Metal free, "click and click–click" conjugation of ribonucleosides and 2′-OMe oligoribonucleotides on the solid phase†

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A fast and practical metal free conjugation of ribonucleosides and 2′-OMe 4-mer oligoribonucleotides has been accomplished by a nitrile oxide alkyne click cycloaddition reaction on the solid-phase, the methodology is suited to modification at either, or both, the 3′- or the 5′-terminus of the oligoribonucleotide substrate.

Chemically modified oligonucleotides are finding increasing application as therapeutic agents and as tools for genomic research.[1,2] The conjugation to an oligonucleotide of a group capable of cellular association or surface recognition is hailed as one potential solution to the problem of inefficient delivery of these therapeutic molecules.[3,4] Whilst a variety of methods describe conjugation to DNA,[5] modification to the chemically more sensitive RNA provides a greater challenge.[1] We wish here to report nitrile oxide alkyne click cycloaddition chemistry as an effective, fast and high yielding method for conjugation to ribonucleoside and 2′-OMe oligoribonucleotide substrates directly on a solid support. The Cu(I) catalysed azide–alkyne click reaction discovered by Meldal[6] and Sharpless[7] has become one of the most ubiquitous chemical ligation methods. It has recently been applied to monitor RNA transcription[8] and has been developed as a reliable method for DNA modification.[9] As a bioconjugation tool it has numerous advantages over the postsynthetic reaction of amino- or thioalkyl modified oligonucleotides with active esters; conjugate yields are often higher and product purification more facile. The azide–alkyne reaction generally introduces the ligating group at the nucleobase, along the backbone, or at the 5′-terminus. Conjugation at the oligonucleotide 3′-terminus is less well studied. However, during the preparation of this work a Cu(I) promoted, microwave assisted, method for 3′-,5′-bi-click DNA conjugation was reported.[10] Since copper mediated DNA functionalisation is sensitive to the availability of a sufficient amount of a proper copper(I) complexing ligand[11] an orthogonal click ligation avoiding the copper catalyst is highly desirable. Recently reported solid phase[12a,b] and solution phase[12c] nitrile oxide click modification of DNA by cycloaddition to alkynes and strained alkenes make important contributions to this field. Further examples of catalyst free click cycloadditions include the azide-ring strained cycloalkyne pairing[13a] and the photoinduced nitrile imine–alkene cycloadditions.[13b] The objective of the current work was to develop a solid phase, catalyst free, click methodology suited to 2′-OMe oligoribonucleotide modification at either the 3′- or the 5′-terminus, and suited to formation of 3′,5′-bisconjugates.
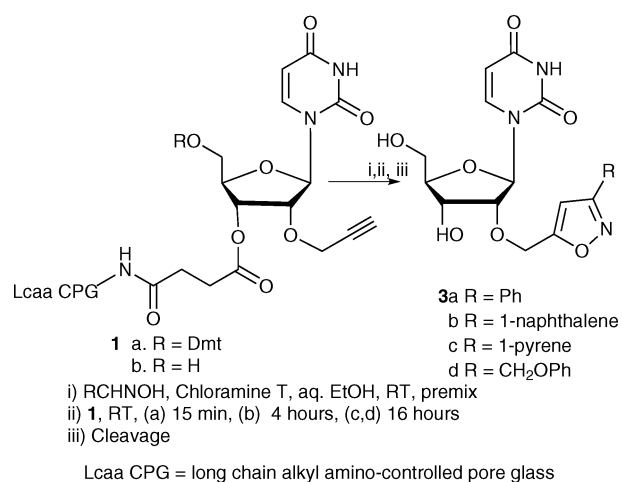
We selected the nitrile oxide–alkyne cycloaddition[14] as the conjugating reaction of choice. In the current context the chemical and biological stability of the resultant aromatic isoxazole nucleus is particularly important. Among the potential sites for oligonucleotide modification the 2′-position is attractive as substitution at this position locks the pentofuranose sugar in the $C3′$-*endo* conformation which is the structure preferred by the RNAi intracellular machinery.[15] Thus, from the outset we were interested in 2′-*O*-alkynylnucleoside substrates. 2′-Oligonucleotide conjugates are not well known, however, recent reports demonstrate 2′-*O*-(4-pentenyl) adenosines[16a] and 2′-*O*-propargyluridines[16b] as versatile building blocks for DNA conjugation; 2′-*O*-propargyluridine is also a successful click partner with azidoboron clusters.[17]

500 Å CPG-Succinyl 2′-*O*-propargyl nucleosides, with 5′-DMT protection and with standard base protecting groups were commercially available and the proposed nitrile oxide–alkyne click chemistry was initially tested on uridine. Benzaldehyde oxime was selected as the nitrile oxide precursor and chloramine-T as the dipole generating agent.[18] These components were premixed in aqueous EtOH (1:1) prior to exposure to an eppendorf tube containing the supported alkyne **1a**. After agitation at room temperature for 15 min the CPG was thoroughly washed prior to cleavage from the resin. The HPLC profile of the resulting reaction mixture indicated the desired click chemistry had failed. We considered steric demands of the 5′-DMT protecting group may have contributed to the lack of reaction. Gratifyingly, following deprotection, **1b** readily participated in reaction, and after 15 min reaction at room temperature, and following work-up, HPLC analysis indicated near quantitative conversion to the isoxazole–nucleoside conjugate **3a**, Scheme 1. Thus, the retention time of the ligated adduct **3a** is approximately 6 min longer than that of the parent propargyl uridine **2a** (Fig. 1). The MS data is in full agreement with the expected product [Found 402.1310; $(M + H)^+$ requires 402.1296].
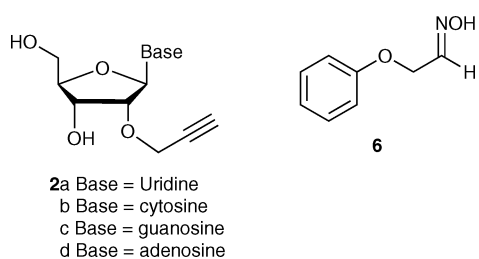
It is necessary to demonstrate the compatibility of the nitrile oxide alkyne click reaction with all natural RNA bases and before focusing on the purine bases we explored ligation of the supported N^{Bz}-2′-*O*-propargyl cytosine **4a**. Click conjugation of **4a** proceeded very efficiently under conditions identical to those described above for **1b**, Scheme 2. However, to show the future potential of this methodology for commercial RNA modification on an automated synthesizer the reaction was attempted directly on a synthesis column. Following DMT deprotection of the commercial material,

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Scheme 1 Solid supported click conjugation of 2′-O-propargyluridine.

Fig. 1 Structures of the cleaved and deprotected 2′-O-propargyl nucleosides **2a–d** and the aliphatic oxime **6**.

a near quantitative reaction. N^{Bz}-2′-O-Propargyl adenosine **4c** was treated similarly, again optimal conversion was found after 15 min at room temperature. HPLC analysis of the unpurified click product indicated that the major product is the isoxazole conjugate **5c** and there is little evidence for the presence of unreacted alkyne **2d**.

To demonstrate the scope of the reaction in terms of the acceptable range of nitrile oxide partners we experimented with naphthalene oxime and pyrene oxime, potentially capable of delivering ligated nucleosides valuable as molecular beacons.[19] Thus, support bound 2′-O-propargyl uridine **1b** was treated, in turn, with a premixed solution of each oxime and chloramine-T, Scheme 1. With an increase in the aromatic character and the steric bulk of naphthonitrile oxide the click reaction required 4 h to reach completion. Almost full conversion to the naphthalene ligated uridine **3b** was evidenced from the HPLC profile of the crude reaction product. With a further increase in hydrocarbon character 1-pyrene oxime was difficult to solubilise in 50% aq. EtOH, however, in 90% ethanol the click reaction progressed well. The rate of the reaction was again slow and HPLC analysis indicated complete reaction required 16 h at room temperature. MS analysis confirmed the structural integrity of both **3b** and **3c**.

Reaction with the nitrile oxide derived from 2-phenoxyacetaldehyde oxime **6** (Fig. 1) represents the first example of click modification of oligoribonucleotides with an aliphatic nitrile oxide thus revealing the true latitude of the approach, Scheme 1. After 16 h agitation of **1b** with premixed **6** and chloramine-T in aq. EtOH (3 : 2) conversion to the isoxazole ligated product was complete as evidenced by HPLC analysis of the cleaved product; product identity is confirmed by MS analysis. Given that lengthy synthetic procedures may be required to prepare desired biomarkers or bioconjugating groups bearing a masked aromatic nitrile oxide moiety the success of the reaction with an aliphatic nitrile oxide click partner is a significant advance over examples reported to-date.[12]

To address the need for the click reaction to be compatible with oligoribonucleotides the 5′-alkyne functionalized 2′-OMe 4-mer oligoribonucleotide **8** was targeted, Scheme 3. Thus, CPG-bound 2′-OMe-U_4 (U_4) was purchased from the market and following 5′-DMT deprotection the pendant alkyne was introduced by phosphoramidite coupling of **7**.[12a] HPLC analysis on the cleaved **9** indicated effective formation of the oligoribonucleotide–alkyne **8**. Exposure of **8** to premixed benzaldehyde oxime and

the column was thoroughly washed and dried. The resulting **4a** reacted on passing a pre-mixed solution of benzaldehyde oxime and chloramine-T between two syringes over a 15 min period. This procedure was repeated with a second, portion of reactants. Following deprotection and cleavage from the resin (NH₄OH), HPLC analysis showed a complete absence of any starting alkyne **2b** and near quantitative conversion to the isoxazole conjugate **5a**.
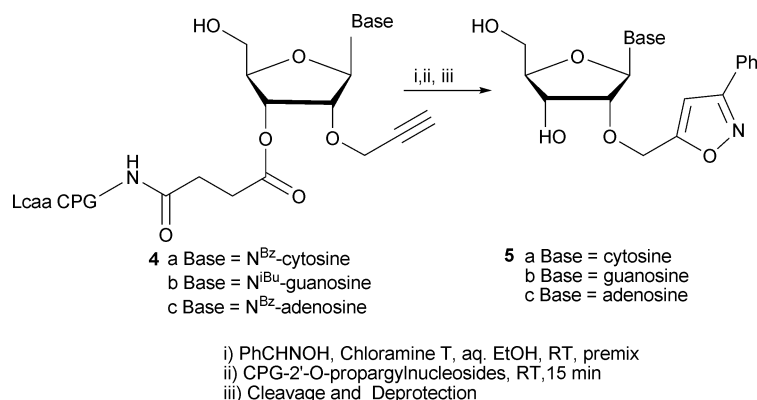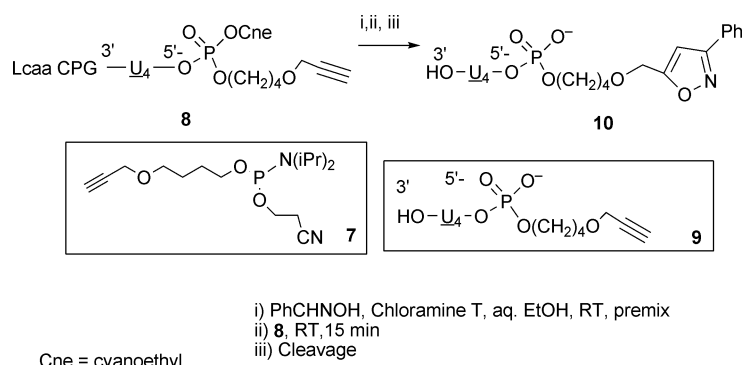
Gratifyingly, the purine nucleobases adenosine and guanosine also submit to the nitrile oxide alkyne click protocol, Scheme 2. Thus, reaction of N^{iBu}-2′-O-propargyl guanosine **4b** delivered the click product **5b** as evidenced from MS analysis. The presence of a small peak characteristic of the parent guanosine–alkyne **2c**, in the HPLC of the unpurified product is consistent with

Scheme 2 Solid supported click conjugation of 2′-O-propargylribonucleosides.
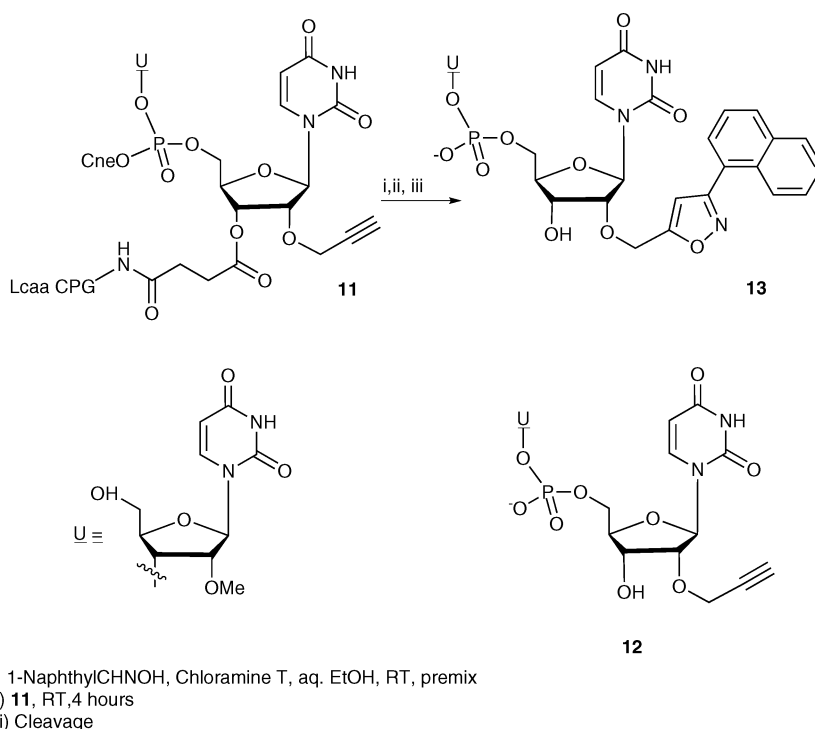
Scheme 3 Solid supported 5′-click conjugation of RNA–alkyne **8**.

chloramine-T in aq. ethanol (3:2) lead to formation of the isoxazole click product **10** in near quantitative yield as shown on HPLC analysis of the unpurified reaction mixture. MALDI TOF MS analysis confirmed the success of the reaction.

Whilst the modification of oligoribonucleotides at the 5′-terminus by nitrile oxide alkyne click cycloaddition represents an improvement over known methods, some applications will desire modification at the 3′-terminus,[1,20] this presents a greater synthetic challenge. Accordingly, the possibility to click functionalize an RNA at the terminus of choice is an important marker for the generality of our protocol. To this end, the support bound dinucleotide **11**, prepared by manual solid phase synthesis, was exposed to the click conjugation protocol, Scheme 4. The formation, after 4 h at room temperature, of the isoxazole ligated product in quantitative yield, was judged by comparison of the HPLC profile the parent alkyne **12** with the cleaved, but unpurified product **13**. LCMS TOF analysis confirmed the structural integrity of both the alkyne **12** and the conjugate **13**. The reaction is

important as it demonstrates the versatility of the approach for the preparation of 2′-OMe oligoribonucleotide conjugates functionalized at 5′- or the 3′-terminus.

The potential for high density functionalisation to tune the material properties of modified oligoribonucleotides is attractive[11b] and in this context, we wished to demonstrate the value of the nitrile oxide alkyne conjugation toolbox for click–click bisfunctionalisation with the substrate **14**, Scheme 5. The required bisalkyne was prepared by coupling of the phosphoramidite **7**[12a] to **1b**, HPLC analysis on **15** indicated effective formation of the support bound bisalkyne **14**. Subsequent exposure to the standard oxime, chloramine-T protocol resulted in near quantitative conversion of **14** to the click–click product as evidenced by HPLC analysis of the cleaved, but unpurified product **16**. LCMS TOF analysis confirmed the success of the double functionalisation reaction. With the high level of cycloaddition efficiency retained in the click–click reaction the potential of the nitrile oxide alkyne pairing for future application to high density functionalisation is apparent.

Scheme 4 Solid supported 3′-click conjugation of RNA–alkyne **11**.

i) PhCHNOH, Chloramine T, aq. EtOH, RT, premix
ii) **14**, RT, 15 min
iii) Cleavage
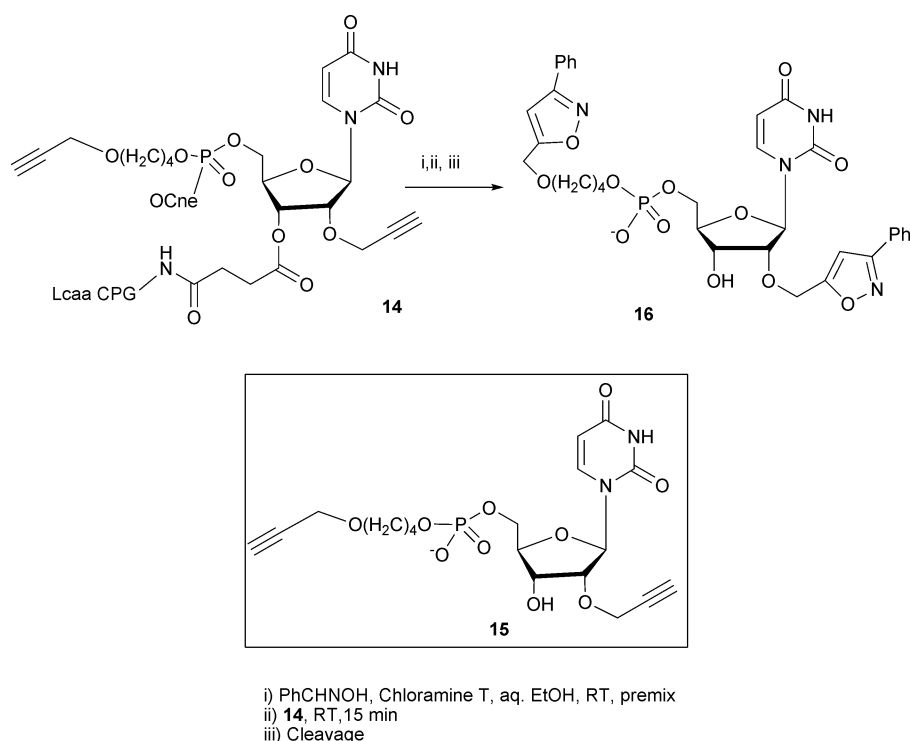
Scheme 5 Solid supported 3′,5′-click–click conjugation of RNA **14**.

In conclusion, we present a series of model reactions which illustrate that solid-phase nitrile oxide–alkyne click chemistry represents a convenient and fast approach towards a variety of ribonucleoside and short 2′-OMe oligoribonucleotide conjugates in an environment free of any metal catalyst. It is compatible with all four natural nucleobases bearing the standard protecting groups. It lends itself to the preparation of either 5′- or 3′-click-conjugates or 3′,5′-click–click conjugates. The reaction is very simple and could be applied to more complex conjugates so unveiling potential for future application in preparation of high density functionalised RNA conjugates, and in provision of substrates for genomic research.

Experimental

General experimental

Analytical TLC was performed on precoated (250 μm) silica gel 60 F254 plates from Merck. All plates were visualized by UV irradiation, and/or staining with 5% H_2SO_4 in ethanol followed by heating. Flash chromatography grade silica gel 60 (230–400 mesh) was obtained from Merck. Mass analysis was performed on an Ettan MALDI-TOF Pro from Amersham Biosciences with 2′,4′,6′-trihydroxyacetophenone as matrix or LCMS TOF from Agilent Technologies. The NMR spectra were obtained (¹H at 300 MHz and ¹³C at 75 MHz) on a Bruker instrument at 25 °C. Chemical shifts are reported in ppm downfield from TMS as standard. HPLC was carried out using a Gilson instrument equipped with a UV detector or a diode array detector and a Nucleosil C18 column. CPG alkynes were bought from Chem Genes. 5′-(4,4′-Dimethoxytrityl)-uridine-2′-O-methyl-3′-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite was pur-

chased from Link Technologies, UK. When required the 5′-DMT group was removed by treatment with dichloroacetic acid (2.5% in DCM) for 5 min followed by washed with acetonitrile and drying. All other chemical agents were purchased from Aldrich Chemical Company unless otherwise noted.

2-Phenoxyacetaldehye oxime 6

To a round bottomed flask containing 2-phenoxyacetaldehyde[21] (1.4 g, 10.2 mM) was added sodium acetate (1.8 g, 21.9 mM) and hydoxylamine hydrochloride (1.5 g, 21.4 mM) in ethanol : water (9 : 1, 40 ml) and the mixture was heated at reflux for 12 h. The product was isolated following extraction with dichloromethane (3 × 80 ml). The organic layer was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography over silica gel using hexane and ethyl acetate (8 : 2) as eluant to give the title compound as a white sticky solid in 56% yield (910 mg).

δ_H (300 MHz; CDCl₃) 8.69 (brs, 1H), 7.33–7.30 (m, 2H), 7.04–6.89 (m, 4H), 4.90 (d, J = 3.6 Hz, 2H); δ_C (75 MHz; CDCl₃) 158.0, 157.9, 150.1, 147.6, 129.6, 121.5, 121.4, 114.7, 114.4, 64.7, 61.8; HRMS (ESI) calcd for $C_8H_9NO_2$ 152.0706 [M + H]⁺; found 152.0704.

General procedure for phosphitylation reactions: preparation of 8, 11 and 14

To manually couple the alkyne phosphoramidite **7**[12a] or 5′-(4,4′-dimethoxytrityl)-uridine-2′-O-methyl-3′-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite to the oligoribonucleotide/nucleoside, 500 μL of phosphoramidite (100 mM in anhydrous CH₃CN) was added to the CPG–RNA or nucleoside (1 μmol)

along with 500 µL of benzylmercaptotetrazole in CH$_3$CN (0.3 M). The mixture was allowed to react for 15 min at RT with mixing between two syringes. This procedure was repeated with a second portion of each of a new solution of phosphoramidite and benzylmercaptotetrazole. The CPG was washed with CH$_3$CN (5 × 2 ml), oxidizer (700 µL, 0.1 M iodine solution in THF : pyridine : water; 78 : 20 : 2) and CH$_3$CN (2 × 5 mL) and dried yielding CPG–RNA–alkynes **8**, **11** and **14**. Cleavage from the resin proceeded by the method described below furnishing samples of **9**, **12** and **15** respectively.

General cleavage–deprotection procedure

For analytical purposes a portion of the RNA or nucleoside was deprotected and cleaved from the CPG by incubating the supported material in 40% aqueous CH$_3$NH$_2$ (500 µL) at 65 °C for 30 min or in the case of N^{Bz}-cytidine with conc. aqueous NH$_3$, ethanol 3 : 1 for 24 h at 25 °C. The CH$_3$NH$_2$/NH$_3$ was evaporated using a concentrator. The CPG was washed with H$_2$O (5 × 200 µL aliquots), all solutions and washings were combined to afford an aqueous solution of the RNA/nucleoside alkynes or cycloaddition products.

General method for HPLC analysis

The ribonucleosides and RNA conjugates were analyzed by reverse-phase HPLC with an analytical column (Nucleosil) under the following conditions; 200 µL injection loop. For RNA alkynes and RNA conjugates buffer A: 0.1 M TEAAc, pH 6.5, 5% (v/v) MeCN. Buffer B: 0.1 M TEAAc, pH 6.5, 65% (v/v) MeCN). Gradient; 0–5 min, 5% B; 5–20 min, 95% B; 20–28 min, 95% B, flow rate: 1.0 mL min^{-1}. For ribonucleoside alkynes and click conjugates of ribonucleosides the eluant was water and acetonitrile (0–5 min, 5% B; 5–15 min, 95% B, 15–25 min, 100% B, flow rate: 1.0 mL min^{-1} and detection at 260 nm or absorbance was monitored in the range 220–500 nm with a diode array detector and recorded at 260 nm.

General procedure for benzonitrile oxide click reactions on CPG–nucleoside–alkynes/CPG–RNA–alkynes 1a,b, 4a–4c, 8 and 14

To solid supported alkynes **1a,b**, **4a–4c**, **8** and **14** (0.5 µM) in an eppendorf tube was added a premixed solution of benzaldehyde oxime (40 mg) and chloramine-T monohydrate (75 mg) which had been premixed in ethanol (500 µL) and water (500 µL). The combined mixture was agitated at room temperature for 15 min. Following settling the supernatant liquid was removed by syringe and the CPG washed firstly with CH$_3$CN (5 × 300 µL) and then H$_2$O (5 × 300 µL). Deprotection–cleavage and HPLC analysis followed by the procedures described above.

Click on the column-preparation of isoxazole conjugate 5a

The click conjugation on N^{Bz}-2′-O-propargyl cytosine **4a** was performed directly on the column subsequent to the 5′-DMT deprotection of the commercial material. The resulting **4a** was reacted for 15 min at room temperature by passing an aqueous ethanolic, pre-mixed solution of benzaldehyde oxime (40 mg) and chloramine-T monohydrate (75 mg) between two syringes. This procedure was repeated with a second, new, portion of pre-mixed oxime and chloramine-T monohydrate. Following deprotection

and cleavage from the resin (NH$_4$OH), HPLC analysis indicated almost quantitative conversion to the isoxazole–nucleotide conjugate **5a**.

Procedure for naphthalene-1-nitrile oxide click reaction on CPG-alkyne 1b and 11

To solid supported alkynes **1b** or **11** and (0.5 µM) in an eppendorf tube was added a premixed solution (10 min) of 1-naphthylaldehyde oxime[22] (45 mg) and chloramine-T monohydrate (59 mg) in ethanol (500 µL) and water (500 µL). The mixture was agitated at room temperature for 4 h. Following settling the supernatant liquid was removed by syringe and the CPG washed firstly with CH$_3$CN (5 × 300 µL) and then H$_2$O (5 × 300 µL). Cleavage from the CPG and HPLC analysis followed by the procedures described above.

Procedure for pyrene-1-nitrile oxide click reaction on 1b

To solid supported alkyne **1b** (0.2 µM) in an eppendorf tube was added a solution of 1-phenoxyacetaldehyde oxime[23] (16 mg) in ethanol (900 µL) followed by water (100 µL) and chloramine-T monohydrate (15 mg). The combined mixture was agitated at room temperature for 16 h. Workup, cleavage and HPLC analysis followed by the procedures described above.

Procedure for phenoxyacetaldehyde-1-nitrile oxide click reaction on 1b

To solid supported alkyne **1b** (0.2 µM) in an eppendorf tube was added a solution of 1-phenoxyacetaldehydeoxime **6** (15 mg) in ethanol (400 µL) followed by water (600 µL) and chloramine-T monohydrate (23 mg). The combined mixture was agitated at room temperature for 16 h. Workup, cleavage and HPLC analysis followed by the procedures described above.

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