Oligonucleotide Conjugates by Means of Copper-Free Click Chemistry – Expanding the Repertoire of Strained Cyclooctyne Phosphoramidites

Pieter van Delft, Evert van Schie, Nico J. Meeuwenoord, Herman S. Overkleeft, Gijsbert A. van der Marel,* Dmitri V. Filippov*

Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands
Fax +31(71)5274307; E-mail: filippov@chem.leidenuniv.nl; E-mail: marel_g@chem.leidenuniv.nl

Received 7 April 2011

Abstract: A set of four phosphoramidite building blocks containing a strained dibenzocyclooctyne moiety is reported, including one example equipped with a cleavable disulfide linker. Application of these amidites in solid-phase oligonucleotide synthesis yields either 5′- or 3′-cyclooctyne-modified nucleic acids. The strained cyclooctyne-bearing oligonucleotides are used in rapid and clean conjugation reactions with azide-containing (bio)molecules.

Key words: oligonucleotides, strain-induced cycloaddition, conjugates, phosphoramidites, bioconjugates

The unique properties and biological roles of nucleic acids have attracted considerable interest toward this class of biomolecules. Recently, nucleic acids and their derivatives have found applications in the fields of nano[1,2] and materials science.[3,4] In biomedical science, the discovery of antisense oligonucleotides[5] and the process of RNA interference (RNAi),[6] and their associated applications,[7–9] has continued to stimulate the design and synthesis of nucleic acid derivatives. Both antisense and RNAi techniques have been employed as tools for post-transcriptional gene-silencing in both fundamental research and clinical settings. In this respect the classic antisense strategy utilizes stabilized isosters of DNA such as phosphothioate backbones, peptide nucleic acids (PNA)[10] and locked nucleic acids (LNA),[11] while the RNAi-based methodology requires double-stranded small interfering RNAs (siRNAs).[12] Although the present state of the art indicates that protein translation arrest can be achieved in a specific and effective manner, delivery of these compounds across the cell membrane to their targets remains an obstacle. In this framework several research groups have pursued studies in which oligonucleotides are conjugated to fluorescent labels,[13] and/or molecular entities, in order to improve cell permeability, to enable monitoring of cellular uptake, or both.[14,15]

We,[16] and others,[17] have reported the use of copper-free alkyne–azide click chemistry based on the strained dibenzocyclooctyne developed by Boons et al.[18] in oligonucleotide conjugation chemistry. The compatibility of the dibenzocyclooctyne with solid-phase nucleic acid chemistry was demonstrated by the successful synthesis of an RNA 16-mer using 5′ modifier **1** (Figure 1). Subsequent conjugation of the oligomer to a hexadecapeptide and a hyaluronan tetrasaccharide proceeded rapidly and in near-quantitative fashion. These results make this strategy appealing and have guided us to the development of additional phosphoramidite building blocks. We focused on easily accessible building blocks for 5′- and 3′-conjugation. The termini are often used as conjugation sites to keep the primary and secondary structures of the oligonucleotide intact, as is required in the field of post-transcriptional gene-silencing using RNAi or antisense oligonucleotides. Figure 1 depicts the new building

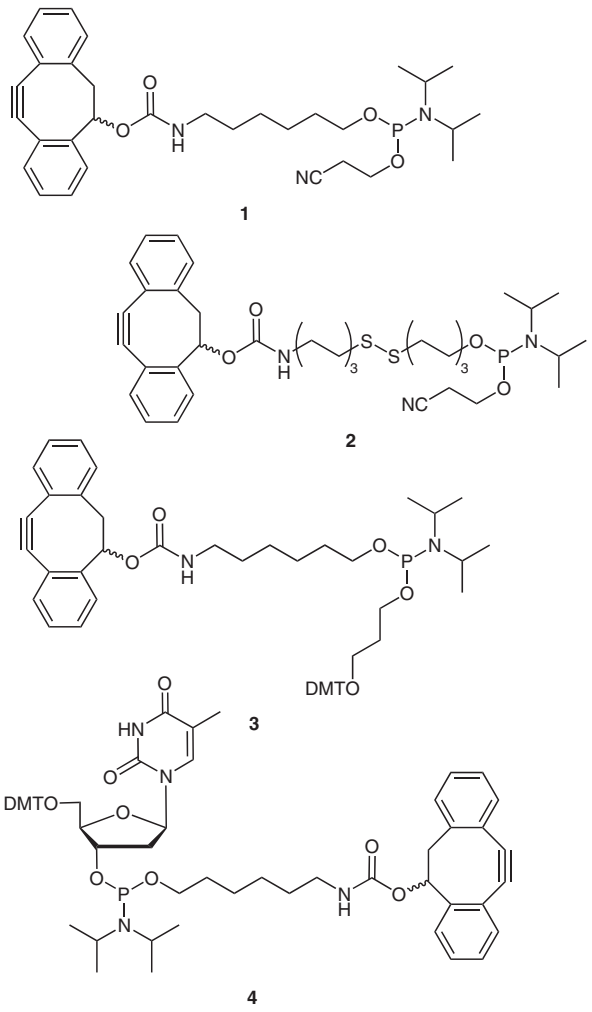
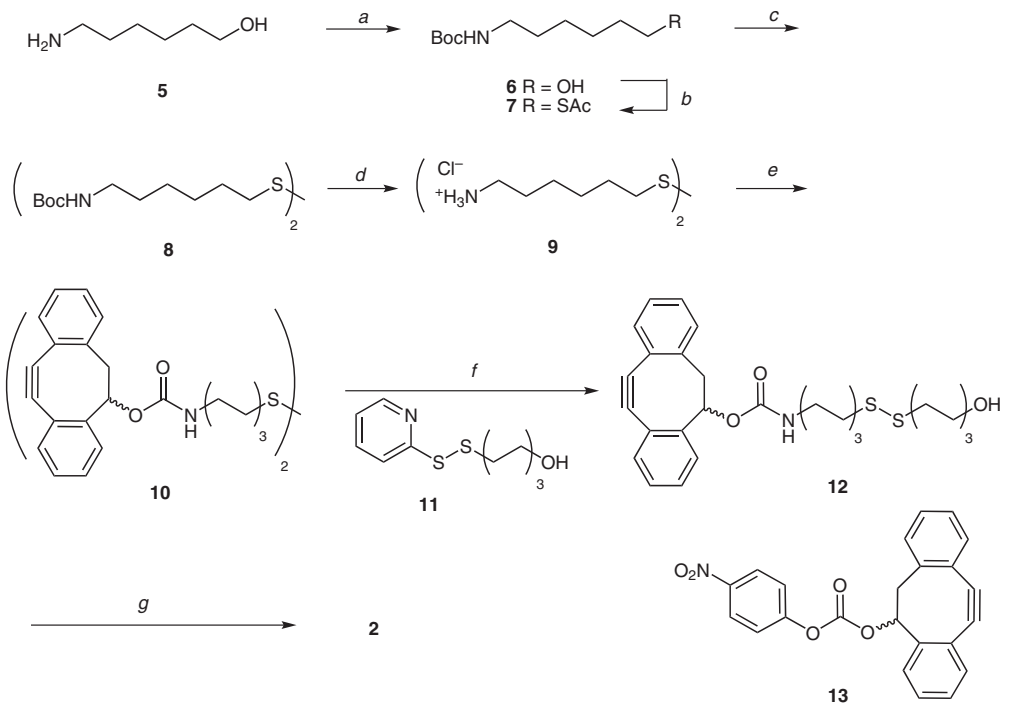


Figure 1 Phosphoramidite building blocks allowing incorporation of strained cyclooctynes into DNA or RNA oligomers (DMT = 4,4′-dimethoxytrityl)

SYNTHESIS 2011, No. 17, pp 2724–2732
Advanced online publication: 08.07.2011
DOI: 10.1055/s-0030-1260104; Art ID: C37811SS



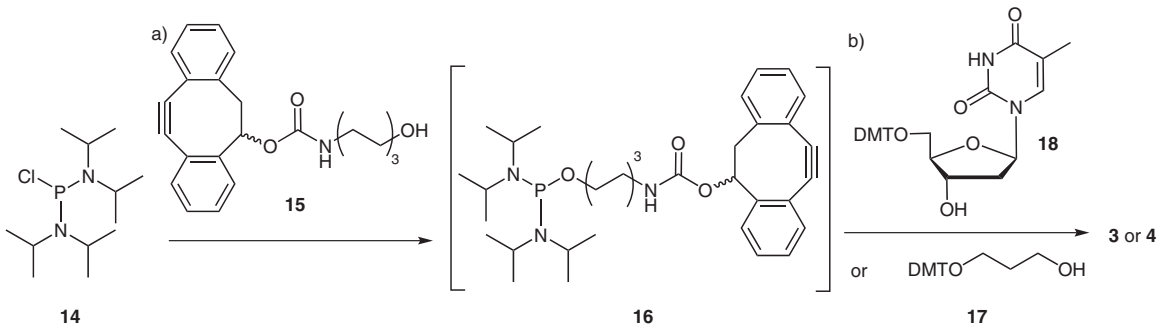
Scheme 1 *Reagents and conditions*: (a) Boc_2O, CH_2Cl_2, sat. aq $NaHCO_3$, 93%; (b) AcSH, Ph_3P, DIAD, toluene, 0 °C, 73%; (c) KOH, EtOH, reflux, then I_2, MeOH, 80%; (d) HCl, 1,4-dioxane, 74%; (e) **13**, Et_3N, DMF, 88%; (f) Me_3P (1 M, toluene), H_2O–THF (1:9), workup, then **11**, THF, 43%; (g) 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine, DIPEA, CH_2Cl_2, 80%.

blocks, **2**–**4** that we have developed for the 3′- and 5′-modification of nucleic acid fragments, and which are the subject of this work.

The new disulfide-containing phosphoramidite **2** allows introduction of the strained cyclooctyne at the 5′-terminus of an oligonucleotide. The presence of the disulfide in the linker arm of **2** allows reductive cleavage of the clicked entities in a cellular environment. Cleavable oligonucleotide conjugates have been found to enhance target gene specificity and lead to a reduced immune response.[19] Phosphoramidites **3** and **4** allow 3′ modification of the oligonucleotide. Phosphoramidite **3**, in combination with a solid support containing a diethoxysulfonyl linker, gives access to DNA fragments functionalized with a cyclooctyne handle at the 3′ end. The design of compound **4** is based on the commonly used 3′-TT overhang in siRNAs for added stability towards enzymatic degradation and enhanced gene-silencing properties.[20,21]

The synthesis of phosphoramidite **2** was started from readily available 6-aminohexan-1-ol (**5**). The amine moiety was protected as the corresponding *tert*-butoxycarbonyl and the hydroxy group was converted into a thioacetate under Mitsunobu[22] conditions to afford compound **7**. Basic hydrolysis using potassium hydroxide in ethanol followed by titration with iodine yielded symmetrical disulfide **8**. Amine deprotection using hydrochloric acid in 1,4-dioxane and treatment of the hydrochloride salt thus obtained with the known *p*-nitrophenylcarbonate of dibenzocyclooctyne **13**[18] yielded compound **10**. Disulfide **10** was cleaved using trimethylphosphine and the resulting crude thiol was reacted with disulfide **11** to furnish asymmetric disulfide **12**. Some of the starting material **10** was recovered as a result of oxidation of the intermediate thiol during workup. Phosphitylation of the free hydroxy group yielded target phosphoramidite **2** in 80% yield.

The synthesis of both 3′ modifiers **3** and **4** started with the reaction of commercially available phosphine **14** with the



Scheme 2 *Reagents and conditions*: (a) Et_3N, 1,4-dioxane, workup; (b) 1*H*-tetrazole, MeCN, 64% (**3**); 61% (**4**).

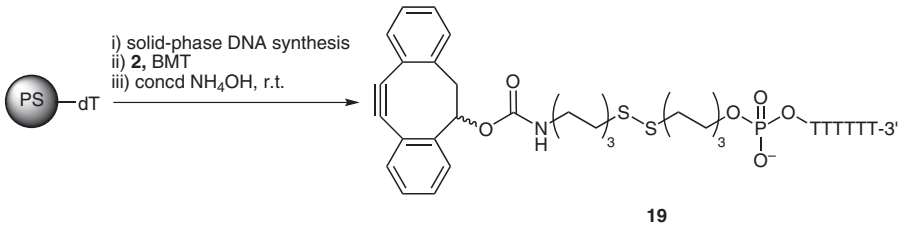
known cyclooctyne **15**.[16] The labile bisamidite intermediate **16** was isolated carefully and further processed by reaction with either mono DMT-protected 1,3-propanediol **17** or 5′-*O*-DMT-dT (**18**) in the presence of 1*H*-tetrazole (0.5 equiv) to yield the strained cyclooctyne building blocks **3** and **4** in 61% and 64% yields, respectively.

With our cyclooctyne amidites **2**, **3** and **4** in hand, we next undertook the synthesis of a thymidine hexamer containing the 5′-disulfide modifier (Scheme 3). Starting from a highly cross-linked polystyrene (PS) resin pre-loaded with DMT-dT, five consecutive coupling cycles were carried out under standard DNA synthesis conditions (five minutes, four equivalents of phosphoramidite, iodine oxidation, acetic anhydride capping and dimethoxytrityl group removal) followed by the final coupling cycle using the disulfide-containing cyclooctyne amidite **2** under optimized conditions (10 minutes, six equivalents). Treatment of the resin with concentrated aqueous ammonium hydroxide and subsequent purification by RP-HPLC yielded functionalized hexamer **19**.

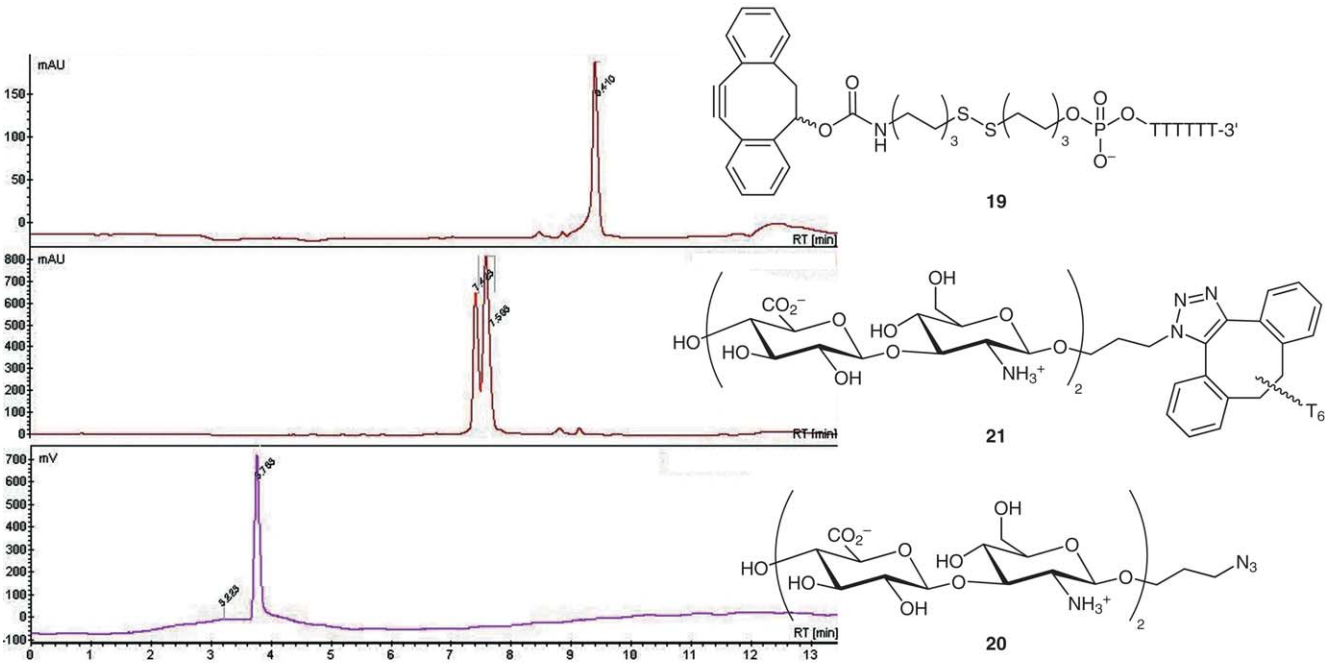
Next, the thymidine hexamer **19** containing the cleavable disulfide linker was conjugated to azide-functionalized zwitterionic tetrasaccharide **20**[23] (Scheme 4). The cycloaddition of strained alkyne **19** and azide **20** was executed in water (at 2 mM concentration), and after 90 minutes, LC–MS analysis revealed the complete disappearance of both starting materials and formation of the conjugate **21** consisting of two putative regioisomers.

Implementation of cyclooctyne phosphoramidites **3** and **4** in oligonucleotide synthesis requires the use of a controlled pore glass (CPG) solid support containing a diethoxysulfonyl linker. Whilst this type of β-eliminating linker is commonly used to introduce a 3′-phosphate monoester, the use of phosphoramidites **3** and **4** would result in 3′-phosphodiesters containing a strained cyclooctyne handle (Scheme 5). Amidites **3** and **4** were coupled under similar conditions as those used for **2**, followed by five consecutive coupling cycles with 5′-*O*-DMT-dT-phosphoramidite, and a final cleavage step using concentrated aqueous ammonium hydroxide to yield the respective 3′-modified thymidine pentamer and hexamer. Purification by RP-HPLC or anion exchange chromatography, or by a combination of both yielded target oligonucleotides **22** and **23**.

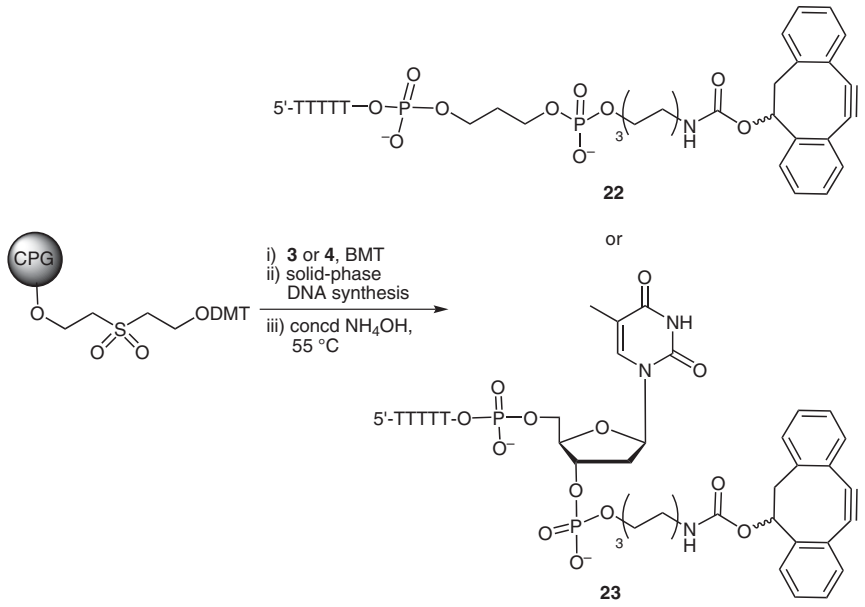
To demonstrate conjugation at the 3′-end of the oligonucleotide we selected the thymidine hexamer **23** as a model



Scheme 3 Solid-phase synthesis of oligonucleotide **19** bearing a strained cyclooctyne at the 5′-end (PS = highly crosslinked polystyrene; dT = thymidine; T = thymidine residue, phosphodiester linked; BMT = 5-benzylmercapto-1*H*-tetrazole)



Scheme 4 Strain-induced alkyne–azide click reaction of thymidine hexamer **19** and tetrasaccharide **20** yielding conjugate **21**, and the corresponding LC–MS traces



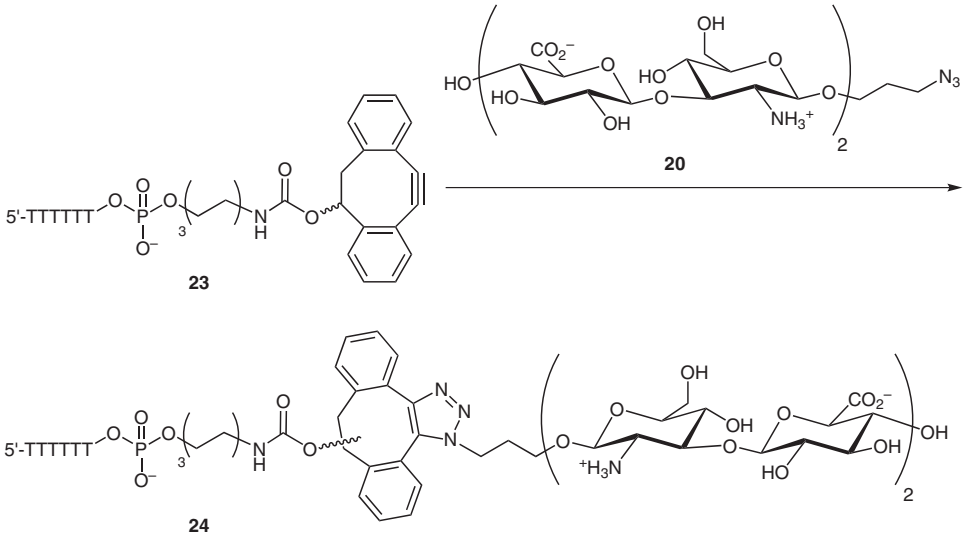
Scheme 5 Solid-phase synthesis of 3′-dibenzocyclooctyne oligonucleotides **22** and **23**

compound. The oligonucleotide **23** was reacted with the zwitterionic oligosaccharide **20** (Scheme 6) under identical conditions as described above for conjugate **21**. LC–MS analysis of the crude reaction mixture showed the presence of the target conjugate **24** and complete disappearance of the starting materials.

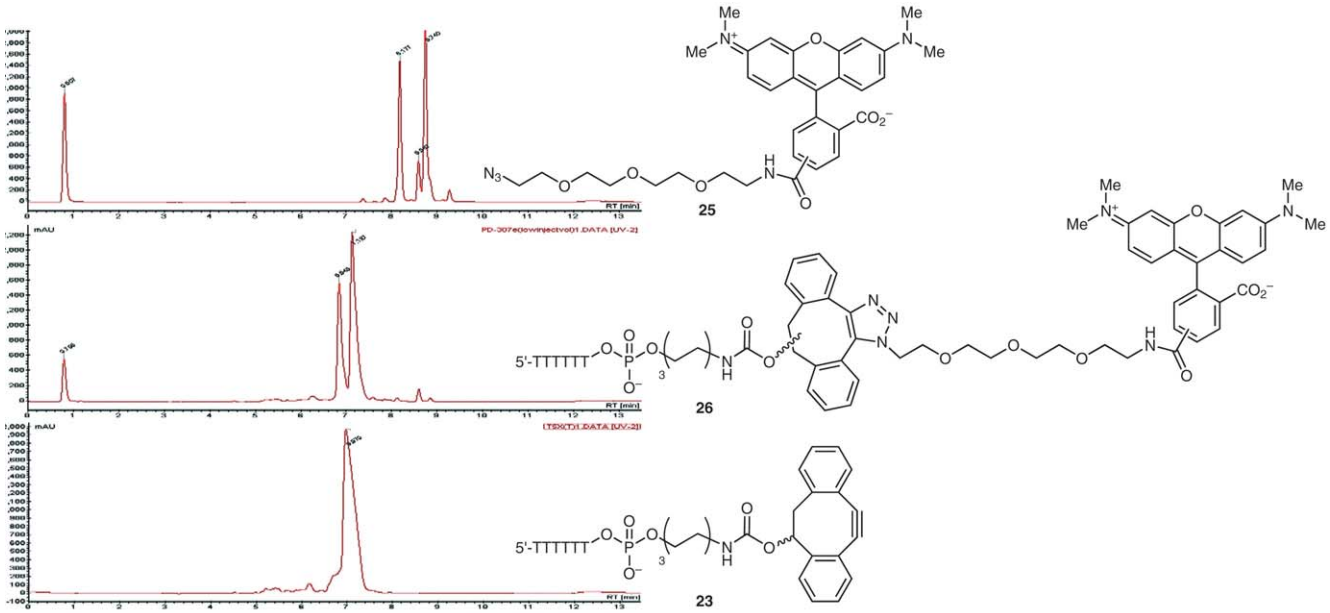
The advent of copper-free click chemistry has led to the commercial availability of several fluorescent labels functionalized with alkyl azides, for example tetramethylrhodamine (TAMRA). As mentioned previously, fluorescently labeled oligonucleotides are often applied in biochemical research, and therefore we conjugated a polyethylene glycol (PEG) spaced tetramethylrhodamine azide (TAMRA-N_3) to both 5′- and 3′-oligonucleotides **19** and **23**. Due to the water-solubility of the tetramethylrhodamine dye, conditions using 2 mM aqueous solutions could be applied without the necessity of adding an additional organic solvent. After 90 minutes, quantitative conversion of the starting materials into the target conjugates was observed. Scheme 7 depicts the LC–MS traces of the reaction between 3′-modified oligonucleotide **23** and tetramethylrhodamine azide (**25**) furnishing fluorescent conjugate **26**.

The conjugation of fluorescent dye **25** was repeated in a similar fashion using oligonucleotide **19** to furnish smoothly the corresponding conjugate **27** (Scheme 8).

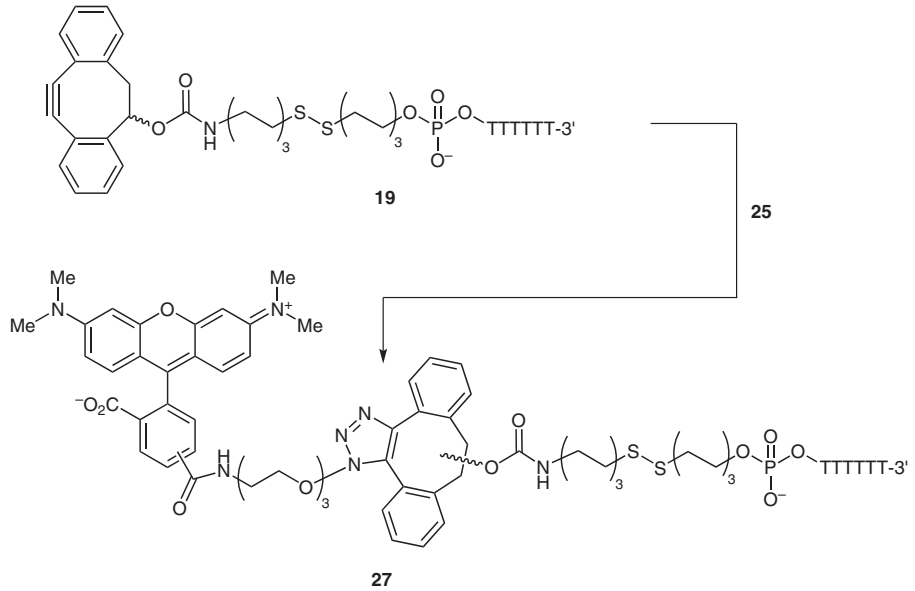
In summary, we have described the synthesis of three new phosphoramidite building blocks containing a strained dibenzocyclooctyne moiety. These amidites, **2**, **3** and **4**, can be used in automated solid-phase synthesis of DNA oligomers to afford oligonucleotides bearing a 3′- or 5′-handle for strain-induced alkyne–azide click reactions. The oligonucleotides obtained were used in conjugation



Scheme 6 Strain-induced alkyne–azide click reaction of thymidine hexamer **23** and tetrasaccharide **20** yielding conjugate **24**



Scheme 7 Strain-induced alkyne–azide click reaction between thymidine hexamer **23** and the fluorescent dye **25** yielding conjugate **26**, and the corresponding LC–MS traces



Scheme 8 Strain-induced alkyne–azide click reaction of thymidine hexamer **19** and the fluorescent dye **25** yielding conjugate **27**

reactions with both a zwitterionic oligotetrasaccharide and a tetramethylrhodamine fluorescent label. The conjugation reactions were rapid and proceeded quantitatively, using equimolar amounts of reagents at low mM concentrations to furnish various conjugates including examples with reductively cleavable disulfide linkers. The easy accessibility of the amidites together with the effectiveness of the strain-promoted alkyne–azide click reaction demonstrates the potential of our approach for future application in the design and synthesis of constructs for application in antisense and RNAi gene-silencing studies.

Chemicals were purchased from Acros Organics, Sigma Aldrich, Proligo and Jena Bioscience and were used as received. CH_2Cl_2 was distilled over CaH_2 and stored over 4 Å MS. Petroleum ether (PE) refers to the fraction boiling in the 40–60 °C range. DIPEA was distilled and stored over KOH pellets. Compounds used in reactions requiring anhyd conditions were co-evaporated with 1,4-dioxane, pyridine or toluene three times. All reactions were performed at ambient temperature under an Ar 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 made visual using UV light (254 nm) or by applying a soln of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L), $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) or 10% H_2SO_4 in H_2O followed by charring (ca. 150 °C). LC–MS analyses were performed on a Jasco HPLC system (UV detection simultaneously at 214 and 254 nm) coupled to a PE/SCIEX API 165 single quadrupole mass spectrometer (Perkin-Elmer). An analytical Gemini C_{18} column (Phenomex, 50 × 4.60 mm, 3 μ) was used in

combination with eluents A: H_2O, B: MeCN and C: aq NH_4OAc (0.1 M). Analytical anion exchange was performed on a GE ÄK-TAexplorer 10 using a Dionex DNA-PAC PA-200 column (4 × 250 mm) with eluents A: NaOAc (500 mM) and $NaClO_4$ (50 mM), and B: NaOAc (500 mM) and $NaClO_4$ (500 mM) using a linear gradient (0–20%). Anion exchange purification was performed on a GE ÄK-TAexplorer 10 using a GE Q-Sepharose HR column (260 × 10 mm) with eluents A: NaOAc (500 mM) and $NaClO_4$ (50 mM), and B: NaOAc (500 mM) and $NaClO_4$ (500 mM), followed by a desalting procedure using a Sephadex G25 column with NH_4OAc (150 mM) as the solvent. Preparative RP-HPLC was performed on a Gilson GX-281 HPLC system. A semi-preparative Altima C_{18} column (Phenomex, 250 × 10 mm, 5 μ) was used in combination with eluents A: aq $Et_3HN{\cdot}OAc$ (50 mM) and B: MeCN. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AV-400 instrument. ^1H and ^{13}C NMR chemical shifts (δ) are quoted relative to tetramethylsilane. ^{31}P NMR chemical shifts (δ) are quoted relative to H_3PO_4; reaction mixture aliquots were measured by means of an acetone-d_6 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 soln in H_2O or MeCN and HCO_2H (0.1%) or NH_4OAc (0.1%)] using a Thermo Finnigan LTQ Orbitrap equipped with an electrospray ion source in positive mode. IR spectra were recorded on a Shimadzu FT-IR 8300 and are reported in cm^{-1}. Melting points were determined using a Stuart Scientific SMP3 melting point apparatus. The yields of the oligonucleotides were determined spectrophotometrically using optical density (OD) measurements on a Varian Cary 50 Bio UV-VIS Spectrophotometer at 260 nm.

tert-Butyl (6-Hydroxyhexyl)carbamate (6)

To a stirred soln of 6-amino-1-hexanol (**5**) (3.5 g, 30 mmol) in CH_2Cl_2–sat. aq $NaHCO_3$ (300 mL, 3:2 v/v) was added Boc_2O (9.8 g, 45 mmol, 1.5 equiv). The reaction mixture was stirred for 40 h, after which the aq layer was separated and extracted with CH_2Cl_2 (100 mL). The combined organic layer was washed with H_2O (2 × 75 mL) and brine (75 mL), then dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was purified by silica gel column chromatography (PE–EtOAc, 7:3→4:6) to afford the title compound **6** as a pale-yellow oil.

Yield: 6.08 g, 28.0 mmol (93%); R_f = 0.56 (PE–EtOAc, 1:1).

IR (neat): 3363, 2930, 1683, 1510, 1362, 1245, 1165, 1059, 996 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 4.65 (s, 1 H), 3.58 (t, J = 6.4 Hz, 2 H), 3.07 (dd, J = 12.8, 6.4 Hz, 2 H), 2.25 (s, 1 H), 1.55–1.48 (m, 2 H), 1.48–1.40 (m, 2 H), 1.40 (s, 9 H), 1.36–1.27 (m, 4 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.0, 79.0, 62.4, 40.2, 32.4, 29.9, 28.3, 26.3, 25.2.

HRMS (ESI): m/z $[M + Na]^+$ calcd for $C_{11}H_{23}NO_3Na$: 240.1570; found: 240.1569; m/z $[M + H]^+$ calcd for $C_{11}H_{24}NO_3$: 218.1751; found: 218.1750.

***S*-{6-[(*tert*-Butoxycarbonyl)amino]hexyl} Ethanethioate (7)**

To a stirred soln of DIAD (5.95 mL, 30 mmol, 1.2 equiv) in anhyd toluene (30 mL) at 0 °C was added a soln of PPh_3 (7.8 g, 30 mmol, 1.2 equiv) in anhyd toluene (30 mL). After 15 min, a pre-cooled (0 °C) soln of **6** (5.4 g, 25 mmol) and AcSH (2.1 mL, 30 mmol, 1.2 equiv) in anhyd toluene (300 mL) was added in one portion. The mixture was allowed to warm to ambient temperature and stirred for 17 h. The mixture was concentrated under reduced pressure and the remaining thick oily residue was purified by silica gel column chromatography (PE–EtOAc, 95:5→91.5:8.5) to afford the title compound (**7**) as an oil.

Yield: 5.04 g, 18.3 mmol (73%); R_f = 0.26 (PE–EtOAc, 9:1).

IR (neat): 2931, 1683, 1506, 1363, 1247, 1169, 1134 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 4.71 (s, 1 H), 3.10 (dd, J = 12.9, 6.4 Hz, 2 H), 2.86 (t, J = 7.3 Hz, 2 H), 2.32 (s, 3 H), 1.63–1.52 (m, 2 H), 1.52–1.41 (m, 11 H), 1.41–1.28 (m, 4 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 195.8, 155.8, 78.7, 40.3, 30.5, 29.7, 29.3, 28.8, 28.3, 28.2, 26.1.

HRMS (ESI): m/z $[M + Na]^+$ calcd for $C_{13}H_{25}NO_3SNa$: 298.1447; found: 298.1448.

Di-*tert*-butyl (6,6′-Dihexyldisulfide)-1,1′-dicarbamate (8)

Thioacetate **7** (4.13 g, 15 mmol) was dissolved in an ethanolic soln of KOH (160 mL, 10% w/v) and heated at reflux temperature for 1 h. The mixture was cooled to 0 °C and quenched by the addition of AcOH [17.6 mL, 1.1 equiv (relative to KOH)]. The mixture was diluted with H_2O (200 mL) and extracted with EtOAc (3 × 200 mL). The combined organic layer was washed with brine (150 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was dissolved in MeOH (100 mL) and titrated with a soln of I_2 in MeOH (0.1 M) until the soln became pale-yellow in color. H_2O (250 mL) was added and the mixture extracted with EtOAc (3 × 200 mL). The combined organic layer was washed with sat. aq $NaHCO_3$ soln containing $Na_2S_2O_5$ (100 mL, 5% w/v) and brine (2 × 100 mL), then dried ($MgSO_4$) and concentrated under reduced pressure. Silica gel column chromatography (PE–EtOAc, 85:15) of the residue afforded the title disulfide **8** as an oil.

Yield: 2.8 g, 6 mmol (80%); R_f = 0.3 (PE–EtOAc, 85:15).

IR (neat): 2928, 1700, 1521, 1363, 1252, 1168, 1120, 872 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 4.59 (s, 2 H), 3.07 (dd, J = 12.8, 6.4 Hz, 4 H), 2.69–2.55 (m, 4 H), 1.71–1.54 (m, 4 H), 1.52–1.43 (m, 4 H), 1.41 (s, 18 H), 1.38–1.21 (m, 8 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 156.2, 79.0, 77.5, 77.3, 77.1, 76.7, 40.5, 38.9, 30.0, 29.1, 28.4, 28.1, 26.4.

ESI-MS: m/z = 464.93 $[M + H]^+$, 487.07 $[M + Na]^+$.

(6,6′-Dihexyldisulfide)-1,1′-diamine Dihydrochloride (9)

To Boc-protected disulfide **8** (1.4 g, 3 mmol) was added a soln of HCl in 1,4-dioxane (4 M, 6 mL). The resulting mixture was stirred for 20 min during which time a white precipitate formed. The mixture was cooled to 0 °C and Et_2O (3 mL) was added. The mixture was filtered and the residue washed with Et_2O (3 × 3 mL) and dried to yield the title salt as a white solid.

Yield: 0.75 g, 2.2 mmol (74%); mp >190 °C (decomp).

IR (neat): 2919, 1608, 1504 cm^{-1}.

^1H NMR (400 MHz, D_2O): δ = 2.98 (t, J = 7.5 Hz, 4 H), 2.75 (t, J = 7.2 Hz, 4 H), 1.77–1.57 (m, 8 H), 1.49–1.33 (m, 8 H).

^{13}C NMR (100 MHz, D_2O): δ = 39.4, 37.9, 28.0, 27.0, 26.5, 25.1.

HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{12}H_{29}N_2S_2$: 265.1767; found: 265.1767.

(6,6′-Dihexyldisulfide)-(1-Carbonic Acid 7,8-Didehydro-1,2:5,6-dibenzocyclooctен-3-yl Ester) 1,1′-Diamide (10)

To a slurry of disulfide **9** (675 mg, 2 mmol) and Et_3N (3.37 mL, 12 mmol) in DMF (20 mL) was added carbonate **13** (4 mmol, 2 equiv) in DMF (20 mL). An additional volume of DMF (40 mL) was added for solubility purposes and the reaction mixture was stirred for 4 d. The mixture was concentrated to ca. 5 mL, diluted with EtOAc (160 mL) and washed with aq NaOH (0.5 M, 2 × 50 mL), brine (2 × 50 mL), then dried ($MgSO_4$) and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (PE–EtOAc, 8:2→6:4) to afford the title disulfide **10** as an oil.

Yield: 1.34 g, 1.76 mmol (88%). R_f = 0.31 (PE–EtOAc, 7:3).

IR (neat): 3326, 2926, 1699, 1516, 1237, 1021, 750 cm^{-1}.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.61 (t, J = 5.7 Hz, 2 H), 7.54 (d, J = 7.7 Hz, 2 H), 7.48–7.34 (m, 14 H), 5.30 (s, 2 H), 3.17 (dd, J = 15.0, 1.9 Hz, 2 H), 3.03–2.92 (m, 4 H), 2.77 (dd, J = 14.9, 3.9 Hz, 2 H), 2.67 (t, J = 7.2 Hz, 4 H), 1.64–1.54 (m, 4 H), 1.47–1.36 (m, 6 H), 1.36–1.22 (m, 6 H).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 155.7, 153.1, 151.4, 130.6, 128.9, 128.8, 127.9, 127.8, 126.6, 126.3, 124.2, 123.4, 120.9, 113.1, 110.4, 75.7, 46.0, 40.7, 40.6, 40.4, 40.2, 40.0, 39.8, 39.6, 39.4, 38.2, 29.7, 29.0, 27.9, 26.3.

HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{46}H_{49}N_2O_4S_2$: 757.3128; found: 757.3132.

6-(Pyridin-2-yldisulfanyl)hexan-1-ol (11)

To a stirred soln of 2,2′-dithiodipyridine (2 g, 9 mmol, 3 equiv) in deoxygenated MeOH (50 mL) was added dropwise 6-mercaptohexanol (0.4 mL, 3 mmol). The mixture was stirred for 1 h followed by evaporation under reduced pressure. Silica gel column chromatography of the residue (CH_2Cl_2–EtOAc, 95:5→8:2) afforded the title compound **11** as an oil.

Yield: 680 mg, 2.8 mmol (93%); R_f = 0.35 (PE–EtOAc, 6:4).

IR (neat): 3378, 2926, 2855, 1575, 1558, 1445, 1416, 1118, 1054, 758 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.46 (dd, J = 4.8, 0.8 Hz, 1 H), 7.73 (d, J = 8.1 Hz, 1 H), 7.65 (td, J = 7.8, 1.8 Hz, 1 H), 7.08 (ddd, J = 7.3, 4.9, 0.9 Hz, 1 H), 3.63 (t, J = 6.6 Hz, 2 H), 2.86–2.73 (m, 2 H), 1.87 (s, 1 H), 1.76–1.65 (m, 2 H), 1.60–1.49 (m, 2 H), 1.48–1.30 (m, 4 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 160.5, 149.5, 137.0, 120.5, 119.6, 62.7, 38.8, 32.5, 28.8, 28.2, 25.3.

HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{11}H_{18}NOS_2$: 244.0824; found: 244.0823.

6′-(1-Carbonic Acid 7,8-Didehydro-1,2:5,6-dibenzocycloocten-3-yl Ester 1′-Hexamide)-disulfanyl-6-hexanol (12)

To a soln of **10** (0.38 g, 0.5 mmol) in THF–H_2O (7 mL, 9:1 v/v) was added a soln of PMe_3 in toluene (1 M, 2 mL, 2 mmol, 4 equiv) and the mixture stirred for 1 h. TLC analysis (PE–EtOAc, 8:2) showed full conversion of the starting material into a less polar product (R_f = 0.49). The mixture was diluted with EtOAc (30 mL), washed with brine (2 × 10 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The crude thiol was coevaporated with deoxygenated THF (2 × 5 mL) followed by addition of a soln of alcohol **11** in deoxygenated THF (0.5 M, 2.1 mL, 1.05 mmol, 1.05 equiv). The mixture was stirred for 18 h and then concentrated under reduced pressure. Silica gel column chromatography (PE–EtOAc, 7:3→6:4) afforded the title compound **12** as an oil.

Yield: 220 mg, 0.43 mmol (43%); R_f = 0.44 (PE–EtOAc, 6:4).

IR (neat): 3324, 2926, 2855, 2363, 1701, 1560, 1522, 1450, 1418, 1254, 1130, 1040 cm^{-1}.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.60 (t, J = 5.6 Hz, 1 H), 7.53 (d, J = 7.7 Hz, 1 H), 7.50–7.34 (m, 7 H), 5.29 (s, 1 H), 4.35 (t, J = 5.1 Hz, 1 H), 3.40–3.35 (m, 2 H), 3.17 (dd, J = 15.0, 1.9 Hz, 1 H), 2.98 (dd, J = 12.8, 6.1 Hz, 2 H), 2.76 (dd, J = 14.9, 3.8 Hz, 1 H), 2.67 (t, J = 7.2 Hz, 4 H), 1.64–1.54 (m, 4 H), 1.46–1.21 (m, 12 H).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 155.2, 152.6, 150.9, 130.1, 128.5, 128.4, 127.4, 127.4, 126.2, 125.9, 123.8, 122.9, 120.4, 112.6, 110.0, 75.2, 60.6, 45.5, 40.2, 40.2, 37.7, 32.4, 29.2, 28.6, 28.5, 27.7, 27.5, 25.8, 25.1.

HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{29}H_{38}NO_3S_2$: 512.2288; found: 512.2286.

6′-(1-Carbonic Acid 7,8-Didehydro-1,2:5,6-dibenzocycloocten-3-yl Ester 1′-Hexamide)-disulfanyl-6-hexyl-1-(2-cyanoethoxy-*N*,*N*-diisopropylamino)phosphoramidite (2)

To a soln of alcohol **12** (200 mg, 0.4 mmol) in anhyd CH_2Cl_2 (4 mL) was added DIPEA (132 μL, 0.8 mmol, 2 equiv) and 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine (87.3 μL, 0.4 mmol, 1 equiv). The reaction mixture was stirred for 3 h, diluted with EtOAc (15 mL, containing 1% Et_3N), washed with brine (2 × 7.5 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Silica gel column chromatography (PE–EtOAc–Et_3N, 94:5:1→91.5:7.5:1) afforded the title compound **2** as an oil.

Yield: 225 mg, 0.31 mmol (80%); R_f = 0.35 (PE–EtOAc–Et_3N, 9:1:0.1).

IR (neat): 2930, 1718, 1508, 1450, 1364, 1240, 1026, 975, 757 cm^{-1}.

^{1}H NMR (400 MHz, CD_3CN): δ = 7.65 (d, J = 7.8 Hz, 1 H), 7.52–7.36 (m, 7 H), 6.02 (t, J = 5.6 Hz, 1 H), 5.43 (s, 1 H), 3.89–3.76 (m, 2 H), 3.73–3.60 (m, 4 H), 3.23 (dd, J = 15.0, 2.0 Hz, 1 H), 3.15 (dd, J = 13.0, 6.6 Hz, 2 H), 2.89 (dd, J = 15.0, 3.9 Hz, 1 H), 2.75 (dd, J = 15.3, 8.1 Hz, 4 H), 2.69 (t, J = 6.0 Hz, 2 H), 1.82–1.67 (m, 4 H), 1.67–1.59 (m, 2 H), 1.59–1.50 (m, 2 H), 1.50–1.34 (m, 8 H), 1.25–1.20 (m, 12 H).

^{13}C NMR (100 MHz, CD_3CN): δ = 156.4, 153.5, 152.3, 131.0, 129.2, 129.2, 128.2, 128.1, 127.1, 126.8, 124.8, 124.3, 121.8, 119.5, 113.5, 110.7, 76.8, 64.3, 64.1, 59.2, 59.1, 47.0, 43.7, 43.6, 41.4, 39.3, 39.3, 31.8, 31.7, 30.4, 29.7, 28.7, 26.9, 26.2, 24.9, 24.9, 24.9, 24.8, 21.0, 21.0.

^{31}P NMR (160 MHz, CD_3CN): δ = 146.8.

HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{38}H_{55}N_3O_4PS_2$: 712.3366; found: 712.3370.

Carbonic Acid 7,8-didehydro-1,2:5,6-dibenzocycloocten-3-yl Ester, 6′-[3-*O*-(4,4′-dimethoxytrityl)-propanoxy-*N*,*N*′-diisopropylamino]phosphinooxy 1′-Amide (3)

Et_3N (180 μL, 1.3 mmol, 2.2 equiv) was added to a stirred soln of **15**[16] (208 mg, 0.57 mmol) in anhyd 1,4-dioxane (5 mL), followed by the addition of bis(diisopropylamino)chlorophosphine (**14**) (300 mg, 1.2 mmol, 1.9 equiv). After stirring for 20 min, an aliquot of the reaction mixture was analyzed by ^{31}P NMR which showed complete conversion into the desired intermediate bis-amidite **16** (δ = 123 ppm). The mixture was filtered under Ar to remove triethylammonium salts and concentrated under reduced pressure. A soln of 1*H*-tetrazole (30 mg, 0.4 mmol, 0.7 equiv) and 3′-*O*-DMT-propanol (**17**) (550 mg, 1.5 mmol, 2.6 equiv) in anhyd 1,4-dioxane (5 mL) was added to a soln of crude **16** in anhyd MeCN (5 mL). After stirring for 30 min, an aliquot of the reaction mixture was analyzed by ^{31}P NMR which showed complete conversion into amidite **3** (δ = 146.5, 146.3 ppm). The reaction mixture was quenched with sat. aq $NaHCO_3$ soln (10 mL) and the aq layer separated and extracted with EtOAc (3 × 10 mL). The combined organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (hexane–CH_2Cl_2–Et_3N, 8:2:0.3→6:4:0.3) to afford the title amidite **3** as an oil.

Yield: 320 mg, 0.37 mmol (64%); R_f = 0.3 (hexane–CH_2Cl_2–Et_3N, 6:4:0.3).

IR (neat): 2930, 1725, 1606, 1506, 1462, 1362, 1300, 1246, 1172, 1032, 970, 826, 753 cm^{-1}.

^{1}H NMR (400 MHz, CD_3CN): δ = 7.56 (d, J = 7.5 Hz, 1 H), 7.45–7.23 (m, 15 H), 7.19 (dd, J = 8.3, 6.2 Hz, 1 H), 6.83 (d, J = 8.9 Hz, 4 H), 5.92 (t, J = 5.7 Hz, 1 H), 5.35 (s, 1 H), 3.73 (s, 6 H), 3.71–3.59 (m, 2 H), 3.59–3.39 (m, 4 H), 3.19–3.12 (m, 1 H), 3.07 (dq, J = 19.4, 6.5 Hz, 4 H), 2.81 (dd, J = 15.0, 3.7 Hz, 1 H), 1.88–1.75 (m, 2 H),

1.64–1.37 (m, 4 H), 1.36–1.20 (m, 4 H), 1.15 (d, *J* = 6.0 Hz, 2 H), 1.10 (d, *J* = 5.8 Hz, 6 H), 1.06 (d, *J* = 6.8 Hz, 6 H).

^{13}C NMR (100 MHz, CD_3CN): δ = 159.4, 153.5, 152.2, 146.5, 137.3, 131.0, 130.8, 129.2, 129.2, 128.8, 128.6, 128.1, 128.1, 127.5, 127.1, 126.8, 124.8, 124.3, 121.8, 113.8, 113.5, 110.7, 86.5, 76.7, 67.6, 63.8, 63.7, 61.1, 61.0, 60.9, 55.7, 46.9, 45.2, 45.1, 43.5, 43.4, 41.5, 32.6, 32.5, 32.0, 30.5, 27.1, 26.4, 25.0, 25.0, 24.9, 24.9, 23.9, 23.9, 22.7, 22.7, 14.5.

^{31}P NMR (162 MHz, CD_3CN): δ = 145.2, 145.1.

HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{53}H_{64}N_2O_7P$: 871.4446; found: 871.4451.

Carbonic Acid 7,8-Didehydro-1,2:5,6-dibenzocycloocten-3-yl Ester, 6′-[5′-*O*-(4,4′-Dimethoxytrityl)-3′-thymidine-*N*,*N*′-diisopropylamino]phosphinooxy 1′-Amide (4)

Et_3N (504 μL, 3.6 mmol, 2 equiv) was added to a stirred soln of **15**[16] (653 mg, 1.8 mmol) in anhyd 1,4-dioxane (5 mL) at r.t., followed by addition of bis(diisopropylamino)chlorophosphine (**14**) (610 mg, 2.4 mmol, 1.3 equiv). After stirring for 20 min, an aliquot of the reaction mixture was analyzed by ^{31}P NMR which showed complete conversion into the desired intermediate bis-amidite **16** (δ = 123 ppm). The mixture was filtered under Ar to remove triethylammonium salts and concentrated under reduced pressure. A soln of 1*H*-tetrazole (70 mg, 1 mmol, 0.7 equiv) and 5′-*O*-DMT-thymidine (**18**) (1.2 g, 2.2 mmol, 1.2 equiv) in anhyd 1,4-dioxane (5 mL) was added to a soln of crude **16** in anhyd MeCN (5 mL) at r.t. After stirring for 30 min, an aliquot of the reaction mixture was analyzed by ^{31}P NMR which showed complete conversion into amidite **4** (δ = 148, 144). A sat. aq soln of $NaHCO_3$ (15 mL) was added to the mixture and the aq layer separated and extracted with EtOAc (3 × 15 mL). The combined organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (hexane–CH_2Cl_2–Et_3N, 5:5:0.3→2:8:0.3) to afford the title compound **4** as an off-white foam.

Yield: 1.12 g, 1.09 mmol (61%); R_f = 0.3 (hexane–CH_2Cl_2–Et_3N, 3:7:0.3).

IR (neat): 2931, 1703, 1505, 1463, 1248, 1176, 1031, 970, 826, 755 cm^{-1}.

^{1}H NMR (400 MHz, CD_3CN): δ = 7.55 (d, *J* = 7.5 Hz, 1 H), 7.50–7.15 (m, 18 H), 6.84 (d, *J* = 8.8 Hz, 4 H), 6.23 (dt, *J* = 6.8, 3.0 Hz, 1 H), 5.95 (dd, *J* = 10.9, 5.9 Hz, 1 H), 5.35 (s, 1 H), 4.58 (ddd, *J* = 10.5, 8.5, 4.4 Hz, 1 H), 4.07 (dd, *J* = 15.6, 3.1 Hz, 1 H), 3.77–3.70 (m, 6 H), 3.63–3.40 (m, 4 H), 3.37–3.20 (m, 2 H), 3.13 (dd, *J* = 15.0, 1.7 Hz, 1 H), 3.05 (dt, *J* = 13.8, 6.8 Hz, 2 H), 2.79 (dd, *J* = 15.0, 3.8 Hz, 1 H), 2.43–2.23 (m, 2 H), 1.67–1.16 (m, 13 H), 1.16–1.06 (m, 8 H), 1.02 (d, *J* = 6.7 Hz, 3 H).

^{13}C NMR (100 MHz, CD_3CN): δ = 164.7, 159.7, 156.5, 153.6, 152.3, 151.4, 145.9, 145.8, 136.7, 136.6, 136.6, 136.5, 136.5, 131.0, 129.2, 129.2, 129.0, 128.9, 128.9, 128.2, 128.1, 127.9, 127.1, 126.8, 124.9, 124.3, 121.8, 114.1, 113.5, 111.4, 111.3, 110.8, 87.4, 86.2, 86.2, 85.9, 85.4, 76.8, 74.1, 73.9, 73.7, 73.6, 64.4, 64.2, 64.2, 64.1, 55.9, 47.0, 43.8, 43.7, 41.5, 40.3, 40.1, 31.9, 30.5, 27.1, 26.4, 26.4, 25.0, 24.9, 24.8, 12.3.

^{31}P NMR (162 MHz, CD_3CN): δ = 146.86, 146.85, 146.31, 146.28.

HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{60}H_{70}N_4O_{10}P$: 1037.4824; found: 1037.4828.

Oligonucleotide 19

The synthesis of oligonucleotide **19** was performed on 8 μmol scale using a polystyrene solid support. Coupling was carried out using commercially available amidites (4 equiv) and phosphoramidite **2** (6 equiv) at a concentration of 0.1 M. 5-Benzylthio-1*H*-tetrazole (0.3 M) was used as the activating agent in 5 min coupling cycles. Oxidation and capping were performed by means of standard procedures using I_2, pyridine, H_2O and Ac_2O, respectively. Deprotection and cleavage from the resin were accomplished using concd aq NH_4OH soln for 1 h at r.t. HPLC purification followed by anion exchange chromatography and consecutive desalting yielded the target oligo-2′-deoxynucleotide (see general methods and materials) (OD = 0.64 μmol).

ESI-MS: *m*/*z* = 1169.4 [M + 2H]$^{2+}$.

LC–MS: 9.41 min [MeCN–aq NH_4OAc (10 mM), 0→50 v/v].

Oligonucleic Acids 22 and 23

The syntheses of oligonucleic acids **22** and **23** were performed on 8 μmol scale using a CPG solid support containing a diethoxysulfonyl linker. Coupling was carried out using commercially available amidites (4 equiv) and phosphoramidite **3** or **4** (6 equiv) at a concentration of 0.1 M. 5-Benzylthio-1*H*-tetrazole (0.3 M) was used as the activating agent in 5 or 10 min coupling cycles (phosphoramidites **3** and **4**). Oxidation and capping were performed by means of standard procedures using I_2, pyridine, H_2O and Ac_2O, respectively. Deprotection and cleavage from the solid support were accomplished using concd aq NH_4OH soln overnight at 55 °C. Concentration and subsequent HPLC purification (see general methods and materials) yielded the target oligo-2′-deoxynucleotides **22** (OD = 0.3 μmol) and **23** (OD = 0.7 μmol).

Oligonucleic Acid 22

ESI-MS: *m*/*z* = 2024.2 [M + H]$^+$, 1012.0 [M + 2H]$^{2+}$.

LC–MS: 6.95 min [MeCN–aq NH_4OAc (10 mM), 0→50 v/v].

Oligonucleic Acid 23

ESI-MS: *m*/*z* = 1095.0 [M + 2H]$^{2+}$.

LC–MS: 6.97 min [MeCN–aq NH_4OAc (10 mM), 0→50 v/v].

Oligonucleic Acid–Tetrasaccharide Conjugates 21 and 24

To an aq soln (50 μL) of strained alkyne oligonucleotide **19** or **23** (200 nmol) was added an aq soln (30 μL) of tetrasaccharide **20** (200 nmol). The reaction mixture was shaken for 90 min after which time LC–MS analysis revealed conversion of all the starting material into the corresponding conjugate.

Conjugate 21

ESI-MS: *m*/*z* = 1557.4 [M + 2H]$^{2+}$.

LC–MS: 7.42 min, 7.60 min [MeCN–aq NH_4OAc (10 mM), 0→50 v/v].

Conjugate 24

ESI-MS: *m*/*z* = 1483.2 [M + 2H]$^{2+}$.

LC–MS: 5.47 min, 5.62 min [MeCN–aq NH_4OAc (10 mM), 0→50 v/v].

Oligonucleic Acid–Tetramethylrhodamine Conjugates 26 and 27

To an aq soln of strained alkyne oligonucleotide **19** or **23** (200 nmol) was added an aq soln (20 μL) of tetramethylrhodamine azide (200 nmol). The reaction mixture was shaken for 90 min after which time LC–MS analysis revealed conversion of all the starting material into the corresponding conjugate.

Conjugate 26

ESI-MS: *m*/*z* = 1410.8 [M + 2H]$^{2+}$.

LC–MS: 6.84 min, 7.13 min [MeCN–aq NH_4OAc (10 mM), 0→50 v/v].

Conjugate 27

ESI-MS: *m*/*z* = 1485.0 [M + 2H]$^{2+}$.

LC–MS: 8.48 min, 8.65 min [MeCN–aq NH_4OAc (10 mM), 0→50 v/v].

Acknowledgment

This work was funded by the Dutch Organization for Scientific Research (NWO).

References

(1) Chiu, T.-C.; Huang, C.-C. *Sensors* **2009**, *9*, 10356.
(2) Endo, M.; Sugiyama, H. *ChemBioChem* **2009**, *10*, 2420.
(3) Kwak, M.; Herrmann, A. *Angew. Chem. Int. Ed.* **2010**, *49*, 8574.
(4) Alemdaroglu, F. E.; Herrmann, A. *Org. Biomol. Chem.* **2007**, *5*, 1311.
(5) Stephenson, M. L.; Zamecnik, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 285.
(6) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998**, *391*, 806.
(7) Opalinska, J. B.; Gewirtz, A. M. *Nat. Rev. Drug Discovery* **2002**, *1*, 503.
(8) Dorsett, Y.; Tuschl, T. *Nat. Rev. Drug Discovery* **2004**, *3*, 318.
(9) Alvarez-Salas, L. M. *Curr. Top. Med. Chem.* **2008**, *8*, 1379.
(10) Koppelhus, U.; Nielsen, P. E. *Adv. Drug Delivery Rev.* **2003**, *55*, 267.
(11) Braasch, D. A.; Corey, D. R. *Biochemistry* **2002**, *41*, 4503.
(12) Whitehead, K. A.; Langer, R.; Anderson, D. G. *Nat. Rev. Drug Discovery* **2009**, *8*, 129.
(13) Astakhova, I. V.; Korshun, V. A.; Jahn, K.; Kjems, J.; Wengel, J. *Bioconjugate Chem.* **2008**, *19*, 1995.
(14) Lönnberg, H. *Bioconjugate Chem.* **2009**, *20*, 1065; and references cited therein.
(15) Marlin, F.; Simon, P.; Saison-Behmoaras, T.; Giovannangeli, C. *ChemBioChem* **2010**, *11*, 1493.
(16) van Delft, P.; Meeuwenoord, N. J.; Hoogendoorn, S.; Dinkelaar, J.; Overkleeft, H. S.; van der Marel, G. A.; Filippov, D. V. *Org. Lett.* **2010**, *12*, 5486.
(17) Jayaprakash, K. N.; Peng, C. G.; Butler, D.; Varghese, J. P.; Maier, M. A.; Rajeev, K. G.; Manoharan, M. *Org. Lett.* **2010**, *12*, 5410.
(18) Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem. Int. Ed.* **2008**, *47*, 2253.
(19) Jung, S.; Lee, S. H.; Mok, H. H.; Chung, J.; Park, T. G. *J. Controlled Release* **2010**, *144*, 306.
(20) Ghosh, P.; Dullea, R.; Fischer, J. E.; Turi, T. G.; Sarver, R. W.; Zhang, C.; Basu, K.; Das, S. K.; Poland, B. W. *BMC Genomics* **2009**, *10 (Suppl. 1)*, S17.
(21) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494.
(22) Li, Y.; Zhang, Y.; Huang, Z.; Cao, X.; Gao, K. *Can. J. Chem.* **2004**, *82*, 622.
(23) Dinkelaar, J.; Codée, J. D. C.; van den Bos, L. J.; Overkleeft, H. S.; van der Marel, G. A. *J. Org. Chem.* **2007**, *72*, 5737.