



Conjugation at the oligonucleotide level based on isoxazole phosphoramidites generated by click chemistry

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ABSTRACT

The versatility of the isoxazole generating nitrile oxide–alkyne Huisgen cycloaddition for provision of chemically modified oligonucleotides has been extended; in a novel approach isoxazole conjugated oligodeoxyribonucleotides have been constructed by phosphoramidite chemistry of isoxazole derivatives previously generated by nitrile oxide–alkyne click chemistry. The conjugation involves manual solid phase synthesis at room temperature in aqueous ethanol and proceeds in high yield.

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1. Introduction

Huisgen cycloaddition reactions performed on DNA substrates represent a powerful approach to chemically modified oligonucleotides, in particular the Cu(I) catalysed azide–alkyne triazole forming reaction has received much attention.¹ However, we and others have reported attributes of the [3+2]-cycloaddition reaction of nitrile oxides and nitrones as a viable, catalyst free, regioselective alternative for the formation of functionalised nucleosides and oligonucleotides.^{2–6} In previously reported work the alkyne partner was introduced to the resin supported oligonucleotide, and subsequent cycloaddition was conducted directly on the solid phase. The objective of the current work was to develop an alternative, modular strategy, whereby modified oligonucleotides could be constructed by coupling of isoxazole phosphoramidite building blocks previously generated by click chemistry. The alternative strategies, where the key cycloaddition reaction is conducted on-, or off-resin, are shown retrosynthetically, in Fig. 1. The attractions of on-resin click chemistry for oligonucleotide conjugation are already well known.¹ Potential benefits of the off-resin approach, (a), may include ease of operation, particularly with regard to scale-up and functional group compatibility. Dipolar cycloadditions present a robust synthetic strategy for heterocycle formation, and it is the hypothesis of this paper that if either the dipole or the dipolarophile have a pendant hydroxy group, then following cycloaddition and phosphoramidite formation oligonucleotide conjugation can be achieved in a catalyst free environment. Certainly off-resin cycloaddition will serve to increase the versatility of ‘click chemistry’ for oligonucleotide conjugation and it could be used as

a complement to solid phase oximation or CuAAC (copper catalysed azide alkyne cycloaddition) approaches to DNA conjugates and, in theory, be applied for the reliable introduction of a variety of reporting or targeting groups into DNA.

Approaches to chemically modified oligonucleotides using building blocks previously prepared by CuAAC chemistry have been reported. In one application, oligodeoxyribonucleotide glycoconjugates have been generated by solid-supported oximation of aminoxy terminated oligonucleotides with glycoclusters constructed by click chemistry.⁷ In other applications phosphoramidite derivatives of click chemistry derived triazole monomers have been utilised, most frequently the triazole moiety is borne at the C-5 position of functionalised pyrimidine bases including deoxyuridines,^{8–10} cytidines¹¹ and thymidines,¹² however, the triazole unit has also been sited at the 5'-position of the sugar¹³ and as a spacer between the base and sugar moieties.¹⁴ Finally, non-canonical¹⁵ and non-nucleosidic¹⁶ triazole phosphoramidites have also been reported. In contrast, whilst isoxazoles are an interesting heterocyclic family with diverse applications, isoxazole bearing phosphoramidites are not well known. To the best of our knowledge, the D-threoninol derived **1**, Fig. 2, prepared for inclusion in a library of cyclic oligonucleotides targeted to the Hepatitis C virus, NS5B, represents the only known example.¹⁷

2. Results and discussions

The retrosynthetic approach set out in Fig. 1(a) requires isoxazole building blocks equipped with a pendant hydroxy functionality to act as a handle for ultimate conversion to phosphoramidite monomers. We intended to prepare the isoxazole from an in situ generated, nitrile oxide–alkyne cycloaddition, (NOAC), and believed the isomeric hydroxyethoxybenzaldehyde oximes **2a–c** represented an ideal platform to explore the concept

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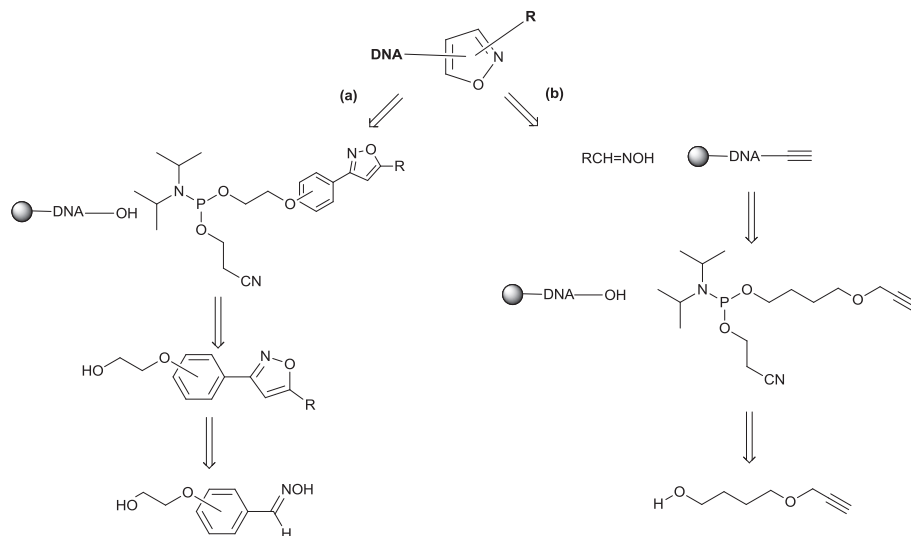


Fig. 1. Retrosynthetic approaches to chemically modified oligonucleotides involving (a) off-resin and (b) on-resin nitrile oxide–alkyne cycloaddition (NOAC) chemistry.

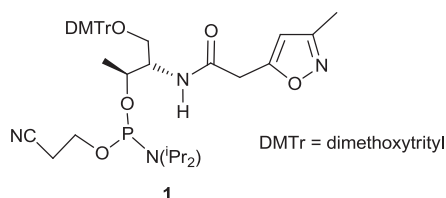


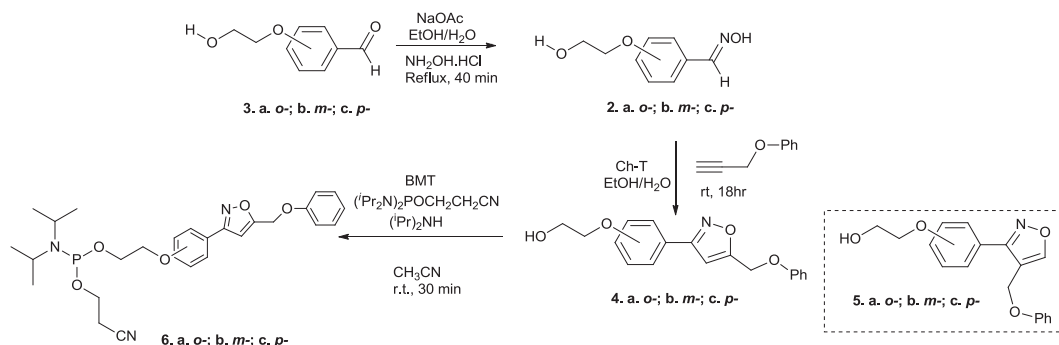
Fig. 2. Isoxazole phosphoramidite, **1**.¹⁷

since all three regioisomers, with their varying steric demands, could be obtained.

The desired oximes **2** were prepared in excellent yields (88–96%) from commercially available aldehyde precursors **3** following reaction with hydroxylamine hydrochloride in the presence of sodium acetate. For each oxime, in situ generation of the corresponding nitrile oxide was achieved through reaction with chloramine-T in aqueous ethanol. The transient dipole was trapped with propargyl phenyl ether and the cycloaddition products, **4a–c**, were obtained in 81–85% isolated yield following stirring at room temperature, Scheme 1. In each case reaction was, as expected, highly selective for formation of the 5-substituted isomer. Regiochemical assignment of the major isomer follows from the resonance position of the isoxazole ring proton, which presents as a singlet in the range 6.59–6.76 ppm. This chemical shift is typical of an isoxazole H-4 proton; a proton at the C-5 position is expected to resonate more downfield, somewhere in the region of

7.50–8.50 ppm,^{18–20} indeed, in each case low intensity signals, at ~8.5 ppm in the ¹H NMR spectrum of the crude cycloaddition products provides evidence for minor amounts of the 4-substituted regioisomers **5**. Further, singlet resonances appearing at ~4.9 ppm, are deemed characteristic of the methylene protons adjacent to the isoxazole ring. The regioselectivity of the reaction ranges from 44:1 to 27:1 in favour of the 5-substituted isomers **4**.

The regioisomeric phosphoramidite monomers **6** were prepared, in reasonable yields, from the corresponding isoxazole building blocks **4** following reaction with 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite in anhydrous acetonitrile. Benzylmercaptotetrazole (BMT) was employed as activator in the presence of diisopropylamine (Scheme 1). Following 30 min at room temperature, TLC analysis indicated complete consumption of the starting alcohol. ³¹P NMR spectroscopy demonstrated phosphorus resonances at ~148–149 ppm, supporting formation of the desired monomers. Purification of phosphoramidites by flash column chromatography can be tricky; to avoid decomposition and erosion in yield, rapid elution under a stream of an inert gas is required. For synthetic purposes we have found **6a–c** were suitable for manual solid phase coupling without purification. The unpurified isoxazole phosphoramidite monomers, **6**, were introduced to resin bound nucleoside/oligonucleotide substrates (Scheme 2). Initial experimentation focused on reaction between CPG-(controlled pore glass)-supported thymidine and the *o*-substituted isoxazole phosphoramidite **6a**. Reaction was achieved by manual solid phase coupling on a 1 μmol scale. The thymidine loaded resin,

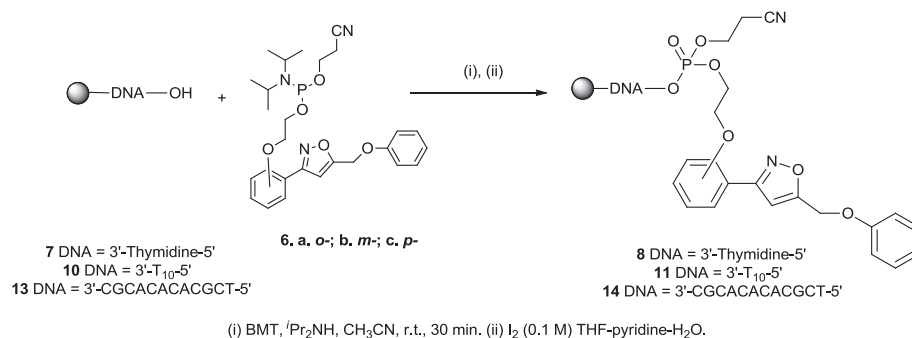


Scheme 1. Synthesis of the phosphoramidite monomers **6**.

7, was placed in a DNA synthesis column and a 1 mL syringe containing an acetonitrile solution of the required phosphoramidite was attached to one end. A second syringe, containing a solution of the activator, benzylmercaptotetrazole, was attached to the other end. The solutions were passed through the column over a period of 15 min with mixing between the two syringes. To ensure complete reaction the procedure was repeated with a second portion of each of a new solution of the phosphoramidite and the activator. Following the second 15 min period of mixing at room temperature oxidation preceded cleavage of the reaction product from the resin **8a**. The coupling yield of crude product **9**, Fig. 3, was judged from the HPLC profile of the raw material to be higher than 95%.

four DNA bases with their standard protecting groups, was selected to establish the general applicability of the concept. As described above, during a 30 min period, separate solutions of the phosphoramidite monomer **6a** and the activator were manually passed over the resin-supported material **13** contained in a DNA synthesis column. The oligonucleotide was then deprotected and removed from the solid support **14a** following treatment with ammonium hydroxide (24 h, room temperature). Significantly, HPLC analysis of the unpurified reaction product indicated quantitative conversion of **13** to **15a**, Fig. 4.

In a further examination of the generality of this approach the regioisomeric phosphoramidites **6b** and **6c** were successfully in-



Scheme 2. Solid phase synthesis of chemically modified oligonucleotides using previously clicked isoxazole phosphoramidite monomers.

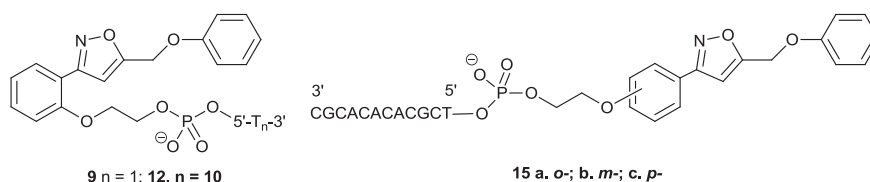


Fig. 3. Structures of isoxazole modified oligonucleotides.

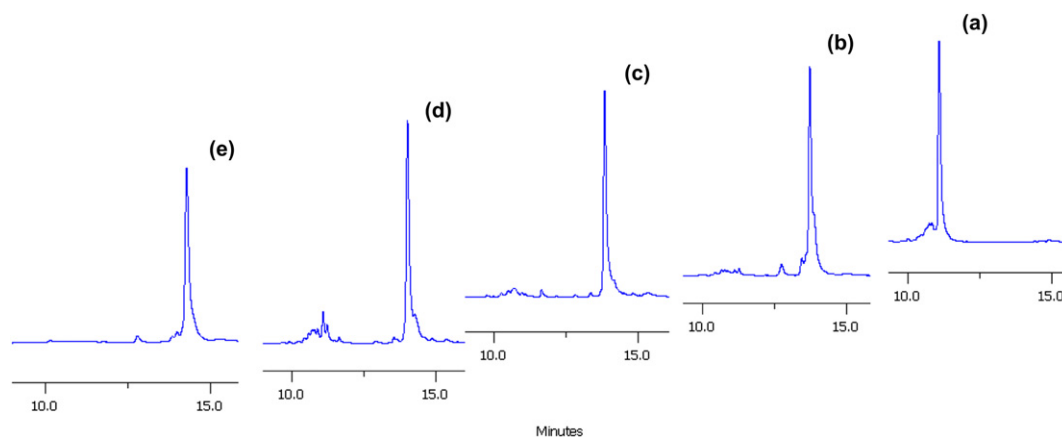


Fig. 4. HPLC profiles of (a) reference sample of 3'-CGCACACACGCT-OH-5', (b) (c) and (d), conjugates **15a**, **15b** and **15c** derived, respectively, from isoxazole phosphoramidites **6a–c** and prepared by manual solid phase synthesis (e) **15c** prepared on a DNA synthesizer.

To establish compatibility with an oligonucleotide, reaction with CPG-loaded decathymidine, **10**, was examined. Gratifyingly, under the same conditions, reaction with the 10-mer substrate was found to proceed smoothly, and following cleavage of the DNA from **11a** HPLC analysis of the unpurified reaction product indicated **12** was present in essentially quantitative yield.

To further probe the scope of the reaction a CPG-supported 12-mer oligonucleotide **13** was examined. The sequence, bearing all

incorporated into the resin-supported mixed base 12-mer **13** by manual solid phase synthesis. In each case, cleavage and deprotection of the supported products, **14b,c** was effected by treatment with ammonium hydroxide. HPLC analysis, Fig. 4, confirmed complete consumption of starting material and selective formation of the products **15b,c**.

The potential to automate the coupling was established on an Expedite DNA synthesizer using purified samples of the isoxazole

phosphoramidites. The conjugate **15c** was formed in excess of 90% yield from reaction between **6c** and resin supported **13** (0.2 μ mol) using the standard instrument protocol, viz. 37 equiv of phosphoramidite and 1.5 min coupling time. However, to achieve the same level of conjugation with the sterically more demanding substrates **6a** and **6b** more generous conditions were required, viz. 50 equiv of phosphoramidite and 15 min coupling time. HPLC analysis shows a favourable comparison between the manual and automated approaches; in all cases, greater than 90% incorporation of the non-natural phosphoramidites was observed; comparative data are shown for samples of **15c** prepared by both approaches, Fig. 4, (d) and (e).

All new conjugates, **9a**, **12a** and **15a–c** derived from the isomeric phosphoramidites **6** were characterized by mass spectrometry (ESI-MS or MALDI-TOF MS), and most importantly, in all cases yields of conjugates derived from isoxazoles generated off-resin, Fig. 1 (a), compare favourably with those obtained for similar conjugates constructed by on-resin click cycloaddition chemistry with alkyne modified DNA substrates, Fig. 1, path (b).⁴

3. Conclusion

Novel isoxazole building blocks were generated by Cu-free nitrile oxide–alkyne cycloadditions. Following conversion to phosphoramidites, incorporation into oligonucleotides has been achieved manually on the solid phase and on a DNA synthesizer employing a standard protocol. The coupling reaction is efficient both at the monomeric level (CPG-Thymidine) and for oligonucleotides (CPG-12-mer DNA). Whilst on-resin functionalisation of alkyne modified oligonucleotides by nitrile oxide or CuAAC click chemistry is already established as a powerful method for oligonucleotide conjugation, for some applications it may be more attractive to assemble the conjugate from phosphoramidite building blocks previously prepared by click chemistry. Possible advantages may ensue in terms of cost, ease of operation and enhanced potential for scale-up.²¹

4. General experimental

Analytical TLC was performed on precoated (250 μ m) silica gel 60 F₂₅₄ plates from Merck. All plates were visualized by UV irradiation, and/or staining with 5% H₂SO₄ in ethanol followed by heating. Flash chromatography was performed using silica gel 32–63 μ m, 60 Å. Mass analysis was performed on an Agilent Technologies 6410 Time of Flight LC/MS. MALDI-TOF mass data was acquired on an Applied Biosystem Voyager instrument or a LASER-TOF LT3 from Scientific Analytical Instruments with 3-hydroxypicolinic acid or 2',4',6'-trihydroxyacetophenone as matrix. NMR spectra were obtained on a Bruker instrument at 25 °C (¹H at 300 MHz; ¹³C at 75 MHz; ³¹P at 121 MHz). Chemical shifts are reported in parts per million downfield from TMS as standard. All NMR spectra are recorded in CDCl₃. HPLC was carried out using a Gilson instrument equipped with a diode array detector and a Nucleosil C18 column (4.0×250 mm). Automated oligonucleotide synthesis was carried out on an Expedite nucleic acid synthesis system.

4.1. General procedure for preparation of oximes 2a–c

A solution of the required aryl aldehyde **3a–c** (1.00 g, 6.02 mmol) and hydroxylamine hydrochloride (1.26 g, 18.1 mmol) in EtOH (35 mL) was stirred at room temperature for 10 min, after which a solution of sodium acetate (1.69 g, 24.1 mmol) in H₂O (15 mL) was added. The mixture was heated under reflux for 40 min. Following solvent removal under reduced pressure the crude product, obtained as a white solid, was taken up in EtOAc

(30 mL), washed with H₂O (3×20 mL) and dried over anhydrous magnesium sulfate. Removal of the solvent under reduced pressure yielded the pure product as a white solid in excellent yield.

4.1.1. 2-(2-Hydroxyethoxy)benzaldehyde oxime, 2a. White solid (96%), mp 98–100 °C, *R*_f=0.44 (hexane/EtOAc, 3:7); ¹H NMR δ 8.39 (s, 1H, HC=N), 7.54 (dd, *J*=7.7, 1.7 Hz, 1H, ArH), 7.38–7.32 (m, 1H, ArH), 7.04–6.95 (m, 2H, ArH), 4.17 (t, *J*=4.2 Hz, 2H, CH₂), 3.98 (t, *J*=4.2 Hz, 2H, CH₂); ¹³C NMR δ 157.0, 147.8, 131.2, 128.9, 121.6, 121.2, 113.7, 70.7, 61.2; IR (KBr) 3388, 3219, 1601, 1251, 764 cm⁻¹; HRMS (ESI) calcd for [M+Na]⁺, C₉H₁₁NO₃Na, 204.0631, found 204.0640.

4.1.2. 3-(2-Hydroxyethoxy)benzaldehyde oxime, 2b. White solid (86%), mp 94–96 °C, *R*_f=0.44 (hexane/EtOAc, 3:7); ¹H NMR δ 8.10 (s, 1H, HC=N), 7.59 (br s, 1H, OH), 7.33–7.28 (m, 1H, ArH), 7.19–7.13 (m, 2H, ArH), 6.96 (dd, *J*=9.0, 2.4 Hz, 1H, ArH), 4.12 (t, *J*=4.2 Hz, 2H, CH₂), 3.98 (t, *J*=4.2 Hz, 2H, CH₂); ¹³C NMR δ 156.9, 147.7, 131.2, 128.8, 121.6, 121.2, 113.6, 70.6, 61.2; IR (KBr) 3350, 3154, 1595, 1262, 907, 795 cm⁻¹; HRMS (ESI) calcd for [M+H]⁺, C₉H₁₂NO₃, 182.0812, found 182.0809.

4.1.3. 4-(2-Hydroxyethoxy)benzaldehyde oxime, 2c. White solid (88%), mp 109–111 °C, *R*_f=0.40 (hexane/EtOAc, 3:7); ¹H NMR δ 8.09 (s, 1H, HC=N), 7.51 (d, *J*=8.8 Hz, 2H, ArH), 7.32 (br s, 1H, OH), 6.93 (d, *J*=8.8 Hz, 2H, ArH), 4.12 (t, *J*=4.8 Hz, 2H, CH₂), 3.98 (t, *J*=4.8 Hz, 2H, CH₂); ¹³C NMR δ 160.1, 149.9, 128.5, 125.2, 114.8, 69.3, 61.4; IR (KBr) 3262, 1605, 1259, 836 cm⁻¹; HRMS (ESI) calcd for C₉H₁₁NO₃Na, [M+Na]⁺, 204.0631, found 204.0622.

4.2. General procedure for preparation of cycloadducts 4

The required oxime **2a–c** (500 mg, 2.76 mmol) was dissolved in EtOH (2.5 mL) and to this solution was added propargyl phenyl ether (182 mg, 1.38 mmol), chloramine T (785 mg, 3.45 mmol) and H₂O (2.5 mL). The mixture was stirred for 2 h at room temperature after which analysis by TLC (hexane/EtOAc, 3:7) indicated complete reaction. After removal of the solvent under reduced pressure, the residue was taken up in EtOAc (30 mL) and the solution was washed with 5% NaOH (3×10 mL). The organic layer was dried over anhydrous magnesium sulfate and the solvent was removed under reduced pressure. The crude products were purified by flash column chromatography (hexane/EtOAc, 3:7) to give the cycloadduct in good yield.

4.2.1. 2-{2-[5-(Phenoxymethyl)isoxazol-3-yl]phenoxy}ethanol, 4a. Colourless oil (81%), *R*_f=0.54 (hexane/EtOAc, 3:7); ¹H NMR δ 7.71 (dd, *J*=7.6, 1.3 Hz, 1H, ArH), 7.42–7.29 (m, 3H, ArH), 7.08–6.97 (m, 5H, ArH), 6.76 (s, 1H, isox-H), 5.20 (s, 2H, CH₂), 4.18 (t, *J*=4.2 Hz, 2H, CH₂), 3.92 (br s, 2H, CH₂), 3.24 (br s, 1H, OH); ¹³C NMR δ 167.4, 160.4, 157.8, 156.7, 131.4, 129.8, 129.7, 121.8, 121.6, 118.4, 114.8, 113.9, 104.2, 70.9, 61.3, 61.1; IR (film) 3400, 1601, 1245, 755 cm⁻¹; HRMS (ESI) calcd for C₁₈H₁₈NO₄, [M+H]⁺, 312.1230, found 312.1242.

4.2.2. 2-{3-[5-(Phenoxymethyl)isoxazol-3-yl]phenoxy}ethanol, 4b. White solid (81%), mp 70–72 °C, *R*_f=0.52 (hexane/EtOAc, 3:7); ¹H NMR δ 7.39–7.28 (m, 5H, ArH), 7.03–6.96 (m, 4H, ArH), 6.61 (s, 1H, isox-H), 5.17 (s, 2H, CH₂), 4.12 (t, *J*=4.2 Hz, 2H, CH₂), 3.96 (br s, 2H, CH₂), 2.42 (br s, 1H, OH); ¹³C NMR δ 168.7, 162.4, 159.1, 157.8, 130.1, 130.0, 129.7, 121.9, 119.8, 116.8, 114.8, 112.5, 101.5, 69.4, 61.4, 61.3 cm⁻¹; IR (KBr) 3208, 1600, 1260, 1231, 885, 755 cm⁻¹; HRMS (ESI) calcd for C₁₈H₁₈NO₄, [M+H]⁺, 312.1230, found 312.1233.

4.2.3. 2-{4-[5-(Phenoxymethyl)isoxazol-3-yl]phenoxy}ethanol, 4c. White solid (85%), mp 95–97 °C, *R*_f=0.49, (hexane/EtOAc, 3:7); ¹H NMR δ 7.74 (d, *J*=8.6 Hz, 2H, ArH), 7.32 (t, *J*=7.7 Hz, 2H, ArH),

7.04–6.98 (m, 5H, ArH), 6.59 (s, 1H, isox-H), 5.20 (s, 2H, CH₂), 4.13 (t, *J*=4.2 Hz, 2H, CH₂), 3.99 (t, *J*=4.2 Hz, 2H, CH₂), 2.04 (br s, 1H, OH); ¹³C NMR δ 168.4, 162.1, 160.1, 157.8, 129.7, 128.3, 121.9, 121.7, 114.9, 114.8, 101.1, 69.3, 61.5, 61.4; IR (KBr), 3438, 1613, 1258, 1238, 842 cm⁻¹; HRMS (ESI) calcd for C₁₈H₁₈NO₄, [M+H]⁺, 312.1230, found 312.1245.

4.3. General procedure for preparation of phosphoramidites 6

The required cycloadduct **4a–c** (100 mg, 0.321 mmol), and benzylmercaptotetrazole (31 mg, 0.159 mmol) were placed in a dried round bottomed flask under an argon atmosphere.

Anhydrous acetonitrile (5 mL) was added to the flask followed by diisopropylamine (23 μL, 0.162 mmol) and 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite (112 μL, 0.353 mmol). The reaction mixture was stirred for 30 min at room temperature after which TLC analysis (hexane/EtOAc, 3:7) showed complete consumption of the starting alcohol. The reaction mixture was diluted with ethyl acetate (25 mL) and washed with aqueous sodium bicarbonate (10×3 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to give the crude phosphoramidite, which was purified by flash column chromatography (hexane/EtOAc, 3:7) with elution under a positive pressure of nitrogen gas.

4.3.1. 2-Cyanoethyl 2-[2-[5-(phenoxymethyl)isoxazol-3-yl]phenoxy]ethyl *N,N*-diisopropylphosphoramidite, **6a.** Colourless oil, *R*_f=0.83 (hexane/EtOAc, 3:7); ¹H NMR δ 7.94 (dd, *J*=7.7, 1.7 Hz, 1H, ArH), 7.43–7.31 (m, 3H, ArH), 7.07–6.98 (m, 6H, ArH and isox-H), 5.19 (s, 2H, CH₂), 4.25–4.22 (m, 2H), 4.16–3.54 (m, 6H), 2.55–2.51 (m, 2H), 1.19–1.14 (m, 12H); ³¹P NMR δ 148.8.

4.3.2. 2-Cyanoethyl 2-[3-[5-(phenoxymethyl)isoxazol-3-yl]phenoxy]ethyl *N,N*-diisopropylphosphoramidite, **6b.** Colourless oil, *R*_f=0.83 (hexane/EtOAc, 3:7); ¹H δ NMR 7.39–7.32 (m, 5H, ArH), 7.05–6.98 (m, 4H, ArH), 6.64 (s, 1H, isox-H), 5.21 (s, 2H, CH₂), 4.20 (t, *J*=5.1 Hz, 2H), 4.10–3.49 (m, 6H), 2.63 (t, *J*=6.4 Hz, 2H), 1.20 (dd, *J*=6.8, 2.1 Hz, 12H); ³¹P NMR δ 149.1.

4.3.3. 2-Cyanoethyl 2-[4-[5-(phenoxymethyl)isoxazol-3-yl]phenoxy]ethyl *N,N*-diisopropylphosphoramidite, **6c.** Colourless oil, *R*_f=0.83 (hexane/EtOAc, 3:7); ¹H NMR δ 7.73 (d, *J*=8.7 Hz, 2H, ArH), 7.32 (t, *J*=7.6 Hz, 2H, ArH), 7.04–6.95 (m, 5H, ArH), 6.59 (s, 1H, isox-H) 5.20 (s, 2H, CH₂), 4.29 (t, *J*=5.1 Hz, 2H, CH₂), 4.10–3.77 (m, 4H), 3.70–3.57 (m, 2H), 2.63 (t, *J*=6.5 Hz, 2H), 1.20 (dd, *J*=6.8, 2.9 Hz, 12H); ³¹P NMR δ 149.0.

4.4. General procedure for the phosphitylation of CPG-DNA-OH 5', preparation of **8**, **11** and **14a–c**

Option 1. To manually couple the phosphoramidites **6a–c** to the resin supported nucleoside/oligonucleotide, **7**, **10** or **13**, separate solutions of the phosphoramidite (500 μL, 100 mM in anhyd CH₃CN) and of benzylmercaptotetrazole (500 μL, 0.3 mM in anhyd CH₃CN), both in 1 mL syringes were attached to either end of a DNA synthesis column containing CPG-DNA (1 μmol). The mixture was reacted for 15 min at room temperature with mixing between the two syringes. This reaction was repeated with a second portion of each of a new solution of the phosphoramidite and benzylmercaptotetrazole. The CPG was washed with CH₃CN (5×2 mL) prior to treatment with oxidizer (700 μL, 0.1 M Iodine solution in THF/pyridine/water; 78:20:2). Further washing with CH₃CN (2×5 mL) and drying yielded CPG-DNA-isoxazole conjugates **8**, **11** and **14a–c**.

Option 2. Automated coupling of the phosphoramidites **6a–c** to the resin supported oligonucleotide, **13**, was achieved using an Expedite Nucleic Acid Synthesis System using standard instrument

protocols. All reactions were conducted on a scale involving CPG-DNA (0.2 μmol). For coupling **6a,b** 50 equiv of phosphoramidite were used and 15 min coupling time. For coupling **6c** 37 equiv of phosphoramidite were used and 1.5 min coupling time.

4.5. General deprotection procedure, preparation of **9**, **12** and **15a–c**

For analytical purposes a portion of the DNA was deprotected and cleaved from the CPG by incubating the CPG-DNA in either

- 40% aqueous CH₃NH₂ (500 μL) at 65 °C for 30 min (for substrates **8** and **11**)
- 28% aqueous NH₄OH (1 mL) at 25 °C for 24 h (for substrates **14a–c**)

As appropriate, CH₃NH₂/NH₄OH was evaporated using a concentrator. The CPG was washed with H₂O (3×150 μL aliquots), all solutions and washings were combined to afford an aqueous solution of DNA-alkynes **9**, **12** and **15a–c**. On analysis by HPLC retention times (*t*_R) of the starting nucleosides/oligonucleotides and the modified conjugates are as follows.

Thymidine *t*_R=22.7 min, **9** *t*_R=16.5 min.

Decathymidine *t*_R=12.3 min, **12** *t*_R=16.0 min.

12-mer 3'-CGCACACGCT-OH-5' *t*_R=10.4 min, **15a** *t*_R=13.8 min, **15b** *t*_R=13.9 min, **15c** *t*_R=14.0 min.

Compound **9** HRMS (ESI) *m/z* calcd for 638.1510 [M+Na]⁺; found 638.1534.

Compound **12** MALDI-TOF-MS *m/z*: calcd 3353; found 3353.

Compound **15a** MALDI-TOF-MS *m/z*: calcd 3948; found 3949.

Compound **15b** MALDI-TOF-MS *m/z*: calcd 3948; found 3949.

Compound **15c** MALDI-TOF-MS *m/z*: calcd 3948; found 3949.

4.6. General method for HPLC analysis

Nucleoside and oligonucleotide conjugates were analyzed by reverse-phase HPLC under the following conditions; 200 μL injection loop; 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: 0–4.3 min, 5% B; 4.3–16.6 min, 5–100% B; flow rate: 1.0 mL/min and detection at 260 nm.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2011.06.096. These data include MOL files and InChIKeys of the most important compounds described in this article.

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