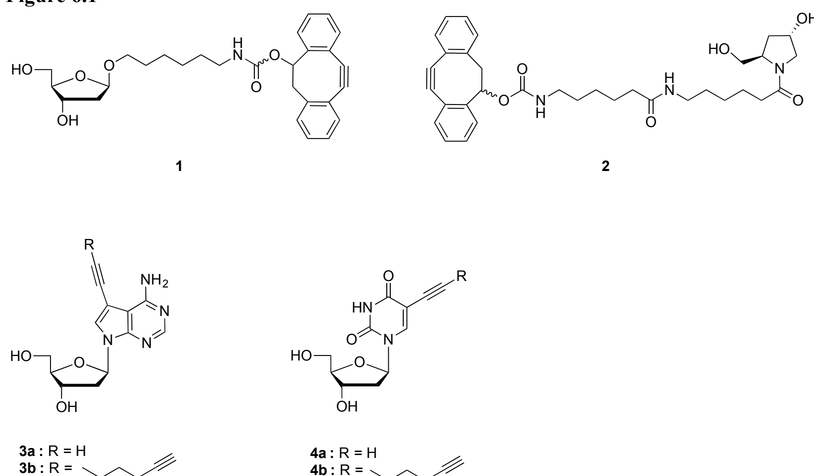
Author: Delft, Pieter van
Title: Synthetic studies towards oligonucleotide derivatives and conjugates
Issue Date: 2013-09-03

6 RITRICO, a Purine Derived Cyclooctyne for Copper Free Click Chemistry in a RNA-DNA Duplex

3.1 Introduction

The previous chapters have demonstrated the use of cyclooctyne derivatives in synthetic oligonucleotide conjugation. Based on the dibenzocyclooctyne reported by Boons *et al*[1] several phosphoramidites were designed, synthesized and appended on the 3'- and 5'-ends of synthetic DNA and RNA oligomers. These oligomers were all efficiently labeled with a variety of azide functionalized compounds including fluorescent labels, oligopeptides, oligosaccharides and proteins. In addition, the synthesis of in-strand modifier **1** was described in Chapter 3. The phosphoramidite derived from riboside **1** proved to be useful to incorporate the strained dibenzocyclooctyne moiety at any position of an oligonucleotide leaving a "clickable" abasic site.

Figure 6.1

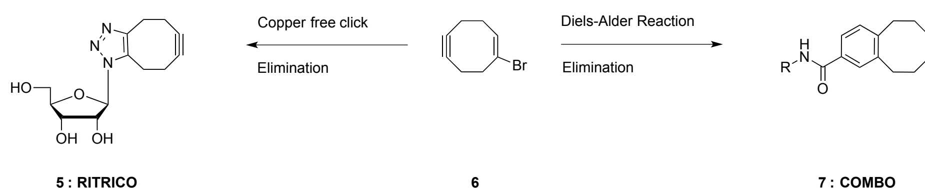


A similar strategy using a 3-hydroxy proline based in-strand modifier **2** was published by others[2]. In both structures **1** and **2,** the cyclooctyne entity is appended to the oligonucleotide by means of a spacer moiety. The use of such linkers is generally applied to avoid negative steric interactions between the functional group appended (in this case the octyne) and its conjugation counterpart contributing to improved conversion(rates).
Reactive handles attached via a spacer to the nucleobase are also employed. For instance in the field of copper catalyzed click chemistry, alkynes are introduced on either the purine or pyrimidine[3, 4]. The need for a spacer entity to allow efficient labeling in single stranded DNA seems largely dependent on the amount of conjugation handles incorporated.[5] In oligonucleotide duplexes the influence of the handles and spacers are more complex. The

position of a modification on the nucleobase is important and ideally, the reaction handle should point outward the major groove to facilitate conjugation. Hence C-7 alkyne modified 7-deaza-purines **3**[6] and the more straightforward C-5 modified 2'-deoxy uridines **4**[7,8,9] have been used (Figure 6.1)[10]. Besides the conjugation reaction itself, the structural change and stability of the modified duplex are also of interest. With modifications residing in the major groove, the duplex perturbation is kept to a minimum. Additionally, Watson and Crick hydrogen bonding is left unaffected which is beneficial for the duplex stability.

In order to explore the use of small cyclooctyne derivatives as substitute of a purine nucleobase, ribosyl triazole annulated cyclooctyne **5** (RITRICO, Scheme 6.1) was designed.

Scheme 6.1: design of D-ribosyl triazole annulated cyclooctyne **5**.

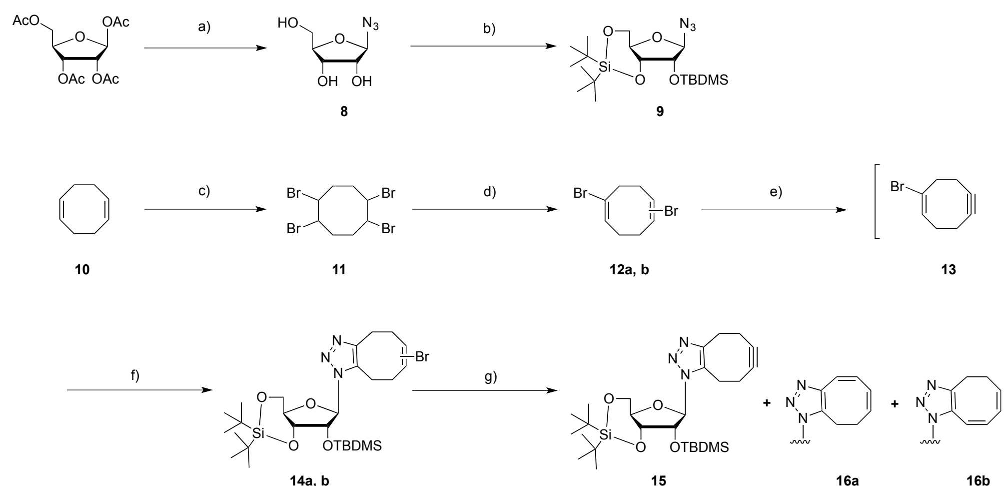


It was recently shown[11] that monobenzocyclooctyne **7** showed exceptionally fast reaction rates in strain promoted cycloadditions. This indicated that triazole derivative **5** would also show favorable kinetics in cycloaddition reactions with azides given the structural similarities.
This chapter describes the synthesis of a RITRICO phosphoramidite building block and its application in the fully automated synthesis of a modified DNA 5-mer and a RNA13-mer sequence. Subsequently the reactivity of RITRICO, incorporated both in single strand oligonucleotides and in an RNA-DNA hybride duplex, towards azides was investigated. The structure of the modified hybride duplex and its labeled product was assessed using circular dichroism spectroscopy.

Results and Discussion

Protected azide **8** was obtained in a four step sequence starting from commercially available β-1,2,3,5-tetra-*O*-Acetyl-D-ribose.[12] The latter was treated with TMS-azide and $SnCl_4$ as previously described to stereoselectively introduce the azide moiety at the anomeric position. Saponification of the acyl protective groups yielded the crude unprotected ribosylazide **8** which was then fully silylated[13]. Bis-vinylbromide **12** was synthesized as described[14] starting with bromination of cycloocta-1,4-diene (**10**) to yield tetrabromides **11**. Subsequent elimination using KOtBu yielded a mixture of regioisomeric dibromides **12a** and **12b**.

Scheme 6.2 : Synthesis of a ribose triazole fused cyclooctyne.



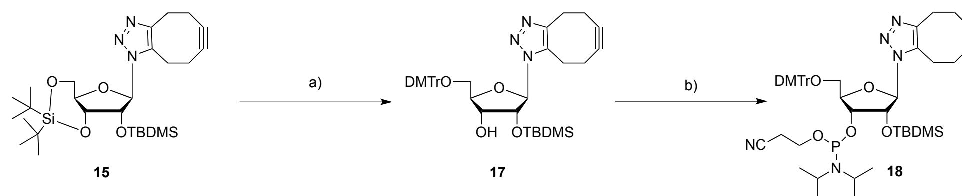
Reagents and conditions : *a)* TMS-N_3, $SnCl_4$, DCM then NaOMe, MeOH *b)* *t*-$Bu_2Si(CF_3SO_3)_2$, DMF then Imidazole, TBDMS-Cl, 60 °C, 61% (4 steps, *a* and *b*). *c)* Br_2, DCM, -78 °C, 66%. *d)* KOtBu, Et_2O, -78 °C to r.t., 72%. *e)* KOtBu, 18-crown-6, hexane, yield n/a. *f)* **9**, hexane/MeCN/EtOAc, 96%. *g)* KOtBu, 18-crown-6, hexane, 45% (based on recovered 14).

Treatment of this mixture with potassium *tert*-butoxide at room temperature in hexanes yielded the labile bromocyclooctyne **13** that deteriorates upon concentration by the evaporation of solvents. Fortunately, crude **13** proved to be sufficiently stable in solution to allow the [2+3]-dipolar cycloaddition with anomeric azide **9** to proceed. With an estimated fourfold excess of crude cyclooctyne **13** added, the azide starting material was fully converted into triazole fused bromooctane **14** as a mixture of two regioisomers (**14a** and **14b**) which were separated by silica gel column chromatography.

Upon treatment of the mixture of regioisomeric vinylbromides **14a** and **14b** with KOtBu TLC/MS analysis showed formation of the alkyne product co-eluting with the higher running regioisomer of the starting bromide. Additionally, TLC analysis revealed formation of two high-running side products as well as base-line material. The two high-running byproducts could be isolated and were identified by NMR analysis as di-enes **16a** and **16b.** To minimize the side product formation, optimization experiments[15] were performed showing that best results are obtained using short reaction times (< 30 minutes) at room temperature. Although under these conditions both degradation and di-ene formation were suppressed the reaction at this point did not proceeded to completion. To avoid the isolation of an inseparable mixture the most polar region isomer (**14)** was used in the ensuing elimination reaction yielding the target alkyne **15** in 45% based on recovered starting material.[16]

Next, the silylidene protective group was selectively removed by treatment with pyridine•HF in pyridine/DCM[13] yielding the corresponding 3',5'-diol that was converted into partially protected cyclooctylriboside **17** by dimethoxytritylation of the 5'-hydroxyl. Subsequent phosphitylation of the free 3'-hydroxyl gave target phosphoramidite **18** in a moderate yield.

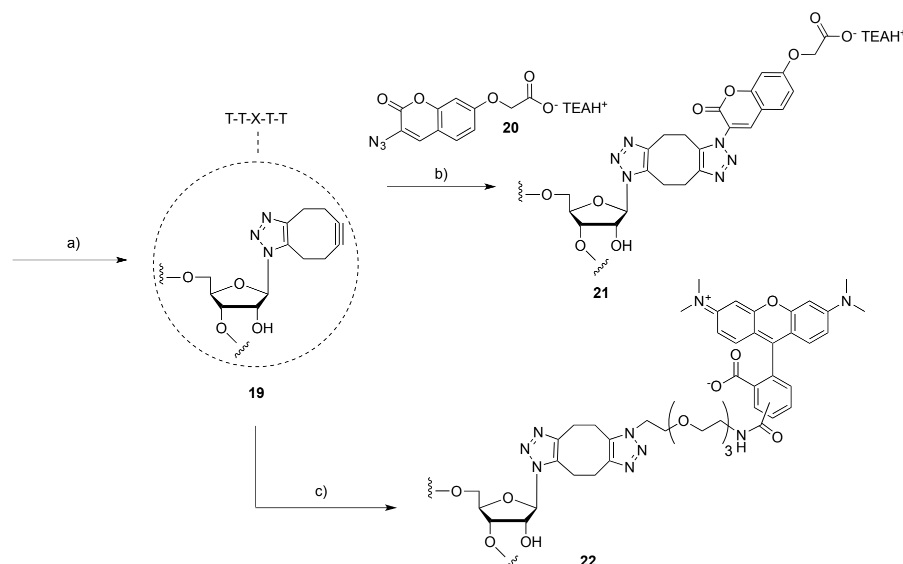
Scheme 6.3 : Conversion of protected ribose-cyclooctyne **15** into phosphoramidite building block **18**.



Reagents and conditions : *a*) HF·pyridine, pyridine/DCM, 0 °C then DMT-Cl, pyridine, 0 °C, 93% over two steps. *b)* 2-cyanoethyl-*N,N*-diiso-propylamino-chlorophosphoramidite, DIPEA, DCM, 50%.

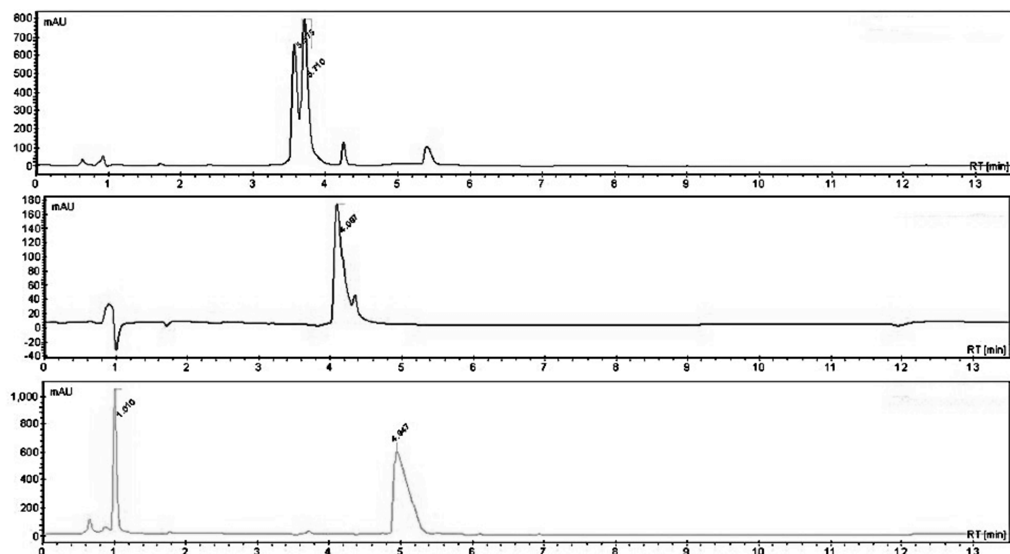
Adopting the synthetic procedures for fully automated DNA and RNA synthesis described in Chapter 2 and 3, pentanucleotide **19** (Scheme 6.4) was synthesized using the newly developed RITRICO phosphoramidite **18** the building block. The RITRICO moiety proved to be fully compatibility with the applied conditions of solid phase oligonucleotide synthesis. Next the viability in the copper free click reaction of RITRICO incorporated oligonucleotides was examined by reacting oligonucleotide **19** with azido-modified tetramethylrhodamine (TAMRA-N_3) and 3-azido-coumarine **20.**[17] The click reaction was found to be complete after 1 hour using a small excess of azide as monitored by HPLC analysis (Figure 6.2).

Scheme 6.4 : Incorporation of ribose cyclooctyne **18** in DNA 5-mer and its copper free click reactions with azides.

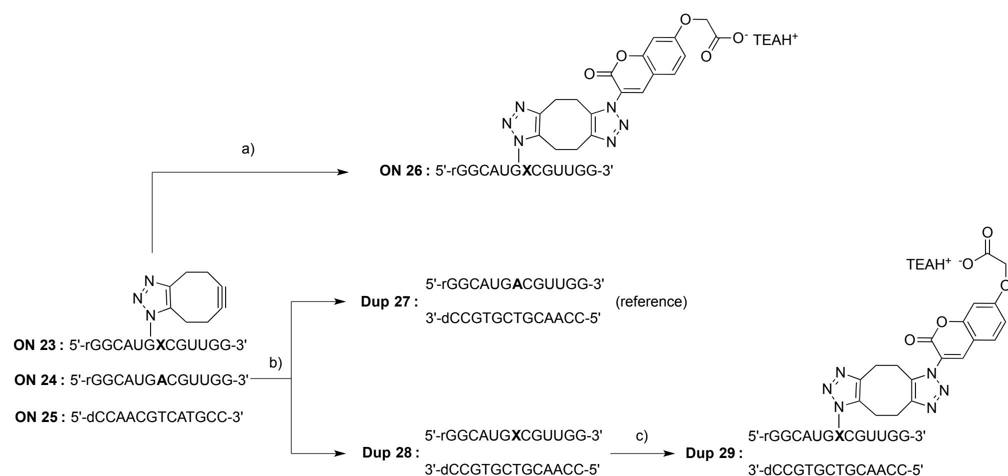


Reagents and conditions : *a)* solid phase oligonucleotide synthesis. *b)* **20**, H_2O. *c*) TAMRA-N_3, H_2O.

Figure 6.2 : LC traces of the copper free click reaction of pentamer **19** and azido-coumarine **20**. *From top to bottom: reaction mixture after 1 hour; oligomer **19**; azido-coumarine **20**.*



Scheme 6.5 : Synthesis of cyclooctyne modified RNA 13-mer and copper free click reaction with 3-azidocoumarine in single stranded and DNA heteroduplex form. (X represents the RITRICO modification)

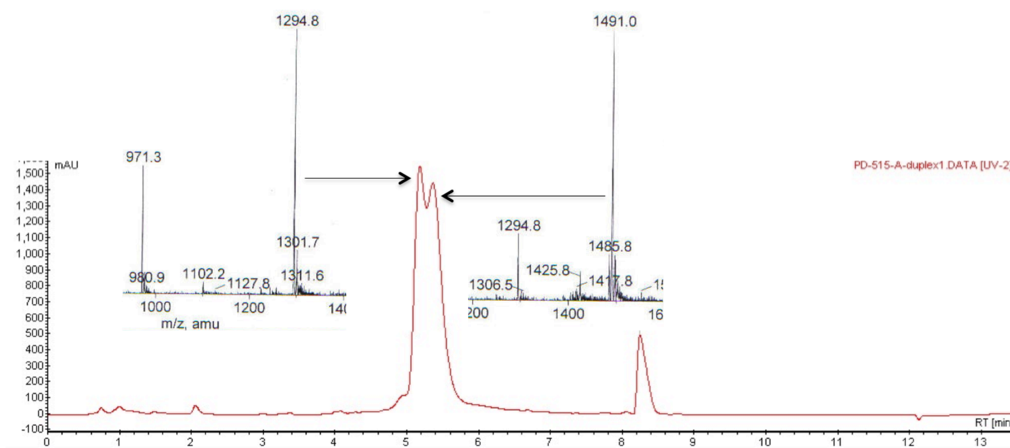


Reagents and conditions : *a)* 2-((3-azido-coumaryl-7-yl)oxy) acetic acid, phosphate buffer saline pH 7.3 (0.1 M/0.2 M). *b*) phosphate buffer saline pH 7.3 (0.1 M/0.2 M) *c)* 2-((3-azido-coumaryl-7-yl)oxy) acetic acid. (X represents the RITRICO modification)

Stimulated by these results, non-self-complementary RNA 13-mer (**ON 23**), having the cyclooctyne modification incorporated in the middle of the sequence, was successfully synthesized (Scheme 6.5). Subsequently oligomer **ON 23** was treated with an excess of azidocoumarine **20** resulting in fast conversion to the labeled oligomer **ON 26**.

Synthesis of the complementary DNA strand (**ON 24**) allowed the formation of a DNA-RNA duplex**.** Additionally, unmodified RNA strand (**ON 25**) was synthezised to be used in the formation of a reference duplex (**Dup 27**). Modified RNA 13-mer (**ON 26**) and the complementary DNA oligomer (**ON 25**) were mixed in equal amounts, forming **Dup 28,** which was subjected to an excess of azidocoumarine **20**. LC/MS analysis of the reaction mixture revealed reaction rates similar to those found with the single stranded RNA and after 3 hours, complete conversion of the RNA-DNA duplex to the labeled duplex **Dup 29** was observed. LC analysis of the modified duplex was executed under denaturing conditions and showed the DNA and modified RNA strand as partially resolved (Figure 6.3) thereby allowing the extraction of the corresponding MS information. The modified RNA **ON 27** ($[M + 3H]^{3+}$ = 1491.0) and DNA **ON 25** ($[M + 3H]^{3+}$ = 1294.8) were detected while the absence of starting **ON 23** ($[M + 3H]^{3+}$ = 1404) signal indicated complete conjugation.

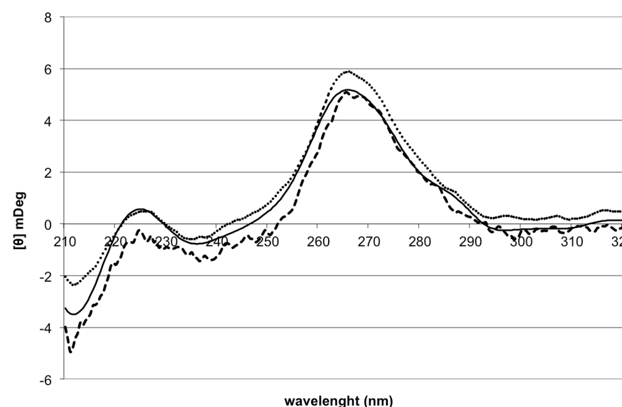
Figure 6.3 : LC trace of modified RNA-DNA duplex **Dup 29** and corresponding MS-spectrum extracts.



To determine the secondary structure of nucleic acids, circular dichroism (CD) spectroscopy is a widely used method due to its sensitivity and distinct spectral signals for the different secondary structures.[18] As a result the CD spectra of the secondary structures of most common forms of nucleic acid, including RNA-DNA hetero duplexes have been determined. It has been established that this latter type of duplex forms the right-handed A-form duplex. In CD spectroscopy this is observed by a characteristic positive peak slightly red-shifted from the 260 nm absorption maximum.[19]

In order to gauge the influence of both the RITRICO modification and its cycloaddition adduct on the secondary structures of the RNA-DNA duplexes, the corresponding circular dichroism (CD) spectra of **Dup 28 and Dup 29** as well reference duplex **Dup 27** were recorded. All three duplexes are characterized by the distinctive A-helix positive absorption peak around 265nm (figure 6.4).

Figure 6.4 : Circular dichroism spectra of reference duplex **Dup 27** (dotted line), cyclooctyne modified duplex **Dup 28** (solid line) and coumaryl derivatized duplex **Dup 29** (dashed line).



The base substitution appears to have a small effect on the nature of the helix. However upon cycloaddition there seems a larger perturbation of the helix conformation, in fact, the A-duplex character looks more defined. This can be deduced from the similarity of the CD spectrum of **Dup 29** with the CD spectra of an RNA-RNA homo duplex in the region between 210 – 240 nm.[19] Which is consistent with recent findings of Engels *et al.*[20] In their studies a spin label was appended to N-2 of adenosine on the RNA strand of a DNA RNA duplex and as a consequence A-like helices were observed.

Conclusion

A nucleoside mimic, named RITRICO, in which the nucleobase is replaced by a new bicyclic triazole annulated cyclooctyne was designed, synthesized and successfully incorporated in a DNA 5-mer and a RNA 13-mer. The RITRICO moiety incorporated in these single strand oligonucleotides permitted efficient [2+3] dipolar azide alkyne cycloadditions with an azide containing TAMRA label or a 3-azido coumarine derivative. A similar cycloaddition with 3-azido substituted coumarine proceeded equally efficient when the RITRICO modified RNA fragment was part of a RNA-DNA duplex. CD spectroscopy was used to resolve the secondary structures of the RNA-DNA duplexes before and after cycloaddition. A-form duplexes were formed which were influenced upon modification as a result of putative base pairing perturbation due to added steric bulk. The high reactivity, synthetic accessibility and compactness of RITRICO make it an attractive alternative for existing cyclooctynes.

Experimental section

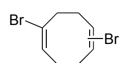
General methods and materials

Chemicals were purchased from Acros Organics, Sigma Aldrich, Proligo and Jena Bioscience and used as received. Dichloromethane was distilled over CaH_2 and stored on 4 Å molecular sieves. DIPEA was distilled and stored on KOH pellets. Compounds used in reactions requiring anhydrous conditions were co-evaporated with 1,4-dioxane, pyridine or toluene three times. All reactions were performed at ambient temperature under an argon atmosphere unless stated otherwise. Oligonucleotides were synthesized on an ÄKTA Oligopilot Plus oligonucleotide synthesizer (GE Healthcare Life Sciences). Reactions were monitored by TLC on Kieselgel 60 F254 (Merck). Compounds were visualized by using UV light (254 nm) or applying a solution of $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ 25 g/L, $(NH_4)_4Ce(SO_4)_4\cdot 2\ H_2O$ 10 g/L, 10% H_2SO_4 in H_2O followed by charring (+/- 150 °C). LC/MS analysis was performed on a Jasco HPLC system (UV detection simultaneously at 214 and 254 nm) coupled to a PE/SCIEX API 165 single quadruple mass spectrometer (Perkin-Elmer). An analytical Gemini C_{18} column (Phenomex, 50 x 4.60 mm, 3 micron) was used in combination with eluents A: H_2O; B: MeCN and C: 0.1 M aq. NH_4OAc as the solvent system. Analytical anion-exchange was performed on a GE ÄKTAexplorer 10 using a Dionex DNA-PAC PA-200 4x250 mm column with eluents A: 500 mM NaOAc and 50 mM $NaClO_4$ and B: 500 mM NaOAc and 500 mM $NaClO_4$ using a linear gradient (0 - 20%). Anion exchange purification was performed on a GE ÄKTAexplorer 10 using a GE Q-Sepharose HR 26 x 10 column with eluents A: 500 mM NaOAc and 50 mM $NaClO_4$ and B: 500 mM NaOAc and 500 mM $NaClO_4$ followed by a desalting procedure using a Sephadex G25 column with 150 mM NH_4OAc as the solvent system. Preperative RP HPLC was performed on a Gilson GX-281 HPLC system. A semipreparative Altima C_{18} column (Phenomex, 250 x 10 mm, 5 micron) was used in combination with eluents A: 50 mM aq. NH_4OAc and B: MeCN as the solvent system. ^{1}H, ^{13}C and ^{31}P NMR were recorded on a Bruker AV-400 instrument. Chemical shifts (δ) of ^{1}H and ^{13}C spectra are relative to tetramethylsilane. ^{31}P chemical shifts are relative to phosphoric acid, reaction mixture aliquots measured by means of an acetone-d6 capillary. $CDCl_3$ was neutralized by filtration over neutral Al_2O_3 (Merck). HRMS spectra were recorded by direct injection (2 µL of a µM solution in H_2O or MeCN and 0.1% formic acid) on a Thermo Finnigan LTQ Orbitrap equipped with an electro spray ion source in positive mode. IR spectra were recorded on a Shimadzu FT-IR 8300 and are reported in cm^{-1}. The yields of the oligonucleotides were determined specrophotometrically using Optical Density measurements on a Varian Cary 50 Bio UV-VIS Spectrophotometer at 260 nm. Circular Dichroism spectra were recorded on a Jasco J-810 CD spectrometer using 5 µM solutions at ambient temperature with a 2.0 mm optical pathway.

(1R,2R,5R,6R)-1,2,5,6-tetrabromocyclooctane + (1R,2R,5S,6S)-1,2,5,6-tetrabromocyclooctane (11)

1,5-Cyclooctadiene (12.3 mL, 100 mmol) was dissolved in dichloromethane (360 mL), molecular sieves were added and the solution was cooled to -78ºC. Bromine (11.53 mL, 225 mmol, 2.25 eq.) in dry dichloromethane (360 mL) was added dropwise via a dropping funnel. The mixture was stirred for 15 min at -78ºC, allowed to warm to r.t. and stirred overnight. The reaction was quenched with sat. aq. Na_2SO_3, the layers were separated and the organic layer was washed twice with H_2O. The organic layer was dried over $MgSO_4$, filtrated and concentrated *in vacuo*. The crude product was recrystalised from dichloromethane/n-hexane twice to obtain compound **11** as a mixture of two stereoisomers (**11a** and **11b**) as off-white crystals (28.1 g, 65.7 mmol, 66%).

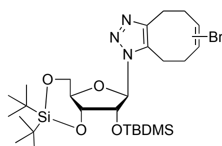
^{1}H NMR (400 MHz, $CDCl_3$) δ 4.76 (d, J = 4.0 Hz, 4H), 4.61 – 4.54 (m, 4H), 2.81 (d, J = 13.5 Hz, 4H), 2.61 – 2.49 (m, 4H), 2.48 – 2.34 (m, 4H), 2.12 (d, J = 12.7 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 58.31, 57.28, 31.42, 26.49. IR (neat) cm^{-1}: 2932; 1424; 1216; 994; 668.



(1E,5E)-1,6-dibromocycloocta-1,5-diene (3a) and (1E,5E)-1,5-dibromocycloocta-1,5-diene (12)

Compound **11** (12.83 g, 30 mmol) and KOtBu (13.47 g, 120 mmol, 4 eq.) were cooled to -78ºC while stirring. Dry diethyl ether was added and the mixture was allowed to warm to r.t. slowly and stirred for 40 h, almost full conversion was indicated by TLC. The reaction was quenched with sat. aq. NH_4Cl (200 mL), stirred vigorously and the layers were separated. The organic layer was washed with brine (200 mL), filtrated, separated again, dried over $MgSO_4$, filtrated and concentrated *in vacuo*. The crude product was distilled *in vacuo* (93 ºC, < 0.5 mbar) and the product was obtained as a thick oil which solidified upon standing at -20 ºC (5.75 g, 21.6 mmol, 72%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.09 (t, *J* = 7.1 Hz, 2H), 6.06 – 6.01 (m, 2H), 2.91 (s, 4H), 2.88 – 2.79 (m, 4H), 2.42 (q, *J* = 6.9 Hz, 4H), 2.37 – 2.29 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 129.8, 129.5, 124.4, 123.7, 38.2, 38.0, 27.6, 27.4. IR (neat) cm^{-1}: 3000; 2898; 2360; 1646; 1414; 856; 808.



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-1H-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-1H-cycloocta[d]-[1,2,3]triazole 14a + 14b

Compound **12** (2.66 g, 10 mmol) was dissolved in dry n-hexane, 18-crown-6 (661 mg, 5 mmol, 0.5 eq.) and KOtBu (4.49 g, 40 mmol, 4 eq.) were added. The solution turned into a brown suspension upon addition of KOtBu. The reaction mixture was stirred for 3.25 h. The reaction was quenched by the addition of sat. aq. NH_4Cl (130 mL) and H_2O (100 mL). The layers were separated and the organic layer was washed with sat. aq. NH_4Cl (100 mL) and H_2O (100 mL) twice. Compound **9** (1.05 g, 2.45 mmol, 0.25 eq.) was added to the organic layer, 100 mL acetonitrile and 50 mL EtOAc were added and the mixture was stirred overnight. The reaction mixture was concentrated *in vacuo* to a volume of 150 mL and washed with 50 mL H_2O. The organic layer was dried over Na_2SO_4, filtrated and concentrated on celite *in vacuo*. The crude product was purified by automated silica gel column chromatography (EtOAc/pentane 3/97-20/80) yielding the two isomers as a thick oil and a crystalline solid (1.43 g, 2.34 mmol, 96% (2 steps)).

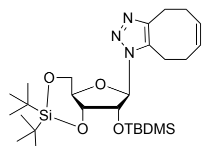
14a

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.05 (t, *J* = 8.3 Hz, 1H), 5.54 (s, 1H), 5.04 (d, *J* = 4.6 Hz, 1H), 4.48 (dd, *J* = 9.7, 4.6 Hz, 1H), 4.35 (dd, *J* = 9.1, 5.1 Hz, 1H), 4.18 (td, *J* = 10.1, 5.1 Hz, 1H), 3.88 (dd, *J* = 10.3, 9.3 Hz, 1H), 3.19-3.15 (m, 2H), 3.01 – 2.87 (m, 3H), 2.87 – 2.75 (m, 1H), 2.65 – 2.55 (m, 2H), 1.06 (s, 9H), 1.02 (s, 9H), 0.88 (s, 9H), 0.13 (s, 3H), 0.11 (s, 3H). ^{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. IR (neat) cm^{-1}: 2933; 2858; 1472; 1249; 1124; 1081; 1052; 1002; 950; 900; 828. HRMS calculated for $[C_{27}H_{48}BrN_3O_4Si_2 + H]^+$: 614.24450; found: 614.24439 $[M + H]^+$

14b

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.99 (t, *J* = 6.0 Hz, 1H), 5.60 (s, 1H), 5.00 (d, *J* = 4.6 Hz, 1H), 4.50 (dd, *J* = 9.6, 4.6 Hz, 1H), 4.36 (dd, *J* = 9.0, 5.1 Hz, 1H), 4.19 (td, *J* = 10.1, 5.1 Hz, 1H), 3.93 – 3.82 (m, 1H), 3.22 – 3.07 (m, 3H), 3.07

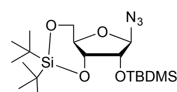
– 2.85 (m, 3H), 2.52 – 2.42 (m, 2H), 1.06 (s, 9H), 1.02 (s, 9H), 0.89 (s, 9H), 0.13 (s, 3H), 0.11 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.5, 132.6, 122.0, 131.5, 92.0, 76.3, 75.3, 75.0, 67.9, 35.0, 29.2, 27.5, 27.0, 25.9, 24.0, 22.6, 22.1, 20.3, 18.3, -4.3, -5.2. IR (neat) cm^{-1}: 2933; 2859; 1472; 1254; 1171; 1130; 1054; 1002; 896; 828. HRMS calculated for $[C_{27}H_{48}BrN_3O_4Si_2 + H]^+$: 614.24450; found: 614.24445 $[M + H]^+$



1-(1-2-O-(tert-butyldimethylsilyl)-3,5-O-(di-tert-butylsilanediyl)-β-D-ribofuranosyl)-4,5,8,9-tetrahydro-1H-cycloocta[d]-6-yn-[1,2,3]triazole (15)

The lower running isomer of compound **14** (TLC EtOAc/petroleum ether 10/90 Rf. 0.34 and 0.54) (615 mg, 1 mmol) was coevaporated with 1,4-dioxane three times, kept under argon and dissolved in 30 mL n-hexane. 18-crown-6 (159 mg, 0.6 mmol, 0.6 eq.) was coevaporated with 1,4-dioxane three times, kept under argon and dissolved in 50 mL dry n-hexane. KOtBu (337 mg, 3 mmol, 3 eq.) was added and the solution of compound **14** was added to the resulting suspension. The reaction mixture turned brown and was stirred for 25 min. The reaction mixture was diluted with EtOAc (80 mL), washed with water (2 x 65 mL), washed with brine (65 mL), dried over $MgSO_4$, filtrated and concentrated on celite. Silica gel column chromatography (EtOAc/petroleum ether 4/96-4.75/95.25) afforded starting material compound **14** (272 mg, 0.44 mmol, 44%) and the title compound **15** as a thick oil (125 mg, 0.23 mmol, 23%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.63 (s, 1H), 5.19 (d, J = 4.5 Hz, 1H), 4.49 (dd, J = 9.6, 4.6 Hz, 1H), 4.34 (dd, J = 9.0, 5.1 Hz, 1H), 4.20 (dd, J = 10.1, 5.1 Hz, 1H), 3.91 – 3.82 (m, 1H), 3.27 – 3.04 (m, 4H), 2.49 – 2.19 (m, 4H), 1.07 (s, 9H), 1.03 (s, 9H), 0.89 (s, 9H), 0.14 (s, 3H), 0.11 (s, 3H). ^{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. IR (neat) cm^{-1}: 3444; 2028; 1472; 1053; 835; 827. HRMS calculated for $[C_{27}H_{47}N_3O_4Si_2 + H]^+$: 534.31834; found: 534.31732 $[M + H]^+$



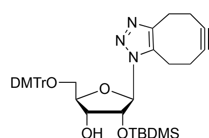
1-azido-2-O-(tert-butyldimethylsilyl)-3,5-O-(di-tert-butylsilanediyl)-β-D-ribofuranose (9)

1,2,3,5-Tetra-*O*-acetyl-β-D-ribose (4.77 g, 15 mmol) was dissolved in 30 mL dichloromethane. Trimethylsilyl azide (2.17 mL, 16.5 mmol, 1.1 eq.) was added and $SnCl_4$ (0.088 mL, 0.75 mmol, 0.05 eq.) in 15 mL dichloromethane was added slowly over 2 minutes. The reaction mixture was stirred overnight and quenched with $NaHCO_3$ (40 mL). The resulting layers were separated and the water layer was extracted with dichloromethane three times. The combined organic layers were dried over $MgSO_4$, filtrated and concentrated under reduced pressure.

The crude product was dissolved in 150 mL MeOH, NaOMe (810 mg, 15 mmol, 1 eq.) was added and the reaction mixture was stirred overnight. The reaction was quenched with Amberlite™ IR-120 H^+ (pH ~ 7), filtrated and concentrated *in vacuo*. The crude product was coevaporated with 1,4-dioxane three times and dissolved in 30 mL N,N-dimethylformamide. The solution was cooled to 0ºC and di-tert-butylsilyl bis(trifluoromethanesulfonate) (5.34 mL, 16.5 mmol) was added dropwise over 15 min and stirred for 30 min at 0 ºC. Imidazole (5.11 g, 75 mmol, 5 eq.) was added and the mixture was stirred for 5 min at 0 ºC. The mixture was allowed to warm to r.t. and stirred for 25 more min. *Tert*-butyldimethylchlorosilane (2.71 g, 18 mmol, 1.2 eq.) was added and the reaction mixture was stirred

overnight and concentrated *in vacuo*. The crude product was diluted with EtOAc and washed four times with $NaHCO_3$ and with brine. The second and third water layers were extracted with dichloromethane (2 x 50 mL). The title compound (3.91 g, 9.11 mmol, 61%) was obtained by silica gel column chromatography purification (Et_2O/petroleum ether 0.1/99.9-0.35/99.65).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.06 (s, 1H), 4.42 (dd, *J* = 9.0, 5.1 Hz, 1H), 4.12 (td, *J* = 10.2, 5.1 Hz, 1H), 4.01 (d, *J* = 4.1 Hz, 1H), 3.96 – 3.86 (m, 2H), 1.05 (s, 9H), 1.00 (s, 9H), 0.92 (s, 9H), 0.13 (s, 3H), 0.12 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 96.1, 76.2, 75.9, 74.5, 68.3, 27.5, 27.0, 25.8, 22.7, 20.3, 18.4, -4.4, -5.2. IR (neat) cm^{-1}: 2935; 2860; 2107; 1472; 1049; 826.

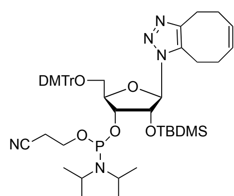


1'-(1'-2'-*O*-(tert-butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl)-4,5,8,9-tetrahydro-1*H*-cycloocta[d]-6-yn-[1,2,3]triazole (17)

Protected **15** (480 mg, 0.9 mmol) was taken up in anhydrous DCM and cooled (0 °C). A freshly prepared and cold (0 °C) solution solution of pyridine•HF (83 µL) in pyridine (0.54 mL) was added and the mixture was stirred for 90 minutes. TLC analysis revealed complete conversion of the starting material to a more polar compound and the mixture was diluted with DCM (50 mL). The organic layer was washed with H_2O (15 mL), sat aq $NaHCO_3$ (2 x 15 mL) and dried ($MgSO_4$). The organic layer was concentrated to half the volume, diluted with toluene and then further concentrated at 30 °C.

The concentrate was dissolved in cold (0 °C) pyridine (4 mL), DMT-Cl (436 mg, 0.99 mmol, 1.1 eq) and stirred o.n. at 0 °C. The reaction mixture was quenched with MeOH (0.5 mL), diluted with EtOAc (50 mL), washed with ice water (3 x 15 mL) and cold brine (15 mL) and dried (Na_2SO_4). The crude product was co-evaporated with toluene (2 x 100 mL) at 30 °C as described above prior to complete concentration. silica gel column chromatography (TEA pre-neutralized) purification (petroleum ether/ethyl acetate, 85 : 15 → 60 : 40) yielded the title compound as a white foam (580 mg, 0.83 mmol, 93% over two steps).

^{1}H NMR (400 MHz, CD_3Cl δ 7.43 (d, *J* = 7.5 Hz, 2H), 7.37 – 7.15 (m, 8H), 6.82 (dd, *J* = 8.8, 1.9 Hz, 4H), 5.76 (d, *J* = 5.0 Hz, 1H), 5.59 (t, *J* = 5.0 Hz, 1H), 4.37 – 4.35 (m, 1H), 4.30 – 4.19 (m, 1H), 3.82 – 3.76 (m, 6H), 3.37 (dd, *J* = 10.5, 3.4 Hz, 1H), 3.34 – 3.16 (m, 4H), 3.13 (dd, *J* = 10.5, 4.2 Hz, 1H), 2.82 (d, *J* = 3.9 Hz, 1H), 2.58 – 2.25 (m, 4H), 0.91 (s, 9H), 0.18 (s, 3H), 0.00 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.3, 145.6, 144.6, 135.8, 135.1, 130.0, 128.1, 127.7, 126.6, 113.0, 97.9, 95.4, 89.4, 86.2, 84.8, 74.5, 72.1, 63.6, 55.2, 29.2, 26.4, 25.6, 20.3, 19.7, 17.9, -5.0, -5.2. IR (neat) cm^{-1}: 3285; 2949; 2930; 1607; 1508; 1462; 1445; 1302; 1250. HRMS calculated for $[C_{40}H_{49}N_3O_6Si + H]^+$: 696.34634; found: 696.34705 $[M + H]^+$.



1’-(1’-3’-*O*-(2-cyanoethyl)-*N,N*-disopropylamino-(2’-*O*-(tert-butyldimethylsilyl)-5’-*O*-(4,4’-dimethoxytrityl)-β-D-ribofuranosyl-phosphoramidite)-4,5,8,9-tetrahydro-1*H*-cycloocta[d]-6-yn-[1,2,3]triazole (18)

To a stirred solution of protected alkyne **17** (580 mg, 0.84 mmol) in anhydrous DCM (8.4 mL) was added DIPEA (364 μL, 2.1 mmol, 2.5 eq) and (2-cyanoethyl)-*N,N*-di-isopropyl-chlorophoshoramidite (206 μL, 0.92 mmol, 1.2 eq). After 3 hours TLC analysis (EtOAc/petroleum ether) indicated full conversion of the starting material. This was assessed by taking an aliquot of the reaction mixture and dissolving it with DCI in wet MeCN since product and starting material run at the same hight. The reaction mixture was diluted with EtOAc (2% TEA, 40 mL) and washed with sat aq $NaHCO_3$ (3 x 10 mL), brine (10 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Silica gel column chromatography purification (Toluene / ethyl acetate / TEA, 8 : 2 : 0.2 → 75 : 25 : 0.2) yielded the title compound as a white foam (375 mg, 0.42 mmol, 50%).

^{1}H NMR (400 MHz, CD_3CN) δ 7.47 – 7.34 (m, 2H), 7.33 – 7.12 (m, 8H), 6.89 – 6.72 (m, 4H), 5.87 – 5.74 (m, 1H), 5.41 – 5.29 (m, 1H), 4.64 – 4.45 (m, 1H), 4.40 – 4.20 (m, 1H), 3.97 – 3.76 (m, 2H), 3.72 (s, 7H), 3.69 – 3.49 (m, 3H), 3.47 – 3.29 (m, 2H), 3.23 (t, J = 5.4 Hz, 2H), 3.15 (t, J = 5.2 Hz, 2H), 3.06 – 2.93 (m, 1H), 2.76 – 2.55 (m, 1H), 2.49 – 2.34 (m, 2H), 2.34 – 2.28 (m, 2H), 1.13 (d, J = 6.8 Hz, 8H), 0.99 (d, J = 6.8 Hz, 4H), 0.83 (s, 9H), 0.08 (m, 3H), -0.03 (m, 3H). ^{13}C NMR (101 MHz, CD_3CN) δ 160.05, 146.91, 146.56, 146.49, 137.42, 137.30, 137.21, 137.07, 137.04, 131.55, 129.53, 129.47, 129.25, 129.21, 128.16, 119.91, 119.60, 114.51, 114.46, 99.10, 97.26, 97.18, 90.59, 90.42, 87.55, 87.45, 85.27, 85.23, 76.42, 76.39, 76.01, 75.96, 74.47, 74.37, 74.33, 74.19, 73.40, 71.90, 64.59, 64.50, 60.54, 60.36, 59.82, 59.62, 56.36, 44.66, 44.54, 44.33, 44.21, 30.28, 30.24, 27.48, 27.41, 26.75, 26.70, 25.69, 25.62, 25.50, 25.44, 21.63, 21.57, 21.52, 21.34, 20.98, 20.86, 19.19, 1.79, -3.72, -4.10. IR (neat) cm^{-1}: 2961; 2930; 1607; 1508; 1248; 1177; 1034. HRMS calculated for $[C_{49}H_{66}N_5O_7PSi + H]^+$: 896.45419; found: 896.45569 $[M + H]^+$.

5’-T-T-(RITRICO)-T-T-3’ (19)

Standard DNA solid phase was performed on a 5 μM scale using a polystyrene thymidine pre-loaded resin, commercially availably thymidine phosphoramidite building block and amidite **18** in the third coupling cycle. Cleavage from the solid support was performed using conc. aq. NH_4OH for 1 hr at room temperature. The 2’ silyl protective group was cleaved o.n. by treatment of 3 mL neat Et_3NHF. Concentratation and subsequent purification on RP-HPLC yielded the title oligonucleotide. (1.3 μmol).

LC/MS: (MeCN : 10 mM aq NH_4OAc 5 → 50 v/v) retention time: 4.09 min. HRMS calculated for $[C_{53}H_{69}N_{11}O_{32}P_4 + H]^+$:1496.31333; found: 1496.31615 $[M + H]^+$.

5’-T-T-(RITRICO-TAMRA)-T-T-3’ (22)

To a solution of pentamer **19** (250 nmol) in H_2O (17 μL) was added a solution of TAMRA-N_3 (100 nmol) in H_2O (10 μL). The mixture was shaken for 1 hour. LC/MS analysis showed full conversion of all TAMRA label to the

TAMRA-nucleic acid oligomer conjugate. The remaining pentamer **19** was converted by a second addition of azide (150 nmol) in H_2O. The reaction mixture was evaluated after 8 hours showing disappearance of all TAMRA label and starting pentamer. HPLC purification isolated the two regio-isomers as a mixture.

LC/MS: (MeCN : 10 mM aq NH_4OAc 5 → 50 v/v) retention time: 4.89 min (ESI-MS: 1064.5 $[M + 2 H^+]^{2+}$) and 5.25 (ESI-MS: 1064.5 $[M + 2 H^+]^{2+}$). HRMS calculated for $[C_{86}H_{107}N_{17}O_{39}P_4 + 2 H]^{2+}$:1063.80040; found: 1063.80164 $[M + 2 H]^{2+}$.

5’-T-T-(RITRICO-Coumaryl)-T-T-3’ (21)

Pentamer **19** (200 nmol) in H_2O (55 µL) and 3-azido-7-(oxy-acetic acid)-coumarine triethylammonium salt **20** (200 nmol) in H_2O/EtOH (1 : 2, 15 µL) were shaken for 1 hour. LC/MS analysis revealed complete conversion of all starting oligonucleotide into two more polar products. HPLC purification isolated the two regio-isomers as a mixture.

LC/MS: (MeCN : 10 mM aq NH_4OAc 5 → 50 v/v) retention time: 3.57 min (ESI-MS: 879.7 $[M + 2 H]^{2+}$) and 3.71 (ESI-MS: 879.7 $[M + 2 H]^{2+}$). HRMS: Calculated for $[C_{64}H_{76}N_{14}O_{37}P_4 + NH_4]^+$:1774.37845; found: 1774.38007 $[M + NH_4]^+$. HRMS calculated for $[C_{64}H_{76}N_{14}O_{37}P_4 + H]^+$:1758.35488; found: 1758.35699 $[M + H]^+$.

5'-dCCAACGTCATGCC-3' (ON 25)

Standard DNA synthesis protocols were employed employed on a 10 µmol scale using commercially available phosphoramidites dA(Bz), dC(Ac), dG(iBu) and T. Cleavage and deprotection of the protective groups was accomplished by treatment with conc. aq. NH_4OH over night at 50 °C. Purification on a Source Q column as described in the general section followed by desalting on HPLC yielded the DNA-13 mer (1 µmol, OD).

LC/MS: (MeCN : 10 mM aq NH_4OAc 00 → 25 v/v) retention time: 5.75 min (ESI-MS: 1294.8 $[M + 3 H]^{3+}$) and HRMS calculated for $[C_{124}H_{159}N_{47}O_{75}P_{12} + 3 H]^{3+}$: 1293.90774 ; found: 1293.90564 $[M + 3 H]^{3+}$.

5’-rGGCAUGACGUUGG-3’ (ON 24)

Standard RNA synthesis protocols were employed employed on a 10 µmol scale using commercially available phosphoramidites A(tAc), C(Ac), G(tAc) and U. Capping was performed using a solution of phenoxyacetic acid anhydride solution in THF. Cleavage and deprotection of the acyl protective groups was accomplished by treatment with conc. aq. NH_4OH (10 mL) at room temperature for 2 hours. 2’ Silyl protective groups were removed using neat triethyl ammonium hydrogen fluoride (4 mL) at room temperature over night. Purification on a Source Q column as described in the general section followed by desalting on HPLC yielded the DNA-13 mer (0.85 µmol, OD).

LC/MS: (MeCN : 10 mM aq NH_4OAc 00 → 25 v/v) retention time: 5.37 min (ESI-MS: 1400.3 $[M + 3 H]^{3+}$) and HRMS calculated for $[C_{125}H_{154}N_{52}O_{90}P_{12} + 3 H]^{3+}$: 1399.54092 ; found: 1399.53894 $[M + 3 H]^{3+}$.

5'-rGGCAUG(X)CGUUGG-3' (ON 23)

Standard RNA synthesis protocols were employed on a 10 µmol scale using commercially available phosphoramidites A(tAc), C(Ac), G(tAc), U and **18**. Capping was performed using a solution of phenoxyacetic acid anhydride solution in THF. Cleavage and deprotection of the acyl protective groups was accomplished by treatment with conc. aq. NH_4OH (10 mL) at room temperature for 2 hours. 2' Silyl protective groups were removed using neat triethyl ammonium hydrogen fluoride (4 mL) at room temperature over night. Purification on a Source Q column as described in the general section followed by desalting on HPLC yielded the DNA-13 mer (0.07 µmol, OD).

LC/MS: (MeCN : 10 mM aq NH_4OAc 00 → 25 v/v) retention time: 5.9 min ESI-MS: 1400.3 $[M + 3 H]^{3+}$ HRMS calculated for $[C_{128}H_{158}N_{50}O_{90}P_{12}+ 2 H]^{2+}$: 2104.31641 ; found: 2104.31603 $[M + 2 H]^{2+}$.

5'-rGGCAUG(RITRICO-Coumaryl)CGUUGG-3' (26)

To a solution of RNA **ON 23** (11 nmol) in 55 µL PBS buffer (pH 7.3, 100 mM phosphate, 200 mM NaCl) containing EDTA (1 µM) was added 3-azido-7-(oxy-acetic acid)-coumarine triethylammonium salt **20** (33 nmol, 3 eq) and swirled for 1 hour. LC/MS analysis showed full conversion to the conjugated RNA derivative.

LC/MS: (MeCN : 10 mM aq NH_4OAc 00 → 25 v/v) retention time: 5.9 min (ESI-MS: 1400.3 $[M + 3 H]^{3+}$)

Modified duplex (Dup 29)

To a solution of modified RNA **ON 23** (11 nmol) and complementary DNA **ON 25** (11 nmol) in 54 µL PBS buffer (pH 7.3, 100 mM phosphate, 200 mM NaCl) containing EDTA (1 µM) was added 3-azido-7-(oxy-acetic acid)-coumarine triethylammonium salt **20** (33 nmol, 3 eq) and shaken for 1 hour. LC/MS analysis showed full conversion to the conjugated RNA derivative

LC/MS: (MeCN : 10 mM aq NH_4OAc 00 → 25 v/v) retention time 5.2 min : **ON 27** ($[M + 3H]^{3+}$ = 1491.0) and 5.4 min : **ON 25** ($[M + 3H]^{3+}$ = 1294.8).

References and notes

(1) Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem. Int. Ed.* **2008**, *120*, 2285–2287.

(2) Jayaprakash, K. N.; Peng, C. G.; Butler, D.; Varghese, J. P.; Maier, M. A.; Rajeev, K. G.; Manoharan, M. *Org. Lett.* **2010**, *12*, 5410–5413.

(3) Gramlich, P. M. E.; Wirges, C. T.; Manetto, A.; Carell, T. *Angew. Chem. Int. Ed.* **2008**, *47*, 8350–8.

(4) El-Sagheer, A. H.; Brown, T. *Chem. Soc. Rev.* **2010**, *39*, 1388–405.

(5) Gierlich, J.; Burley, G. A.; Gramlich, P. M. E.; Hammond, D. M.; Carell, T. *Org. Lett.* **2006**, *8*, 3639–3642.

(6) Gramlich, P. M. E.; Wirges, C. T.; Gierlich, J.; Carell, T. *Org. lett.* **2008**, *10*, 249–51.

(7) Seela, F.; Sirivolu, V. R. *Helv. Chim. Acta* **2007**, *90*, 535–552.

(8) Seela, F.; Sirivolu, V. R. Nucleos. Nucleot. & Nucleic Ac. **2007**, *6-7*, 597-601.

(9) Ding, P.; Wunnicke, D.; Steinhoff, H.-J.; Seela, F. *Chemistry Eur. J.* **2010**, *16*, 14385–96.

(10) Salic, A.; Mitchison, T. J. *Proc. Nat. Ac. Sc. USA* **2008**, *105*, 2415–20.

(11) Varga, B. R.; Kállay, M.; Hegyi, K.; Béni, S.; Kele, P. *Chemistry Eur. J.* **2012**, *18*, 822–8.

(12) Stimac, A.; Kobe, J. *Carbohydr. Res.* **1992**, *232*, 359–365.

(13) Serebryany, V.; Beigelman, L. *Tetrahedron Lett.* **2002**, *43*, 1983–1985.

(14) Detert, H.; Rose, B.; Mayer, W.; Meier, H. *Chem. Ber.* **1994**, *127*, 1529–1532.

(15) In these experiments increasement of the amount of the KO*t*Bu base from 3 to 6 eq did not lead to an increase in yield. Increasing the temperature from r.t. to 33, 40 or 60 °C led to a decrease in isolated yield. At 35 min, 6 eq KOtBu 60 °C the isolated yield dropped to 10% with no recovered starting material.

(16) 23% **15**, 44% **14**.

(17) Dirks, A. J.; Cornelissen, J.; Nolte, R. J. M. *Bioconj. Chemi.* **2009**, *20*, 1129–1138.

(18) Kypr, J.; Kejnovská, I.; Renciuk, D.; Vorlícková, M. *Nucleic Acids Res.* **2009**, *37*, 1713–25.

(19) Urata, H.; Shimizu, H.; Akagi, M. *Nucleos. Nucleot. & Nucleic Ac.* **2006**, *25*, 359–367.

(20) Romainczyk, O.; Endeward, B.; Prisner, T. F.; Engels, J. W. *Molecular Biosystems* **2011**, *7*, 1050–2.

* Manuscript in preparation, contributing authors: P. van Delft, W. de Witte, N. J. Meeuwenoord, H. S. Overkleeft, G. A. van der Marel, D. V. Filippov.