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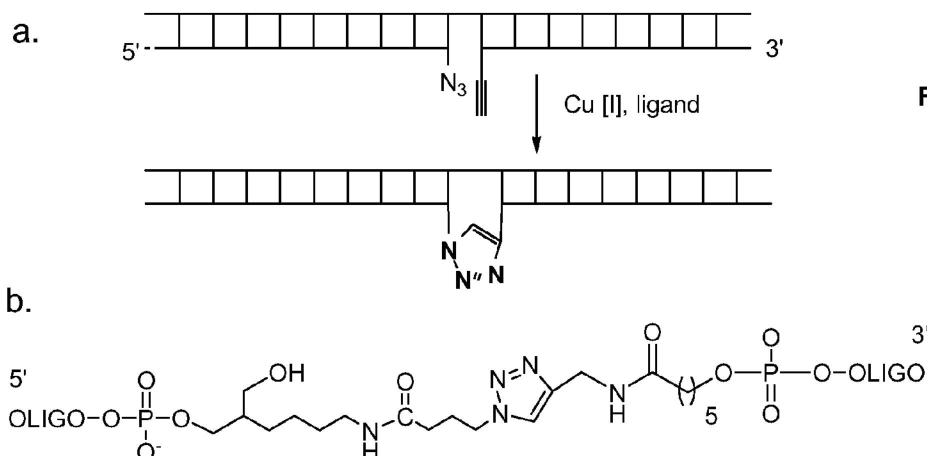

(57) Abstract: Use of azide/alkyne coupling (click ligation) for oligonucleotide circularisation is proposed for both therapeutic and nanotechnology applications. Such non-templated single-strand circularisation may be the first step in building a more complex catenane by further templated-directed click ligation of olignucleotide sequences, e.g. a double- stranded cyclic oligonucleotide or pseudo-hexagon. Circularisation using click ligation may be used to improve stability of therapeutic oligonucleotides to enzyme degradation *in vivo.*

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Modified Nucleic Acids

This invention relates to modified nucleic acids and processes for making modified
5 nucleic acids. More particularly, it relates to circularisation of oligonucleotides by use of
an azide-alkyne coupling reaction. The circularised oligonucleotides made according to
this invention have utility in biological and nanotechnological applications. Azide-alkyne
coupling is proposed for stabilisation of therapeutic oligonucleotides against in vivo
degradation, including improved serum stability, and for use in improving provision of
10 oligonucleotides into cells.

Background to the invention

There is currently considerable interest in the properties and applications of
15 oligonucleotide (ODN) constructs with unusual and unique topologies (see for example
S.H. Park et al., Angewandte Chemie-International Edition, 45, 735 and 6607 (2006); P.
W. K. Rothemund, Nature, 440, 29 (2006); N.C. Seeman, Nature, 421, 427 (2003); N.C.
Seeman and A.M. Belcher, Proc. Natl. Acad. Sci., 99, 6451 (2002)). This interest stems
partly from the fact that simple DNA nanostructures that are held together only by
20 Watson-Crick base-pairing suffer from the disadvantage that they can be easily
disassembled by heat and/or various denaturing agents. Therefore they have to be
analysed under non-denaturing conditions and this can make their characterisation and
purification problematic (F.A. Aldaye and H.F. Sleiman, Angewandte Chemie-
International Edition, 45 (14), 2204 (2006)).

25

In nanotechnology, the term "bottom-up" fabrication or synthesis is a term coined to refer
to techniques in which molecules are constructed not ab initio using complex, external
technology to build chemical systems from small molecules but, rather, to assemble
chemical systems by making use of the ability of certain (particularly biological)
30 molecules to direct their own construction and organisation. Polynucleotides and
oligonucleotides, in particular DNA, are particularly well-suited to exploitation in bottom-
up nanotechnological application. This is, of course, because of the almost unlimited
ability of DNA to carry information as a consequence of its sequence programmability,
and the ability to exploit this sequence programmability by making use of the selective
35 molecular recognition of one strand of DNA by another based upon Watson-Crick base-

pairing. Unsurprisingly, therefore, DNA has been the most well-used and successful component in bottom-up applications to date.

Whilst DNA is superficially of limited utility in bottom-up nanotechnology (since, being linear, and as a consequence of the relative rigidity of the double-helical form, it appears to lend itself solely to one-dimensional structures), the introduction of branching into synthetic DNAs opens up the possibility for 2-dimensional and 3-dimensional structures. For example, a sticky-ended Holliday junction (four DNA strands bound together about a central branch point to form four double-helical arms each of which terminates in a sticky end (a short single-stranded overhang protruding from the end of a double-stranded helical DNA molecule)) permits the construction of 2-D molecular grids by self-assembly. Even if the four strands of DNA in Holliday junctions are considered to be approximately coplanar, so limiting to two the dimensions accessible in self-assembled structures, it is possible to engineer other artificial DNAs such as triplex structures in which the three strands of DNA are not necessarily in the same plane, and so open up the possibility of bottom-up fabrication into the third dimension. More complex structures have been, and continue to be, developed and advances in nanotechnology continue apace.

A recent example of bottom-up fabrication reported in the scientific literature is of the construction of a DNA hexagon by the self-assembly of six building blocks each comprising a rigid 120° synthetic vertex ligated to two short DNA strands, each of which was designed to hybridise to a strand of DNA on an adjacent building block and so enabling self-assembly (F.A. Aldaye and H.F. Sleiman (ibid)).

A general disadvantage in the prior art is, as is alluded to above, the great reliance placed upon Watson-Crick base-pairing in order to hold DNA-based nanostructures together. In order to analyse such structures, therefore, it is necessary to do so under non-denaturing conditions and this complicates their characterisation and purification. It also, of course, limits the uses to which such nanostructures may ultimately be put, as building blocks that can be stored for subsequent use in the assembly of larger more complex nanostructures.

As with nanotechnology, there is also great interest in the use of oligonucleotides in therapeutic applications and as research tools. There is often difficulty in turning so-called biologies, including oligonucleotides, into drugs because of the degradative effect of exo- and endo-nucleases in vivo amongst other difficulties.

Chemical methods for the synthesis of DNA and other polynucleotide constructs in which the strands are covalently interlocked, either intramolecularly e.g. so as to circularise a single strand of oligonucleotide, or intermolecularly, for example to covalently bond two oligonucleotide strands together (e.g. at an internal point or points within a double-helix), or by tying the ends of the helices together so as to form a cyclic structure wherein there is a closed loop of DNA joined at either end by covalent bonds, offer a potential solution to the deficiencies of the prior art alluded to above.

Saito et al. have reported (K. Fujimoto et al., Tetrahedron Letters, 41 (33), 6451 (2000); K. Fujimoto et al., Journal of the American Chemical Society, 122 (23), 5646 (2000); I. Saito et al., Tetrahedron Letters, 46 (1), 97 (2005)) the use of photo-induced reversible cycloaddition reactions to generate covalently modified cyclic DNA constructs by employing vinyl-containing nucleotides in photoligation reactions.

Template-mediated oligonucleotide circularisation has been carried out previously by enzymatic and chemical methods, but non-template-mediated circularisation of long oligonucleotides has proven difficult (E. T. Kool, Accounts of Chemical Research (1998) 31 (8), 502-510; E. T. Kool, Annual Review of Biophysics and Biomolecular Structure (1996) 25, 1-28).

There is, therefore, considerable scope for developing chemistries to modify oligonucleotides for construction of nanostructures based on circularised oligonucleotides and for stabilisation of oligonucleotide therapeutics such as siRNAs, antisense oligonucleotides, aptamers and decoy oligos against degradation in vivo.

The various aspects of the invention exploit the known participation of azide and alkyne functional groups in a 1,3-dipolar cycloaddition reaction that provides 1,2,3-triazoles This is referred to herein as "click ligation". Such reaction has previously been known for linking an oligonucleotide to a surface, e.g. to immobilize DNA on electrode surfaces and chips, or for linking one oligonucleotide strand to another (see e.g. Published International Application WO2004/055160 (The Trustees of Columbia University), Collman et al. Langmuir (2004) 20 (4) 1051-1053, Seo et al. Proc. Natl Acad Sci. U.S.A. (2004) 101 (15) 5488-5493 and Kanan et al. (2004) Nature 545-549), but has now been newly-applied by the inventors to achieve cyclic oligonucleotides, e.g. by non-templated circularisation of single-stranded oligonucleotides, which may then be utilised to build more complex catenanes by addition of one or more further oligonucleotides in a

templated-fashion such as a double-stranded cyclic DNA. Kanan et al. (ibid) describes only end-to-end hybridisation of two oligonucleotides where azide and alkyne functional groups are used to join adjacent ends in a short hairpin structure incorporating a disulphide bond. This was entirely for proof of concept of a hairpin-type oligonucleotide
5 structure as a research tool in reaction discovery. WO 2004/055160 discusses more generally use of such functional groups for covalently joining one biomolecule to another but again does not teach any form of oligonucleotide circularisation nor suggest azide-alkyne coupling as a route to achieving greater in vivo stability of any therapeutic oligonucleotide.

10

Summary of the invention

In one aspect, the present invention provides a method of oligonucleotide circularisation comprising reacting at least one azide group with at least one alkyne group, wherein
15 said at least one azide group and said at least one alkyne group are carried by the same or different oligonucleotide strands, such that a circularised oligonucleotide is obtained with one or more strands. The circularised oligonucleotide may preferably be single-stranded, e.g. a cyclic single-stranded DNA where the 1,2,3-triazole-containing covalent linkage is the sole means of desired stabilisation. As indicated above such non-
20 templated single-stranded circularisation may be the first step in building a more complex catenane, preferably again employing click ligation to add one or more additional oligonucleotides in a template-directed manner. Such a route might, for example be used to provide a double-stranded cyclic oligonucleotide for delivery of a single-stranded oligonucleotide in cells, in which case one or both cyclic strands will
25 additionally contain a linker which is broken intracellular^, e.g. a disuphide bond. Such a route can be used to build double-stranded, closed oligonucleotide catenanes of other forms such as a double-stranded pseudo-hexagon (see Example 5). Moreover, as indicated above, it is also envisaged that circularisation according to the invention may be applied to therapeutic double-stranded oligonucleotides such as aptamers, antisense
30 oligonucleotides, siRNAs and decoy DNAs with a view to providing protection against in vivo enzymatic degradation.

Thus, by reacting appropriate oligonucleotide substrates for a click ligation reaction, the invention provides methods for preparing stabilised oligonucleotide constructs either
35 where the 1,2,3-triazole-containing covalent linkage is the only additional means of stabilisation (for example in cyclic single-stranded DNA constructs), or where the 1,2,3-

triazole linkage provides stabilisation additional to that provided by Watson-Crick base-pairing. Examples of circularised oligonucleotide constructs in which one or more 1,2,3-triazole linkages provide additional stability in accordance with the invention are shown in Figure 10. These include "capped" double-stranded oligonucleotide constructs in
5 which the two termini of the double-stranded helix are each sealed by effecting two 1,2,3-triazole-forming reactions between two pairs of alkyne and azide functional groups located at the four termini of the two strands in the double-helix.

Oligonucleotide comprising constructs resultant from carrying out circularisation
10 methods as described above constitute a further aspect of the invention. As indicated above, circularised single- or double-stranded oligonucleotides in which there are present one or more 1,2,3-triazole-containing linkages represent preferred embodiments. Thus, viewed from one preferred aspect, the invention provides a double-stranded oligonucleotide in which there is present a single 1,2,3-triazole-containing
15 covalent linkage between internal nucleotides of each strand.

The invention provides azide-and/or alkyne-carrying oligonucleotides for use in carrying out circularisation methods as described above. More particularly, viewed from a further aspect, therefore, the invention provides an oligonucleotide comprising precursor for use
20 in carrying out a method of the invention presenting two alkynes, two azides or an alkyne and an azide. In one embodiment these are disposed from the terminal nucleotides of the oligonucleotide.

As indicated above, the azide/alkyne cycloaddition reaction has not previously been
25 exploited for stabilisation of therapeutic oligonucleotides against in vivo degradation and this provides a further aspect of the invention. Thus, the invention also provides use of at least one such coupling between an alkyne group and an azide group for this purpose.

30 The azide/alkyne cylcoaddition reaction has been shown to proceed, albeit slower, in the absence of catalyst and this opens the way for use of such coupling to join oligonucleotide sequences in cells. This is of interest in joining oligonucleotides after cellular uptake up, e.g. for oligonucleotide probing for an expressed mRNA and intracellular antisense or siRNA production.

35

Aspects and embodiments of the present invention are discussed further below with reference to the figures as now detailed.

Brief description of the figures

5

Fig. 1 shows the preparation of a 1,4-disubstituted, 1,2,3-triazole from the copper (I)-catalysed click ligation reaction of an azide and an alkyne present in adjacent nucleotides on different strands of a double-stranded oligonucleotide construct. Fig. 1 (a) shows this schematically and Fig. 1 (b) shows the chemical structure at the ligation
10 point.

Fig. 2. Denaturing anion-exchange HPLC chromatograms (UV absorbance vs time) of the reaction between ODN-2 and ODN-4 under different conditions. (a). Oligonucleotide degradation caused by uncomplexed Cu[I] if no ligand is used. (b). No reaction in the
15 absence of Cu[I] catalysis. (c). All three ODNs at 0.4 μM + Cu[I] + ligand, the reaction is almost complete within 2 hours. (d). ODN-2 + ODN-4 at 0.4 μM + Cu[I] + ligand in the absence of template ODN-5, no significant reaction was observed.

Fig 3. Formation of double-stranded pseudo-hexagon from single-stranded cyclic
20 template ODN-10 and linear ODN-1 1. In this representation the two strands are intertwined 6 times.

Fig 4. HyperChem representation of the formation of double-stranded pseudo-hexagon from single stranded cyclic template ODN-10 and linear ODN-1 1 showing the single-
25 strands intertwined 6 times. # indicates click ligated region. * indicates unpaired TpT/TpT hinge segments.

Fig 5. Denaturing 8% PAGE gel. Lane a. Formation of single stranded closed circular ODN-10 (upper spot) from ODN-9 (lower spot) at 0.4 μmol ODN concentration. Lane b.
30 Formation of covalently closed pseudo-hexagonal duplex (upper spot) from ODN-10 and ODN-1 1. Acyclic ODNs and single-stranded closed circles are also present. Unreacted ODN-9 (no Cu[I] catalysis) is shown on the left for reference.

Fig 6. Reversed-phase HPLC analysis of click ligation reaction (UV absorbance at 275
35 nm vs time). a. Linear oligo (ODN-13), b. Reaction mixture to produce circular oligo (ODN-1 5), c. Mixture of linear oligo (ODN-1 3) and reaction mixture (ODN-1 5).

Fig 7. Denaturing 8% polyacrylamide gel. Lane a. ODN-1 1 (control sample prepared exactly as the sample in lane b but without the addition of the restriction enzyme). Lane b. Digestion of linear oligo ODN-1 1 with Mboll. Lane c. Circular oligo ODN-14 (control
5 sample prepared exactly as the sample in lane d but without the addition of the restriction enzyme). Lane d. Digestion of circular oligo ODN-14 with Mboll.

Fig 8. Example of preparation oligonucleotides with an azide and alkyne group ready for circularisation. Reagents and conditions for provision of the azide group: (i) DMF, NaN_3,
10 (ii) NaOH, THF/H_2O, 20% (two steps) (iii) NHS, DCC, DCM, 63%; (iv) 0.5 M $Na_2CO_3/NaHCO_3$ buffer (pH 8.75), DMSO.

Fig 9. Further example of preparation of a modified oligonucleotide suitable for circularisation in accordance with the invention. Reagents and conditions for
15 preparation of alkyne-modified nucleotide: (i) TBSCI (1.2 eq), imidazole, DMF, 53% (ii) propargylamine, EDC, HOBt,, DIPEA, DCM, 75%; (iii) ; (iv) 2-cyanoethyl-N,N-diisopropyl chlorophosphoramidite, DIPEA, DCM, 55%; (v) coupling during solid phase oligonucleotide synthesis.

20 Fig 10. Examples of various oligonucleotide structures obtainable in accordance with the invention.

Fig 11. Further examples of oligonucleotide structures obtainable in accordance with the invention.

25

Fig 12. Examples of various alkyne-containing nucleotides or other structures that can be used to form modified oligonucleotides in accordance with the present invention: (a) internal alkyne on base, (b) internal alkyne , no base, (c) 5'-alkyne.

30 Fig 13. Examples of various amines on DNA bases/nucleosides: (a) other amines (no base) for internal attachment, (b) threoninol, serinol. These can be added as phosphoramidite monomers during oligonucleotide synthesis: (c) amine at 3'-end of olignucleotide and amine at 5'-end of oligonucleotide. The azide active ester can then be added to the deprotected oligonucleotide to label the amine with the azide group.

35

Fig 14. shows an example of the formation of an azide-comprising oligonucleotide by conversion of an amine group when displayed from an oligonucleotide. Any of the amines shown in Figure 13 can be labelled in this way, for example with the azide ester shown in Fig. 8.

5

Fig. 15 shows cyclic and linear alignment of ODN-9 and ODN-1 1 (K=alkyne, N=azide).

Fig. 16 shows the preparation of a 1,4-disubstituted, 1,2,3-triazole from the copper (I)-catalysed click ligation reaction of an azide and an alkyne present at the termini of a
10 hairpin oligonucleotide with a loop region formed from hexaethylene gycol (HEG) units. A very stable DNA cyclic duplex was formed with a greatly increased melting temperature (C'2 with 19 atom HEG and 24 atom triazole (Tz) linkers as referred to in Table 2 of Example 7). Fig. 1 (a) shows such circularization schematically and Fig. 1 (b) shows the circularization of the hairpin duplex, illustrating the base pairing that increases
15 the rate of circularization. The circularization occurs in oligonucleotides that do not have intramolecular base pairing, albeit at a slower rate.

Fig. 17 shows click ligation of oligo ODN-Ia and oligo ODN-2a to give the end-sealed duplex EsD-1 constructed for investigation of serum stability as described in Example 8.

20

Fig. 18 shows a scheme for the synthesis of an alkyne-presenting oligonucleotide starting with Hex-5-yn-1-ol as used for Example 9. Reagents and conditions: (i) 2-0-cyanoethyl-N,N-diisopropylamino chlorophosphoramidite (1.1 eq), DIPEA (2.5 eq), CH_2Cb, 0.7 hr at room temperature (rt), 20%; for Example 8 , the reaction mixture was
25 left to stir for 2 hrs at rt (ii) oligonucleotide synthesis.

Fig. 19 shows a scheme for the synthesis of azide-presenting oligonucleotide as used for Example 9 and conversion to precursors for alkyne/azide coupling. Reagents and conditions: (i) NaN_3 (2eq), K_2CO_3 (1.5 eq), DMF, 5.75 h, 50 °C, 86%; (ii) NaOH 2 M,
30 H_2O: dioxane 1:2 v/v, 0.5 h, rt, 91%; (iii) N-hydroxysuccinimide (1.3 eq), EDC (1.4 eq), 6.5 h, rt, 52%; (iv)Na_2CO_3/$NaHCO_3$ 0.5 M (pH 8.75), DMSO.

Fig. 20: Cross-linking between azide and alkyne-modified uracil bases.

35 Fig. 21: (a) Alkynyl, amino and azido reagents, nucleosides and mononers used in carrying out cross-linking as shown in Figure 20 and described in Example 10; (b)

scheme for synthesis of protected aminopentynyl dU phosphoramidite monomer. (i) Hydrazine hydrate, EtOH, then CF_3COOEt, DMAP, THF, 38% (ii) DCM, DIPEA, 2-cynaoethoxy-N, N, diisopropylaminochlorophosphine, 60%.

5 Fig. 22: (a) structure of hairpin construct having an oligonucletide duplex of three base pairs joined by a loop region comprising HEG units and having terminal azide and alkyne groups as tested for AAC coupling both in the presence of Cu(I) as a catalyst and in the absence of any catalyst (K =alkyne, Z= azide, X=HEG linkers) (b) analysis of cyclised product formation by denaturing gel electrophoresis using 20% PAGE (reaction 10 conditions and time tested: lane 1: buffer, 50°C, 1 day; Lane 2: buffer, room temperature(rt), 1 day; Lane 3: buffer, 37°C, 1 day, lane 4: 0.2M NaCl, rt, 1 day; Lane 5: 0.2M NaCl, 37°C. 1 day; Lane 6: buffer, rt, with Cu(I), 2hrs; Lane 7: 0.2M NaCl, rt, with Cu(I) , 2hrs; buffer= 1OmM Na phosphate, 0.2M NaCl, pH7; 10µM oligo concentration; T_m = 58°C). Lane 5 shows AAC reaction proceeding in the absence of catalyst 15 (highlighted by arrow).

Detailed description of the invention

The reaction between an azide and an alkyne to form a disubstituted, 1,2,3-triazole is an 20 example of a 1,3-dipolar cycloaddition and has recently been developed by K. B. Sharpless and others at the Scripps Research Institute (see V. V. Rostovtsev et al., Angewandte Chemie-International Edition, 41.(14), 2596 (2002) and WO 03/101972). Whilst the regioselectivity (1,4- versus 1,5-) was problematic in the prior art, Sharpless et al. reported that the use of copper (I) catalysis regiospecifically unites azides and 25 terminal alkynes to afford only 1,4-disubstituted 1,2,3-triazoles, although the cycloaddition reaction itself can work with a variety of catalysts as reported in WO 03/101972. Indeed, the use of ruthenium (II) complexes has been reported to catalyse the cycloaddition of both terminal and internal alkynes, resulting in the formation of 1,5-disubstituted 1,2,3-triazoles (as opposed to the 1,4 regioisomers obtained through Cu (I) 30 catalysis) and 1,4,5-trisubstituted-1 ,2,3-triazoles (L. Zhang et al., J. Am. Chem. Soc, 127, 15998 (2005)); also reported in the recent review by Wu & Fokin of the reactivity and applications of catalytic azide-alkyne cycloadditions (Aldrichimica Acta, 40 (1), 7 (2007)).

35 In the context of the present invention, however, the precise control over regioselectivity of the cycloaddition reaction is not particularly critical in view of its purpose, i.e. to

stabilise the structures of specific oligonucleotides, rather than to provide specific 1,2,3-triazoles. Rather, it is important to note that the reaction can proceed with both internal and external alkynes, without catalyst and with catalysis other than by Cu(I). The copper-catalysed reaction has received arguably the greatest attention in the chemical 5 literature where it is referred to as the copper-catalysed azide-alkyne cycloaddition (CuAAC) reaction.

Whilst the CuAAC reaction has been used to immobilise DNA on electrode surfaces and chips (Collman et al. (ibid); Seo et al. (ibid)), to cyclise peptides on resins (Punna et al., 10 Angewandte Chemie-International Edition in English, 44, 2215 (2005)) and to link the termini of oligonucleotide strands as described by Kanan et al. (ibid; see also WO2004/016767 and WO2007/01 1722), as already noted above, the reaction has not been used previously to stabilise oligonucleotides per se either thermodynamically for nanostructure assembly or against enzyme degradation. Effecting the cycloaddition 15 reaction between alkynes and azides for the purpose of the invention is, however, within the competence of the skilled person, given the detailed description in the prior art about this reaction (see the references cited hereinbefore).

It is necessary to use as substrates in the reaction an alkyne group and an azide group 20 which may or may not be attached to the same oligonucleotide strand. The cycloaddition reaction can be performed in aqueous solution and a variety of organic solvents. For the purpose of the present invention, it is convenient and advantageous to conduct the cycloaddition reaction in aqueous solution.

25 All CuAAC ligation reactions reported in the examples hereinafter were carried out at 0.2M aqueous NaCl. Where formation of base-paired duplex was required by hybridisation, this concentration of saline ensured complete formation of duplex and enabled the ligation reactions to be conducted substantially simultaneously with the hybridisation of the sequences for the ligation. However, it will be understood that other 30 solutions might be employed with selection of the appropriate stringency where oligonucleotide strand hybridisation is required. As is known in the art, the parameters of salt concentration and temperature can also be varied to achieve the desired hybridisation stringency. Guidance regarding such conditions is readily available to those skilled in the art and in particular may be found in Sambrook et al., Molecular 35 Cloning: A Laboratory Manual (Third Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pages 7.9 to 7.12 (2001).

The reaction is catalysed by a number of possible agents. Whilst the reaction can be effected with catalytic quantities of catalysts, in other words of the order less than 1 molar equivalent versus the substrate(s), for example 0.001 - 10 mol%, and in one embodiment between about 0.1 to 5%, for example 1 to 3 mol% catalyst, greater
5 quantities of catalyst may be used, if convenient, depending on the scale of any given reaction. For example, in some of the examples which follow, quantities of copper of the order of 100 equivalent vis-a-vis the reactants are used. Nevertheless, dependent upon scale, this can still involve the use of miniscule quantities of copper catalyst.

10 Whilst copper (I) is the catalyst most described in the scientific (and patent) literature, the reaction can also be catalysed by the presence of metallic copper or by a metal ion selected from Au, Ag, Hg, Cd, Zr, Ru, Fe, Co, Pt, Pd, Ni, Rh and W. Further details of appropriate catalysts will be known to the skilled person and are provided in, for example, WO 03/101972.

15

Where the catalyst is copper (I) a variety of sources of copper (I) can be used. Copper (I) is thermodynamically unstable. However; addition of copper (II) salts can be used in combination with a reducing agent, for example ascorbate, so as to generate conveniently the desired copper (I) species in situ. Alternatively, desired quantities of
20 catalytic amounts of copper (I) can be introduced through comproportionation of copper (II) and copper (0). Further details are provided in WO03/101972 and Wu & Fokin (ibid).

Of particular utility in the cycloaddition of azides with internal alkynes, ruthenium (II) complexes may be used to catalyse the cycloaddition reaction, as reported in Zhang et
25 al. (ibid).

The inventors found that the most convenient catalyst to use for the cycloaddition reaction was copper (I) prepared in situ from copper (II) sulfate and sodium ascorbate. Such reaction, particularly when carried out in 0.2 M aqueous NaCl, was found to be
30 most conveniently conducted in the presence of a water-soluble tris-triazolylamine copper (I)-binding ligand, as has been reported previously (T.R. Chan et al., Org. Lett. (2004) 6 (17), 2853). In particular, it was found that the use of HPTA (tris-(hydroxypropyltriazoylylmethyl)amine) is advantageous insofar as it assists in preventing degradation of oligonucleotides by the copper catalyst. When more than a 5-
35 fold excess of triazolylamine ligand (in particular HPTA) relative to the copper (I) catalyst was used, for example ten or twenty equivalents or even more, virtually no

decomposition of the reactant oligonucleotide substrates for the cycloaddition reaction was observed.

However, as indicated above azide-alkyne coupling has been shown to proceed in the absence of catalyst, although slowly (see Example 12). Significantly, this opens the way for such coupling to be utilised intracellular^.

For use of the 1,2,3-triazole-forming reaction in the preparation of oligonucleotides requiring stabilisation to in vivo degradation, this may be engendered by an intermolecular (between two strands) or intramolecular (in the same strand) reaction between an alkyne and an azide moiety to provide the 1,2,3-triazole-containing linkage. This linkage may be connected to the oligonucleotide strand(s) by a linking moiety.

Conducting the reaction has the effect of introducing, either into the backbone of an oligonucleotide or elsewhere, an unnatural region. In the discussion that follows attention is directed towards the aspects of the invention concerned with preparation of modified DNAs. However, it is to be understood that the present invention is not to be considered to be so limited: for example, oligonucleotide constructs described herein can be used as binding agents for double-stranded RNA (dsRNA) binding enzyme. The single stranded DNA or RNA molecules could be substrates for single stranded DNA or RNA binding enzymes.

As indicated above, one aspect of the invention provides a method of oligonucleotide circularisation in which at least one alkyne group is reacted with at least one azide group. The invention also provides oligonucleotides obtainable by such a method and their use in nanotechnological and other applications as described in greater detail hereinafter.

By circularisation is meant that a closed oligonucleotide loop, consisting of covalently bonded atoms, is formed as a result of the alkyne and azide coupling reaction. There are a great number of structures that may be envisaged for oligonucleotides stabilised according to the circularisation method of the invention. Those shown in schematically in Figure 10 are only illustrative.

As is known in the art, a "nucleotide" comprises a nitrogenous base, a sugar and one or more phosphate groups. Nucleotides are the monomeric units that make up

oligonucleotides and polynucleotides. RNAs comprise nucleotides in which the sugar unit is ribose whereas the sugar is deoxyribose in DNAs. There is no clear distinction made between oligonucleotides and polynucleotides in the art; a polynucleotide is a very long chain comprising very many nucleotides; an oligonucleotide is smaller than a
5 polynucleotide but the terms are often used interchangeably in the art and are here. Herein, therefore, the term 'oligonucleotide' is intended to embrace polymers of nucleotides, and so what may be referred to as oligonucleotides and polynucleotides to those skilled in the art. An oligonucleotide construct of the invention may be as short as a cyclic duplex with just two base pairs (see Example 7) or far longer, e.g. 100 or more
10 nucleotides in length.

The present invention is concerned with the provision of modified oligonucleotides, that is to say oligonucleotide derivatives or analogues in which one or more nucleotides differ from a natural nucleotide as a consequence of the inclusion of a 1,2,3-triazole group and
15 possibly associated linking moieties.

Examples of modified nucleotides that may be utilised in carrying out a method of the invention are shown, as nucleosides, in Figures 12 and 13 and include alkyne groups and amine groups for later conversion to the desired azides. Where the base is absent
20 the nucleoside is referred to an abasic nucleoside. Additionally, it is possible to incorporate nucleoside "surrogates" into the oligonucleotides of this invention, which lack the base and sugar of nucleosides, but which can be inserted into the backbone of oligonucleotides.

25 As is also known in the art, nucleotide analogues or derivatives can, of course, comprise other modifications such as the presence of modified phosphorodiester linkages, such as phosphoramidate linkages. Such analogues as with other derivatives and analogues described herein are embraced by the definitions given above for oligonucleotide. The analogues of all polynucleotides, however, are preferably capable of undergoing
30 Watson-Crick base-pairing.

The desired azido-containing oligonucleotide may be prepared by labelling an appropriate 3'- or 5'- amino-modified nucleotide or oligonucleotide with, for example, 4-azidobutyric acid NHS ester as described in Fig. 8. Desired alkyne-containing
35 oligonucleotides may be prepared as shown, for example, in Fig. 9. However, these

methods are just illustrative and other methods may be used, including additional methods specified in the examples.

Examples of appropriate nucleotides comprising alkynes are shown in Figure 12. These include examples of external alkynes displayed from nucleotide bases, internal alkynes in bases and the inclusion of a terminal alkyne attached to the 1'-oxygen atom of a 2'-deoxyribose (i.e. an example of an abasic nucleotide). The figure also shows the 5'-alkyne shown in Figure 9. In all these structures one of the alcohols can be protected with a dimethoxytrityl (DMT) group and the other can be converted to a phosphoramidite for use in DNA synthesis. An example of such a DMT-protected alkyne-containing nucleoside phosphoramidite is 5-ethynyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine 3'-(2-cyanorthyl N,N'-diisopropylphosphoramidite the synthesis of which is described by D. Graham, J.A. Parkinson & T Brown (J. Chem. Soc. Perkin Trans. 1, 1131 (1998)).

Figure 13 shows examples of amines present in DNA bases and nucleosides. These can be introduced as phosphoramidite monomers during oligonucleotide synthesis as is known in the art. The azide active ester (as depicted in Figure 14) can then be added to the deprotected oligonucleotide to label the amine with the azido group as appropriate. Similarly to Figure 13, in addition to depicting examples of amines compatible to the desired azido functionality attached to the bases on the nucleosides shown in Figure 14, there are also examples given of abasic nucleotides in which the amino group is attached to the 1' oxygen atom through an alkylene (hexylene) linker. The four structures at the foot of the figure are molecules that may be used as nucleoside "surrogates" and can be inserted into the backbone of nucleotides and the amines converted to azides to permit click ligation reactions.

Advantageously, since the methods for preparing the azide and alkyne are orthogonal, the methodologies may be used for the preparation of oligonucleotides comprising both functionalities. It is noteworthy that 5'-alkynyl ODNs containing 3'-amino functions can be labelled with azide NHS ester without a major side reaction occurring between the 5'-alkyne and the active ester. This is because triazole formation is generally very slow in the absence of Cu[I].

For example, azide-ODN-2 was prepared as illustrated schematically in Figure 8 by labelling 3'-amino-modified ODN-1 with 4-azidobutyric acid NHS ester in bicarbonate buffer at pH 8.75. When preparing 5'-alkyne-labelled ODNs, it will be noted that an

orthogonal approach was used to that adopted for preparing the 3'-azido ODNs in order to allow a protocol for the convenient labelling of a single oligonucleotide with both alkyne and azide functionalities for use in circularisation of single-stranded oligonucleotide constructs. As indicated above, the chemistries depicted in Figures 8
5 and 9 are merely exemplary of ways in which to incorporate alkynes and azides into nucleotides or oligonucleotides; other methods will be within the ability of the skilled person. For example, modified deoxyuridine residues presenting an alkyne or azide group may also be employed as described in Example 10.

10 As noted above, Figure 10 illustrates various embodiments of the invention which are now described in more detail. Figure 10(a) illustrates an internal circularisation achieved by two azide/alkyne cycloadditions in which the pairs of azide and alkyne are both present at internal positions within a double-stranded oligonucleotide molecule. Such nucleotides are referred to herein as "internal nucleotides", i.e. nucleotides not at either
15 terminus of a single-stranded oligonucleotide. Generally, the covalent linkages so formed will be between alkyne and azide moieties displayed from complementary, i.e. base-paired, internal nucleotides but this need not be the case. For example, depending upon the length of the linker(s) connecting the azide and/or alkyne to the nucleotide it is possible to envisage the reaction between azides and alkynes not
20 displayed from base-paired nucleotides. Generally, however, oligonucleotides are made with the intention that subsequent stabilisation by the alkyne/azide cycloaddition reaction will take place between azide and alkyne moieties displayed from complementary nucleotides that are base-paired upon hybridisation of the two oligonucleotide strands.

25 Figure 10(b) is similar to Figure 10(a) but, instead of obtaining a circularised oligonucleotide by two cycloaddition reactions between two pairs of alkyne and azide reacting species as is shown in Figure 10(a), Figure 10(b) shows the schematic double-stranded oligonucleotide resultant from the reaction where one of the pairs of azide and alkyne is located at one terminus of the double-stranded oligonucleotide. Similarly,
30 Figure 10(c) shows the result of circularising a double-stranded oligonucleotide wherein both pairs of azide and alkyne are located at the two termini of the double-stranded oligonucleotide. Such a "capped" or end-sealed duplex with two terminal triazole linkers is illustrated in Figure 17 and has been shown to exhibit much increased serum stability compared with single stranded oligonucleotide of comparable length as described in
35 Example 8.

Figure 10(d) illustrates a preferred embodiment of the invention wherein the alkyne and azide are attached at either end of a single-stranded oligonucleotide. The ligation reaction therefore forms a single closed loop by non-templated circularisation. Figure 10(e) is similar to Figure 10(d) but after circularisation the single-stranded
5 oligonucleotide contains a tail. The structure arises by either the azide or the alkyne, being present at an internal position within the oligonucleotide prior to circularisation.

Figure 10(f) is a depiction of a circularised hairpin loop in which the termini of the structure are ligated so as to close the hairpin at the end distal to the loop. As is
10 understood by those skilled in the art, the term hairpin relates to oligonucleotides having a stem and loop structure. The stem comprises a duplex of hybridised nucleotides and the loop is a region of unhybridised nucleotides or other moieties that links the two strands of complementary nucleotides. Such a circularised mini-duplex with just two base pairs and a loop of hexaethyene glycol units, as noted above, is shown in Figure
15 16 and also described more fully with other such end-sealed hairpin constructs in Example 7. Such end-sealed hairpin constructs have also been shown to exhibit increased serum stability in serum compared with the parent construct.

Figure 10(g) shows a more complex hairpin structure with four different annealed
20 regions. Again, the termini of the oligonucleotide chain are ligated to circularise the single-stranded chain. Whilst no emboldened region is shown, which where present in the other structures indicates schematically the position of the 1,2,3-triazole linkage, it will be understood that this linkage is present and that its location in a schematic representation such as Figure 10(g) is arbitrary.

25

Figure 10(h) is a schematic representation similar to Figure 10(d) but with wavy off-vertical lines representing extended linking moieties connecting the 1,2,3-triazole moiety to the single-stranded oligonucleotide moiety. This has the advantage of enabling the oligonucleotide portion to assume a near linear structure even although it is part of a
30 closed loop. For the avoidance of doubt, each of the structures depicted in Figure 10(a)-(g) may also, and indeed are likely to, contain linking moieties between the oligonucleotide and 1,2,3-triazole linkage.

By linking moiety is meant herein those atoms which are neither part of the 1,2,3-triazole
35 moiety nor the backbone atoms of the oligonucleotide chain to which the triazole is covalently attached. It is often advantageous to minimise the number of atoms present

in the linking moiety in order that a substrate molecule can resemble as closely as is practicable the natural nucleotide it is intended to replace. However, there are constraints placed upon how small the linking moiety can be simply as a consequence of the chemistry necessary to introduce the azides and alkynes.

5

In contradistinction, in many circumstances it may be disadvantageous to include too small a linking moiety. For example, a degree of flexibility provided by the existence of a linking moiety serves to increase the probability, and so ease, of intermolecular reaction between an azide and an alkyne moiety displayed from either strand in an oligonucleotide duplex. Furthermore, certain embodiments of the present invention deliberately include elongated linking moieties between either the reactant alkyne and/or the reactant azide. As is depicted in Figure 10(h), an example of an advantage of using long linking moieties in this manner in circularised oligonucleotide constructs is that this allows a more straightened oligonucleotide chain to be displayed than would be the case if the oligonucleotide was connected more directly to the 1,2,3-triazole. By enabling the circularised oligonucleotide to assume a near linear helical structure (shown as the vertical wavy line in Figure 10 (h)), even although part of the circularised construct, this enhances hybridisation to a complementary strand in biological and nanotechnological applications.

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Examples of linking moieties useful with this invention are set forth in the examples which follow. However, as mentioned above, often the linking moiety is as a result of the chemistry most conveniently used to introduce the alkyne and azide and is not particularly important in itself.

25

Figure 10(i) shows schematically a double-stranded catenane structure. This can be formed, for example, by an initial formation of a single-stranded closed loop as depicted in Figure 10(d) followed by hybridisation of a complementary oligonucleotide strand bearing an azide and alkyne at either terminus prior to ligation. Whilst no emboldened regions are shown, (which where present in the other structures indicate schematically the position of the 1,2,3-triazole linkage), it will be understood that two such linkages are present in the catenane and that their positions (and relative positions) in a schematic representation are arbitrary.

30

35 As indicated above, such a route might, for example be advantageously used to provide a double-stranded cyclic oligonucleotide for delivery of a single-stranded oligonucleotide

into cells. In this case, one or both cyclic strands will contain a linker which is broken intracellular^, e.g. a disuphide bond whicb will be reduced in the reducing environment of a cell. In this way, the desired intracellular oligonucleotide(s) can be better protected from degradation prior to cellular uptake. The double-stranded form may, for example, 5 be a DNA duplex, DNA: RNA hybrid or RNA duplex

Figure 11(a) shows a double-stranded oligonucleotide with an internal azide/alkyne coupling. Uses of such an oligonucleotide for formation of circularised forms is further depicted in Figures 11(b) and (c). Figure 11(b) is a schematic representation of a 10 double-stranded oligonucleotide hexagon formed from self-assembly of six strands of DNA followed by six intrahelical ligations. Such a structure may be contrasted with that disclosed by Alday and Sleiman (infra): that of the prior art contains no interstrand covalent stabilisations. Figure 11(c) depicts a related hexameric structure formed by hybridisation and subsequent intrahelical covalent bond formation of six triplex 15 substrates. Clearly the use of triplex or higher order multiplex oligonucleotide substrates allows generation of more complex structures with possible applications in nanotechnology, and in particular nanoelectronics.

Methods of constructing circularised oligonucleotides according to the invention may be 20 achieved in a templated and/or template-free manner. In a templated synthesis, an oligonucleotide serves as a template for one or more complementary oligonucleotides to hybridise prior to effecting one or more ligation reactions between azide and alkyne moieties. For example, formation of a double-stranded catenane of the type depicted in Figure 10(i) is typically undertaken by way of a templated procedure in which the second 25 strand is hybridised to the first strand prior to ligation. In place of a single substrate as the second strand, however, it may be envisaged that two or more oligonucleotides could hybridise appropriately to the single stranded cyclic oligonucleotide which can direct the appropriate positioning of the two or more complementary oligonucleotides prior to ligation of appropriately positioned alkyne and azide moieties. Ever more 30 complex structures may be constructed exploiting the power of bottom-up nanotechnological fabrication.

Further examples of templated click ligations may be seen in Figure 10(a)-(c) and (f), and Figure 11(a). In these figures, one of the oligonucleotide strands shown may be 35 considered to serve as a template for a complementary strand (which in the hairpin

shown in Figure 10(f) is part of the same oligonucleotide) prior to effecting one or more click ligation reactions.

In contrast to the templated synthesis of the catenane described immediately above, it is
5 possible through this invention to construct a single-stranded cyclic oligonucletide that can serve as a template for the second strand of the catenane by way of a template-free ligation reaction.

The cyclisation of single-stranded oligonucleotides in a non-templated, or template-free,
10 manner is a particular advantage, and embodiment, of the present invention. It can be carried out on a large scale with oligonucleotides with much structural modifications, for example in the presence of linking moieties such as heaxethylene glycol, because of the high specificity of the click ligation reaction.

15 It will be noted from Example 5 that in the structure of closed duplex catenanes according to the invention non-complementary regions may be provided, e.g. non-hybridising nucleotides such as the TT base pairs at each of the vertices of ODN-12 (the dsDNA pseudohexagon catenane). These serve as hinges since they are not base-paired and punctuate the fully complementary duplexes (e. g. 10 mers) engineered into
20 the catenane. The use of hinge regions is advantageous; otherwise the inherent linearity of oligonucleotides makes formation of a cyclised oligonucleotide of such short length difficult. Other hinges may be introduced. For example F.A. Aldaye and H.F. Sleiman (infra) employ 1,3-bis(4-hyrdoxyphenyl) benzene as the hinge regions in their hexagon, with 17mer duplex sides. Alternatively more flexible hinges such as oligoethylene glycol
25 moieties (e.g. hexaethylene glycol) may be used. Such oligoethylene glycol moieties are advantageous in that their flexibility permits easier hybridisation of the second strand to the first in catenane formation. Oligoethylene glycol linking moieties are commercially available as their phosphoramidites (Glen Research).

30 The invention also provides substrates for use in the 1,2,3-triazole-forming reactions described herein. Thus, for example, the invention provides single-stranded oligonucleotides bearing an alkyne at one terminus and an azide at the other, for subsequent circularisation.

35 The invention also provides, for example, a catenane precursor having one strand of single-stranded cyclic oligonucleotide hybridised to which is an oligonucleotide bearing

an alkyne at one terminus and an azide at the other, for subsequent circularisation to form the second helical loop.

It is emphasised once again that he methods and oligonucleotides of the present
5 invention can have utility in a variety of applications, including provision of therapeutic agents and nanotechnological applications.

The application of DNA, for example, in structural DNA nanotechnology, defined by N.C. Seeman (Chemistry & Biology, 10, 1151 (2003)), as the use of well-structured
10 components, combined by using both affinity and structure to control geometry, or, at least, strand topology, has often consisted only of combining DNA motifs by exploiting structurally specific well-defined cohesive interactions (mainly making use of sticky-ends) to produce target materials with predictable 3D structures. As a result of such efforts, structural DNA nanotechnology has provided a variety of 2-dimensional
15 structures. However, it will be appreciated that the use of the click ligation reaction is of general utility in nanotechnological applications since it has the capability to strengthen all oligonucleotide-based structures including 3D structures.

For biological applications, circularisation and other stabilisations enabled by the present
20 invention can serve to increase resistance to degradation by exo- and endo-nucleuses, particularly exonucleases. This would be a benefit for use of oligonucleotides both in vivo and when contacted in vitro with biological samples in assays.

The prior art is replete with reports of investigations into attempts to prevent nuclease
25 degradation by, for example, serum nucleases, by blocking nuclease attack by modification of base, sugar and phosphate regions of oligonucleotides. Often these methods are not ideal since such chemical modifications can affect the activity of the oligonucleotide against its intended biological target. In contrast, the present invention can afford modified oligonucleotides with substantially intact base, sugar and phosphate
30 regions leading to improvements in the problem of lack of recognition by the intended biological target.

Uses to which the modified oligonucleotides prepared by the present invention may be put involve the oligonucleotide therapies as described, for example in C. Wilson and
35 A.D. Keefe, Current Opinion in Chemical Biology, 10, 607 (2006); and Y. Fichou and C. Ferec, (Trends in Biotechnology, 24(12), 563 (2006)).

For example, singe-stranded antisense and antigene oligonucleotides, which generally comprise 15 to 20 nucleotides, are frequently modified at their termini to try to retard degradation by exonucleases. By practise of the present invention, such modifications can be by way of circularisation. In particular, by employing the extended linker embodiment illustrated in Figure 10(h), this provides for the preparation of improved antisense and antigene oligonucleotides by allowing presentation of the oligonucleotide to its target in a more linearised manner.

Single-stranded DNA and RNA molecules that can form tertiary structures stabilised by intramolecular hydrogen bonds between bases can act as aptamers (specific binding agents). Some aptamers might function better if cyclised as this would help stabilise their secondary structure and provide resistance to enzymic degradation.

Of particular interest is provision of circularised olignucleotide aptamers from hairpin structures with two or more base pairs for use in vivo as decoys to sequester DNA-binding proteins, e.g. cyclic DNA mini-duplexes which are end-sealed by azide/alkyne coupling as described in more detail in Example 7 and illustrated by Figure 10 (f) anf Figure 16. DNA decoys must remain double-stranded in cells and be resistant to DNAses (Giusto et al. Chembiochem (2006) 7, 535-544). Joining the two ends of a hairpin DNA duplex structure by click ligation means that they cannot come apart in cells. Furthermore such cyclisation can again be expected to improve stability to in vivo degradation. Intracellular enzymatic degradation occurs predominantly from the 3'-end of single strands and frayed ends of duplexes, so cyclic DNA duplexes can be expected to be more resistant to this. They are also more readily taken up by cells (Lee et al. Drug Targets (2003) 4, 535-544).

Ribozymes are RNAs characterised by the ability to catalyse a chemical reaction, often catalytic self-cleavage or cleavage of other RNAs. Ribozymes are generally around 40 nucleotides in length and comprise folded RNAs with double-stranded hybridised regions. Again, synthetic ribozymes with stabilisation conferred by circularisation of their termini, or internally, may be prepared according to the methods of this invention. Figure 10(g) is an example of this.

Use of double-stranded circularised oligonucleotide structures of the type shown in Figure 10(c) can be developed as inhibitors/binding agents for binding (and sequestering) of dsDNA or RNA binding enzymes, such as DNA repair enzymes that

recognise damaged dsDNA, e.g. O^6 methG groups. Such stabilised double-stranded oligonucleotides may contain modified bases to provide selective binding by the enzyme target. Other targets could be, for example, methyltransferases, transcription factors and ribosome binding protein.

5

A further biological application for methods of the present invention is in the preparation of modified, and so stabilised, triplex-forming oligonucleotides. As is known in the art, DNA triple-helices are formed when a third nucleic acid strand binds within the major groove of a DNA duplex. The formation of these structures can be used to achieve
10 selective recognition on extended DNA sequences. Whilst triplex formation is relatively straightforward in vitro, one of the difficulties in using DNA triplexes in medical and biotechnological applications is their instability at physiological pH. Formation of a circular oligonucleotide in which one half is the third strand of a triplex and the other half is one strand of the duplex will produce a construct which will bind tightly to the other
15 strand of the duplex. Such a cyclic construct could be used to bind a single stranded piece of DNA or other nucleic acid or analogue.

A still further application of the present invention is to allow the capture of circularised oligonucleotides having tails (shown schematically in Figure 10(e)) onto a solid support
20 by forming a duplex with a complementary single-stranded oligonucleotide that is bound to the solid support.

The present invention is now illustrated by the following examples which, whilst illustrating what may be regarded as preferred features of the invention, are not intended
25 to limit the invention in any way.

Examples

Reagents and techniques

All reagents were purchased from Aldrich, Avocado, Fluka, Link Technologies and used
30 without purification with the exception of the following solvents, which were purified by distillation: THF (over sodium wire and benzophenone), DCM, DIPEA and pyridine (over calcium hydride). All reactions were carried out under an argon atmosphere using oven-dried glassware with purified and distilled solvents. NAP-10 or NAP-25 columns were purchased from GE Healthcare. Water soluble polyhydroxypropyltriazole amine Cu[I]
35 binding ligand was synthesised by the method reported in the literature (T.R. Chan et al., infra). Column chromatography was carried out under pressure using Fisher

scientific DAVISIL 6OA (35-70 micron) silica. Thin layer chromatography was performed using Merck Kieselgel 60 F24 (0.22mm thickness, aluminium backed). Compounds were visualised by staining with potassium permanganate solution.

^{1}H NMR spectra were measured at 300 MHz on a Bruker AC300 spectrometer and ^{13}C NMR spectra were measured at 75 MHz. Chemical shifts are given in ppm relative to tetramethylsilane, and J values are given in Hz and are correct to within 0.5 Hz. All spectra were internally referenced to the appropriate residual undeuterated solvent signal. Multiplicities of ^{13}C signals were determined using DEPT spectral editing technique. ^{31}P NMR spectra were recorded on a Bruker AV300 spectrometer at 121 MHz and were externally referenced to 85% phosphoric acid in D_2O. Low-resolution mass spectra were recorded using electrospray technique on a Fisons VG platform instrument or a Waters ZMD quadrupole mass spectrometer in acetonitrile (HPLC grade). High-resolution mass spectra were recorded in acetonitrile, methanol or water (HPLC grade) using electrospray technique on a Bruker APEX III FT-ICR mass spectrometer. MALDI-TOF MS were recorded using a ThermoBioAnalysis Dynamo MALDI-TOF mass spectrometer in positive ion mode using oligo dT standards (G. Langley et al., Rapid Communications in Mass Spectrometry, J_3 (17), 1717 (1999)).

Synthesis of Succinimidyl-4-azidobutyrate, 2

Dicyclohexylcarbodiimide (DCC) (0.89 g, 4.3 mmol) was added to a suspension of 4-Azidoebutyric acid 1 (0.46 g, 3.6 mmol) (B. Carboni et al., Tetrahedron, 43(8), 1799 (1987); and N-hydroxysuccinimide (0.49 g, 4.3 mmol) in DCM (20 imL) at room temperature and the reaction was left to stir. After 4 h. aqueous KCl (20 mL) was added and the organic layer was separated, washed with water, dried over sodium sulfate, filtered and the solvent removed in vacuo. Upon purification by column chromatography (99:1 , DCM: methanol) the title compound was isolated as a colourless oil (0.51 g, 63%). IR (thin film) v 2091 , 1780, 1731 cm^{-1}; δ_H (300MHz, $CDCl_3$) 3.37 (2H, t, J=6.6Hz, N_3CH_2), 2.77 (4H, m, $COCH_2CH_2CO$), 2.66 (2H, t, J=7.0Hz, $COCH_2$-), 1.94 (2H, m, -CH_2-); δ_c (75 MHz, $CDCl_3$) 168.9 (CO), 167.9 (CO), 50.0 (N_3CH_2), 28.1 ($COCH_2$-), 25.6 ($COCH_2CH_2CO$), 24.1 (-CH_2-); m/z LRMS [ES^+, MeCN] 249 ($M+Na^+$, 100%). HRMS ($M+Na^+$) ($C_8H_{10}N_4NaO_4$) found, 249.0590; required, 249.0594.

Synthesis of alkyne phosphoramidite monomer

6-O-TBS-hexan-1 -propargylamide, 4

EDC (2.45 g, 12.8 mmol) was added to a suspension of 6-O-TBS-1-hexanoic acid 3 (2.1 g, 8.5 mmol) (K.C. Nicolaou et al., J. Am. Chem. Soc, V\2, 3040 (1990)); propargylamine (0.52 g, 9.4 mmol) and N-hydroxylbenzotriazole (HOBt) (1.44 g, 9.4 mmol) in DCM (20 ml.) followed by DIPEA (5.50 g, 42.7 mmol). The reaction mixture was left to stir for 4 hours at room temperature then partitioned between DCM and aqueous NaHCθ3. The organic layer was separated, washed with water, dried over sodium sulfate, filtered and the solvent removed in vacuo. Upon purification by column chromatography (60: 40, ethyl acetate: hexane) the title compound was isolated as a colourless oil (1.80 g, 75%). δ_H (300 MHz, $CDCl_3$) 5.73 (1H, bs, NH), 4.0, 3.56 (4H, m, NCH_2, OCH_2), 2.18 (3H, m, $COCH_2$, alkyne-H), 1.65-1 .32 (6H, m, $3xCH_2$-), 0.84 (9H, s, TBS), 0.0 (6H, s, TBS); δ_c (75 MHz, $CDCl_3$) 172.6 (CO), 79.7, 71.5 (alkyne-C), 64.0, 63.0 (OCH_2, CH_2N), 36.4, 32.5, 29.1 (CH_2), 25.5 ($3xCH_3$, TBS), 25.4 (CH_2), 18.3 (C-TBS) -0.5 ($2XCH_3$JBS); m/z LRMS [ES^+, MeCN] 284 ($M+H^+$, 20%), 306 ($M+Na^+$, 30%), HRMS ($M+Na^+$) ($Ci_5H_{29}NNaO_2Si$) found, 306.1858; required, 306.1860.

6-Propargylamido-1-hexanol, 5

Compound 4 (0.80 g, 2.8 mmol) was dissolved in tetrabutylammonium fluoride/tetrahydrofuran (6 ml_, 1:1) at room temperature, left to stir for 1 hour then concentrated in vacuo and partitioned between DCM and aqueous $NaHCO_3$. The organic layer was separated, washed with water, dried over sodium sulfate, filtered and the solvent removed in vacuo. Upon purification by column chromatography (80: 20, ethyl acetate: hexane) the title compound was isolated as a colourless oil (0.30 g, 64%). δ_H (300 MHz, DMSO-de) 8.20 (1H, bs, NH), 4.28 (1H, bs, OH), 3.84, 3.38 (4H, m, NCH_2, OCH_2), 3.03 (1H, s, alkyne-H), 2.26 (2H, t, J=7.3Hz, $COCH_2$), 1.62-1 .19 (6H, m, $3xCH_2$-); δ_c (75 MHz, DMSO-d_6) 171 .8 (CO), 81.3, 72.7 (alkyne-C), 60.6 (OCH_2,), 35.1 , 32.2, 27.6, 25.2, 25.0 (CH_2N, CH_2); m/z LRMS [ES^+, MeCN] 170 ($M+H^+$, 45%), 192 ($M+Na^+$, 10%). HRMS ($M+Na^+$) ($C_9H_{15}NNaO_2$) found, 192.0999; required, 192.0995.

6-Propargylamidohexan-1-O-(2-cyanoethyl-N,N-diisosopropyl)phosphoramidite, 6

Compound 5 (1.0 g, 5.9 mmol) was dissolved in DCM (10 mL) under an atmosphere of argon. DIPEA (2.05 mL, 11.8 mmol) was then added followed by 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (1.45 mL, 6.5 mmol) dropwise. The reaction mixture was left to stir at room temperature for 2 hours then transferred under argon into a separating funnel containing degassed DCM (20 mL). The mixture was washed with

saturated KCl (20 ml.) and the organic layer was separated, dried over sodium sulfate, filtered and the solvent removed in vacuo. Upon purification by column chromatography under argon pressure (60:40 ethyl acetate:hexane, 0.5% pyridine) the product was isolated as a pale yellow oil (1.2 g, 55%); δ_P (300 MHz, DMSO-d6) 146.5; m/z LRMS
5 [ES$^+$, MeCN] 370 (M+H$^+$, 40%), 392 (M+Na$^+$, 35%). HRMS (M+Na$^+$) ($C_{18}H_{32}N_3NaO_3P$) found, 392.2079; required, 392.2073.

Synthesis of oligonucleotides

Standard DNA phosphoramidites, solid supports and additional reagents including the
10 C7-aminoalkyl cpg were purchased from Link Technologies or Applied Biosystems Ltd. All oligonucleotides were synthesised on an Applied Biosystems 394 automated DNA/RNA synthesiser using a standard 0.2 or 1.0 μmole phosphoramidite cycle of acid-catalysed detritylation, coupling, capping and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation
15 conductivity monitoring facility and in all cases were >98.0%. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal (A,G,C,T) monomers was 25 sec and the coupling time for the alkyne phosphoramidite monomer 6 was extended to 360 sec. 3'-Aminoalkyl oligonucleotides were synthesized starting from C7-
20 aminolink cpg (Link Technologies Ltd). Cleavage of the oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5h at 55 ^{0}C.

25 **Synthesis of azidobutyramide-labeled oligonucleotides**

To incorporate the azido group at the 3'-end of C7-aminoalkyl oligonucleotide, 10-50 nmol of the oligonucleotide in 40 μL of 0.5 M Na_2CO_3/$NaHCO_3$ buffer (pH 8.75) was incubated for 4 h at room temperature with 10 μmol of succinimidyl-4-azidobutyrate, 2 in 12 μL of DMSO. The crude oligonucleotide was purified by reversed-phase HPLC and
30 desalted by NAP-10 gel-filtration according to the manufacturer's instructions (GE Healthcare).

Example 1: Click ligation of oligonucleotides

General

In developing the templated ligation protocol of this invention, various concentrations of oligonucleotide were trialed. As described below, templated ligation reactions of azido ODN-2 and alkyne ODN-4 in the presence of template ODN-5 were carried out at 10.0, 2.0 and 0.4 µM concentrations of each nucleotide, with and without template and with and without copper (I) catalysis. In all cases no reaction is observed without the catalyst (see Figure 2b). At all three concentrations in the presence of both catalyst/ligand and ODN-5 template, the near quantitative conversion to the click ligated ODN-6 was observed by denaturing an ion-exchange HPLC (see Figure 2c). At high ODN concentrations (greater than 10 µM), formation of ODN-6 was observed without need for the presence of template (at up to 50% conversion) with no significant ligation being observed at 2 and 0.4 µM ODN concentration in three hours at room temperature in the absence of template (see Figure 2d). This demonstrates that intermolecular reactions may be easily controlled in the presence of catalyst by varying the concentration of oligonucleotide reactants. Template-mediated reactions may be conducted over a wide concentration range, and, below 2 µM, intermolecular reaction was found not to proceed in the absence of a template oligonucleotide. This information was exploited in conducting effective template-free intra molecular cyclisations.

Experimental

To a 950 µL of 0.2 M aqueous NaCl, tris-hydroxypropyl triazolyl ligand (Chan et al (ibid); 1.38 µmol), sodium ascorbate (2.0 µmol) and $CuSO_4.5H_2O$ (0.20 µmol) were added sequentially. To the above solution, azide ODN-2 (2.0 nmol), alkyne ODN-4 (2.0 nmol) and template ODN-5 (2.2 nmol) were added and the reaction mixture was kept at room temperature for 2 hours. After completion of the reaction, click ligated ODN-6 was desalted on a NAP-10 column (GE Healthcare), purified by anion-exchange HPLC, desalted again on a NAP-10 column, analysed by MALDI-TOF mass spectrometry [$M + H^+$ = 10205.1] and subjected to melting experiments along with short unmodified oligonucleotides ODN-7 and ODN-8 (18 mers). Click ligation reactions of ODN-2, ODN-4 and ODN-5 were also carried out at 10.0, 2.0, and 0.4 µmolar concentrations with and without template oligonucleotide and also carried out with and without copper catalyst. Dilution was carried out by increasing the volume of 0.2 M aqueous NaCl.

Example 2: Preparation of circular ssDNA (ODN-10)

General

ODN intramolecular (self-circularisation) reactions were carried out at 0.4 µM and
5 monitored by denaturing polyacrylamide gel-electrophoresis (see Figure 5 lane a). This clearly showed the formation of a cyclic product (ODN-10) with lower gel-mobility than the linear starting ODN-9 as previously reported for non-covalently closed pseudo-hexagons by non-denaturing gel-electrophoresis (see F.A. Aldaye, and H. F. Sleiman, infra). Only one major product was formed and no further reaction was observed even
10 after extended times. The 70mer ssDNA closed circle ODN-10 was purified by extraction from the gel into sterile water.

Experimental

To 2500 µL of 0.2M aqueous NaCl, triazolyl ligand (1.38 µmol), sodium ascorbate (2.0
15 µmol) and $CuSO_4.5H_2O$ (0.20 µmol) were added sequentially. To the above solution azide-alkyne labelled ODN-9 (1.0 nmole) was added and the solution was stored at room temperature for 2 hours. The reaction mixture was desalted on a NAP-25 column (GE Healthcare) using the manufacturer's instructions and then lyophilized. Click ligated single strand cyclic ODN-10 was purified by denaturing polyacrylamide gel
20 electrophoresis. The circular ssDNA ODN-10 was also prepared in this manner at 1.0 and 4.0 µM concentrations.

Example 3: Preparation of circular ssDNA (ODN-15)

25 To further demonstrate that circular products and not linear or cyclic dimers are formed under the conditions described in Example 2, oligonucleotide ODN-13 (12 mer) was circularised to ODN-15 by following the same procedure described in Example 2 with 0.2 µM concentration. This proceeded with high efficiency, as shown by HPLC (see Figure 6), and the resultant pure circular ODN-15 was characterised by MALDI TOF MS
30 [M + H^+ Calc. 4316, found 4317].

Example 4: Preparation of circular ssDNA (ODN-14)

Following the same procedure again, ODN-1 1 was circularised at 2.0 µM concentration
35 to give ODN-14.

Conclusive proof of circularisation was obtained by annealing the circular ODN-14 (72 mer) to a complementary ODN-16 (24mer) to create a restriction site for Mboll. A partial enzyme digestion produced a single product migrating at the same position on gel as the linear ODN-1 1 (72 mer) (see Figure 7). When the linear ODN-1 1 (72 mer) was 5 annealed to the same ODN-16 (24 mer) and cut with Mboll, two short fragments were obtained as expected.

Example 5: Preparation of dsDNA pseudo-hexagon (ODN-12)

10 **General**

Template-mediated formation of a dsDNA pseudo-hexagon ODN-12 was carried out by mixing the purified circularized ODN-10 (see Example 2) with its linear complement ODN-1 1 (see Figure 3). The click ligation reaction was carried out and a new retarded band appeared on the denaturing polyacrylamide gel (see Figure 5 lane b) attributed to 15 the formation of a covalently closed linked double stranded catenane constructed from the two click-ligated oligonucleotides.

Experimental

To 400 μ_ of 0.2M aqueous NaCl, tiazolyl ligand (1.38 μmol), sodium ascorbate (2.0μM) 20 and $CuSO_4.5H_20$ (0.20 μM) were added sequentially. A solution of circular ssDNA ODN-10 and azide-alkyne labelled ODN-1 1 (1.0 nmol) in 100 μ_ of 0.2 M aqueous NaCl was heated to 90 ^{0}C and then cooled slowly to 20 ^{0}C. This was repeated three times and the solution was then added to the above prepared catalyst solution at 10 ^{0}C. The reaction was continued for 2 h then for a further 30 mins at room temperature. Finally, the 25 reaction product was analyzed by denaturing polyacrylamide gel electrophoresis.

The covalently closed duplex is described as pseudo-hexagonal because it contains non-Watson-Crick TT base pairs at the vertices, i.e. the two complementary strands ODN-10 and ODN-1 1 have six fully complementary 10mer duplexes punctuated by TT 30 hinges (see Fig. 7).

Interestingly, when linear ODN-9 and ODN-1 1 were mixed in aqueous buffer and the tris-triazolylamine Cu[I]-catalyst/ligand was added, significantly less of this catenated double stranded species was formed. This was expected since the linear duplex 35 structure will increase rigidity and reduce intramolecular interactions at the termini of the construct. Clearly the gel-purification of ODN-10 was also important, as it served to

remove any impure ODN-9 that might contain non-functional termini resulting from incomplete alkyne/azide labeling or from alkyne capping due to reaction of the azide moiety of compound 2 with the terminal alkyne of ODN-9 during the azide labeling reaction. Such side reactions of ODN-1 1 might also limit the yield of the pseudo-
5 hexagonal duplex. Lane b of Figure 5 shows the presence of a weak band just above ODN-1 0 that is probably due to circular single stranded ODN-1 1.

Example 6: Preparation of an oligonucleotide comprising hexaethylene glycol (HEG) linking moieties (ODN-1 7)

10

An oligonucleotide is synthesised by standard solid-phase methods on an automated DNA synthesiser starting with 3'amino C7 controlled pore glass solid support followed by 3 additions of hexaethylene glycol phosphoramidite monomer (Glen Research). The oligonucleotide sequence to be circularised is then assembled on the hexaethylene
15 glycols followed by a further 3 additions of hexaethylene glycol phosphoramidite monomer. Finally, the alkyne phosphoramidite (see Figure 9) is added. The oligonucleotide is then cleaved from the solid support in the normal way (using aqueous ammonia), deprotected by heating at 55° C for 5 hours in a sealed tube, purified by gel-filtration and the amino terminus labelled with azide active ester 2. The resultant
20 oligonucleotide containing terminal azide and alkyne groups (for example ODN-1 7) linked through the hexaethylene glycol spacing moieties is then purified by reverse-phase HPLC and the click ligation reaction described in accordance with the procedure of Example 2.

25 **Analysis and purification of oligonucleotides**

Denaturing polyacrylamide gel electrophoresis (PAGE) analysis and purification

Purification of ODN-1 0, ODN-1 4 and ODN-1 5 was carried out by gel electrophoresis. The crude mixture was purified on 8% polyacrylamide/7 M urea gel (up to 20 A260 of crude DNA per gel) at a constant power of 20 watt for 2 hours, using 0.09 M Tris-borate-
30 EDTA buffer (pH 8.0). Following electrophoresis the plates were wrapped with ding-film, placed on a fluorescent TLC plate and illuminated with a UV lamp (254 nm). The bands were excised, and the gel pieces were crushed and incubated in 3 imL of sterile water at 37 ^{0}C (16 hours). The tubes were then vortexed, centrifuged and the supernatants were lyophilized, dissolved in sterile water (1 mL) and desalted using NAP-10 columns (GE
35 Healthcare) using the manufacturer's instructions.

HPLC purification

Reversed-phase HPLC

Purification of oligonucleotides ODN-2, ODN-4, ODN-5, ODN-7 and ODN-8 was carried out by reversed phase HPLC on a Gilson system using an ABI Aquapore column (C8), 8 mm x 250 mm, pore size 300 A. The system was controlled by Gilson 7.12 software and the following protocol was used: Run time 30 mins, flow rate 4 imL per min, binary system, gradient: Time in mins (% buffer B);0 (0); 3(0); 5(20); 2 1 (100); 25(100); 27 (0); 30(0). Elution buffer A: 0.1 M ammonium acetate, pH 7.0, buffer B: 0.1 M ammonium acetate with 35% acetonitrile pH 7.0. Elution of oligonucleotides was monitored by ultraviolet absorption at 295 nm. After HPLC purification oligonucleotides were desalted using disposable NAP 10 Sephadex columns (GE Healthcare), aliquoted into eppendorf tubes and stored at -20 ^{0}C.

Denaturing anion-exchange HPLC analysis of click ligation reactions and purification of ODN-6

Click ligation reactions were analysed on a Gilson HPLC system using a ResourceQ anion-exchange column (6 imL volume, GE Healthcare). The HPLC system was controlled by Gilson 7.12 software and the following protocol was used: run time 30 mins, flow rate 6 imL per min, binary system, gradient: time in mins (% buffer B); 0 (0); 3(0); 4(5); 23 (100); 26(1 00); 27 (0); 30(0). Elution buffer A: 10mM NaOH, 0.05 M NaCl, pH 12.0, buffer B: 10 imM NaOH, 1 M NaCl pH 12.0. Elution of oligonucleotides was monitored by ultraviolet absorption at 270 nm. ODN-6 was purified by this method monitoring at 295 nm. After HPLC purification it was desalted using disposable NAP 10 Sephadex columns (GE Healthcare), aliquoted into eppendorf tubes and stored at -20 ^{0}C.

Enzymatic digestion of linear and circular oligos

A solution of linear oligo ODN-1 1 (10.57 g, 0.47 nmol) and ODN-16 (5.29 g, 0.7 nmol) in sterile water 20 μL was prepared and heated to 90 ^{0}C then cooled slowly to 20 ^{0}C. This was repeated three times to prepare the double strand DNA. The DNA solution was added to a microcentrifuge tube containing 10.6 μL water, 0.4 μL 10X buffer [60 mM Tris-HCl (pH 7.5), 500 mM NaCl, 60 mM $MgCl_2$ and 10 mM DTT] and 0.4 μL acetylated BSA (0.1 mg/mL). To this solution 8 μL of the restriction enzyme Mbo1 1, (purchased from Promega), (6u/μL) was added and mix gently by pipetting, the tube was closed and centrifuged for a few seconds. The sample was incubated at 37 ^{0}C for 2.5 hrs.

Purified circular ODN-14 was digested following the same procedure. Purification of ODN-14) from the circularisation reaction mixture was done using denaturing 8% polyacrylamide gel electrophoresis (as described above).

5 Table 1 below sets out the sequences of the oligonucleotides used in the above Examples with corresponding SEQ. ID. nos.

Table 1.

ODN	Sequences (5'-3') (**K** = alkyne, **Z=** azide, **X** = 1,2,3-triazole linker, **NH_2**= amino C7, **H**= hexaethylenegycol unit)
ODN-1	CTTTCCTCCACTGTTGC**NH_2** (SEQ. ID no. 1)
ODN-2	CTTTCCTCCACTGTTGC**Z** (SEQ. ID no. 2)
ODN-3	GCGATCAATCAGACG (SEQ. ID. No. 3)
ODN-4	**K**GCGATCAATCAGACG (SEQ. ID. No. 4)
ODN-5	TTTTTCGTCTGATTGATCGCGCAACAGTGGAGGAAAGTTTTT (SEQ.ID.NO.5)
ODN-6	CTTTCCTCCACTGTTGCXGCGATCAATCAGACG (SEQ. ID. No. 6)
ODN-7	ATTGATCGCGCAACAGTG (SEQ.ID. no. 7)
ODN-8	CACTGTTGCGCGATCAAT (SEQ. ID. No. 8)
ODN-9	**K**CCATACATACTTCCACAGCATCTTGATTAGCGTCTTCGATGGTATCTT GGCTCTACAGTTGAGGAGGATG**Z** (SEQ. ID. No. 9)
ODN-10	Circular ssDNA form of ODN-9
ODN-11	**K**TGACGCTAATCTTGATGCTGTGGTTGTATGTATGGTTCATCCTCCTCT TCTGTAGAGCCTTGATACCATCGT**Z** (SEQ. ID no.10)
ODN-12	dsDNA pseudohexagonal catenane
ODN-13	**K**TGAGGAGGATGT**Z** (SEQ. ID. No. 11)
ODN-14	Circular ssDNA form of oligo ODN-11
ODN-15	Circular ssDNA form of oligo ODN-13
ODN-16	AGGCTCTACAGAAGAGGAGGATGA (SEQ. ID. No. 12)
ODN-17	**K**HHHAGCTCGCTACACAAATGACGHHH**Z** (SEQ. ID No. 13)

10

Example 7: Synthesis of Cyclic Mini-Duplexes

The aim of the study was to investigate an approach to making stable mimics of DNA duplexes with linkers that do not physically interact with DNA. The increased

thermodynamic stability of the resultant cyclic duplexes arises principally from the intramolecular nature of the construct with minimal impact on the double helical structure. Cyclic duplexes of this nature have potential biological uses (E. T. Kool, Annu. Rev. biophys. Biomolec. Strut. (1996) 25,1-28). For example, as previously noted, they
5 may find application in vivo as decoys to sequester DNA-binding proteins (Giusto et al. Chembiochem (2006) 7, 535-544). Cyclic DNA duplexes also have potential uses in nanotechnology. Complex DNA nano-array scaffolds can be built from smaller hexagonal building blocks (Tumpane et al. Chem. Phys. Lett (2007) 440, 125-129) and these assemblies could be stabilised by inter-strand cross-linking. The studies reported
10 below show that very stable cyclic DNA duplexes with as few as two base pairs can be constructed using carefully designed linkers.

The precursors to the constructed cyclic DNA duplexes were hairpin oligonucleotides with a 5'-terminal alkyne, a 3'-azide and a loop region consisting of one or two HEG
15 units. CuAAC reaction was used to close the circle by triazole formation (see Figure 16). Two different alkynes were used, the first was based on 6-propargylamidohexanol and the other, which was shorter by four atoms, was derived from 5-hexyn-1-ol. They were introduced into the oligonucleotides as phosphoramidites during solid-phase synthesis. 4-Azidobutyrate was added to a 3'-aminoalkyl group after oligonucleotide synthesis and
20 deprotection as a succinimidyl ester. Reversed-phase HPLC purification was carried out on the oligonucleotides after azide-labelling and after cyclisation, and products were analysed by gel-electrophoresis and mass spectrometry. Oligonucleotide cyclisation was very efficient in all cases (~ 60% yield after full purification).

25 **Experimental Section**

All reagents were purchased from Aldrich, Avocado, Fluka, Link Technologies and used without purification with the exception of the following solvents, which were purified by distillation:THF (over sodium wire and benzophenone), DCM, DIPEA and pyridine (over calcium hydride). All reactions were carried out under an argon atmosphere using oven-
30 dried glassware with purified and distilled solvents. NAP columns were purchased from GE Healthcare. Water soluble polyhydroxypropyltriazole amine Cu[I] binding ligand was synthesized by the method reported in the literature. Column chromatography was carried out under pressure using Fisher scientific DAVISIL 6OA (35-70 micron) silica. Thin layer chromatography was performed using Merck Kieselgel 60 F24 (0.22 mm
35 thickness, aluminium backed). Compounds were visualized by staining with potassium permanganate solution.

1H NMR spectra were measured at 300 MHz on a Bruker AC300 spectrometer and 13C NMR spectra were measured at 75 MHz. Chemical shifts are given in ppm relative to tetramethylsilane, and J values are given in Hz and are correct to within 0.5 Hz. All spectra were internally referenced to the appropriate residual undeuterated solvent signal. Multiplicities of ^{13}C signals were determined using DEPT spectral editing technique. ^{31}P NMR spectra were recorded on a Bruker AV300 spectrometer at 121 MHz and were externally referenced to 85% phosphoric acid in deuterated water. Low-resolution mass spectra were recorded using electrospray technique on a Fisons VG platform instrument or a Waters ZMD quadrupole mass spectrometer in acetonitrile (HPLC grade). High-resolution mass spectra were recorded in acetonitrile, methanol or water (HPLC grade) using electrospray technique on a Bruker APEX III FT-ICR mass spectrometer. MALDI-TOF MS were recorded using a ThermoBioAnalysis Dynamo MALDI-TOF mass spectrometer in positive ion mode using oligonucleotide dT standards.

Synthesis of azide labelling active ester

Succinimidyl-4-azidobutyrate: synthesized as above.

Synthesis of alkyne phosphoramidite monomers

6-O-TBS-hexan-1-propargylamide: synthesized as above.

6-Propargylamido-1-hexanol: synthesized as above.

6-Propargylamidohexan-1-O-(2-cyanoethyl-N,N-diisosopropyl)-phosphoramidite: synthesized as above.

5-Hexyn-1-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite

5-Hexyn-1-ol (1.0 g, 10.2 mmol) was dissolved in DCM (10.0 mL) under an atmosphere of argon followed by the addition of DIPEA (4.4 mL, 25.2 mmol). 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite (2.7 mL, 12.4 mmol) was added dropwise at 0°C and the reaction mixture was left to stir at room temperature for 2 h and then transferred under argon into a separating funnel containing degassed DCM (20.0 mL). The mixture was washed with degassed saturated aqueous KCl (20.0 mL) and the organic layer was separated, dried over sodium sulfate, filtered and the solvent removed in vacuo. The phosphoramidite product (see Figure 18) was purified by column chromatography under

argon pressure (90:10; hexane:ethyl acetate, 0.5% triethylamine) to give the title compound as a colourless oil (1.7 g, 56%); (1.7 g, 56%); δ_H (300 MHz, $CDCl_3$) 3.92-3.75 (2H, m, P-O-CH_2); 3.71-3.53 [4H, m, $NCH(CH_3)_2$ and H-1], 2.66 (2H, t, J = 6.4 Hz, CH_2-CN), 2.25 (2H, dt, J = 2.6, 6.8, H-4), 1.96 (1H, t, J = 2.6 Hz, H-6), 1.89-1.75 (2H, m, H-2), 1.71-1.61 (2H, m, H-3), 1.21 [6H, d, J = 7.0 Hz, $NCH(CH_3)_2$], 1.19 [6H, d, J = 7.0, $NCH(CH_3)_2$]; δ_P (300 MHz, $CDCl_3$) 148.1; m/z LRMS [ES^+, MeCN] 299 (M + H^+, 80%); HRMS (M + Na^+) ($Ci_5H_{27}N_2NaO_2P$) calc.321.1702, found 321.1697.

Oligonucleotide synthesis and azide labelling: Oligonucleotides were synthesized on the 10 µmole or multiples of 1 µmole scale on an ABI-394 DNA synthesizer using standard phosphoramidite cycles. The alkyne phosphoramidites were coupled for 10 minutes and 4-azidobutyrate NHS ester (2 mg) was added post-synthetically to each 1.0 µ mole synthesis of the amino-modified oligonucleotides in 120 µl of DMF/0.5M $NaHCO_3$ buffer (1:2) at pH 8.75 (4 hours at RT). The fully-labelled oligonucleotides were purified by reversed-phase HPLC prior to cyclisation.

General methods for cyclisation of the hairpin oligonucleotides: A wide range Cu (II)/sodium ascorbate and ligand concentrations were evaluated in order to establish the minimum quantities to give high yields of cyclic oligonucleotide. Similarly, oligonucleotide concentrations were varied with the aim of minimizing reaction volumes.

The following is the method of choice: To a solution of tris-hydroxypropyl triazole ligand (17.5 µmol in 3.9 mL 200 mM NaCl; Chan et al. *{ibid))* under argon was added sodium ascorbate (25.0 µmol in 50.0 µL 200 mM NaCl) followed by $CuSO_4.5H_2O$ (2.5 µmol in 25.0 µL 200 mM NaCl). The hairpin loops (1.0 µmol in 1.0 mL 200 mM NaCl) were heated at 80 °C for 5 min, cooled down slowly and added to the above solution. The reaction mixture was kept under argon at room temperature for 2 h and two disposable NAP-25 gel-filtration columns were used to remove reagents (4.0 mL was collected from the column instead of the recommended 3.5 mL. The cyclic oligonucleotides were purified by HPLC as described below to give 57-65 % isolated yield. In some cases, after the cyclisation reaction, gel filtration was omitted and the reagents were removed during HPLC purification as shown in HPLC of C-1 (supporting information). In this case an ACTA-purifier (UPC-900) with a G25 Sephadex column (50 mL) was used to desalt the oligonucleotide after HPLC. In a second reaction to cyclise H-1 to C-1, the ACTA-purifier was used to desalt the oligonucleotide before and after HPLC purification.

Reversed-phase HPLC purification of hairpin and cyclic oligonucleotides:

Purification of cyclic and hairpin loops was carried out by reversed-phase HPLC. After HPLC purification oligonucleotides were desalted using NAP-10 Sephadex columns (GE Healthcare). For the 2-mer cyclic and hairpin loops 2.0 mL was collected from the
5 column instead of the recommended 1.5 mL.

Results

The stability and structural characteristics of cyclic oligonucleotide duplexes and their hairpin precursors of different length and base composition were investigated by UV
10 melting, circular dichroism (CD) and NMR spectroscopy. The results are shown in Table 2 below. C'2 with 19 atom HEG and 20 atom Tz linkers is shown in Figure 16.

Table 2

Code	Oligonucleotide sequence	T_m(X% $HCONH_2$)
H-7	$K_{(11)}$-AATATAA-XX-TTATATT-Z (Seq. ID . no. 14)	37
C-7	$Tz_{(24)}$-AATATAA-XX-TTATATT (Seq. ID. No. 15)	58
H-7 + D	H-7 mer + distamycin A	58
C-7 + D	C-7 mer + distamycin A	68
H-5	$K_{(11)}$-CTTAC- XX-GTAAG-Z # (Seq. Id. No. 16)	47
C-5	$Tz_{(24)}$-CTTAC- XX-GTAAG # (Seq. Id. No. 17)	69
H-5*	CTTAC- XX-GTAAG # (Seq. Id. No. 18)	46
H-4	$K_{(H)}$-CGGC- XX -GCCG-Z (Seq. Id. No. 19)	72, 55(20), 39(50)
C-4	$Tz_{(24)}$-CGGC- XX-GCCG (Seq. Id. No. 20)	>75 (20), 60(50)
H-4*	CGGC- XX-GCCG # (Seq. Id. No. 21)	65
H-3	$K_{(11)}$-CGC- XX-GCG-Z # (Seq. Id. No. 22)	58, 44(20), 32(40)
C-3	$Tz_{(24)}$-CGC- XX-GCG # (Seq. Id. No. 23)	>75, 68(20), 58(40)
H-3*	CGC- XX-GCG # (Seq. Id. No. 24)	51
H-3b*	CGC-X-GCG (Seq. Id. No. 25)	68

H-2	$K_{(1}$D-GC-X-GC-Z # (Seq. Id. No. 26)	49, 32(20)
H-2 + A	$K_{(11)}$-GC-X-GC-Z # +7-aminoact-D (Seq. Id. No. 27)	>75, 60(20), 40(40)
C-2	$Tz_{(24)}$-GC-X-GC # (Seq. Id. No. 28)	>75, 58(20)
C-2 + A	$Tz_{(24)}$-GC-X-GC +7-aminoact- D (Seq. Id. No. 29)	>75, 69(20), 48(40)
H'-2	$K_{(7)}$-GC-X-GC-Z # (Seq. Id. No, 30)	45, 27(20)
C-2	$Tz_{(20)}$-GC-X-GC # (Seq. Id. No. 31)	>75, 66(20), 55(40)
C-1	$Tz_{(20)}$-G-X-C # (Seq. Id. No. 32)	Not done

H = hairpin, C = cyclic, $K_{(11)}$ = 6-propargylamidohexan-1-ol, $K_{(7)}$ = 5-hexyn-1-ol, Z=azide, $Tz_{(24)}$ = triazole from $K_{(11)}$, $Tz_{(20)}$ = triazole from $K_{(7)}$, X = hexaethylene glycol. UV melting at 260 nm (mixed AT/CG sequences) and 272 nm (all-GC sequences) in phosphate buffer (10 mM), NaCl(200mM) and X% $HCONH_2$. # sequences studied by ^{1}H NMR. >75 indicates the T_m was too high to measure accurately.

In all cases the CD spectra confirmed the presence of helically stacked nucleotides, consistent with the B-family of conformations. The NMR coupling constants for the interaction of H1' with H2' and H2" are also indicative of the C-2'-endo family of conformations consistent with B-like DNA.

In these small duplexes the UV and CD spectra are somewhat different from polymeric mixed sequence DNA, particularly the short GC duplexes, which have their UV maximum at 272 nm rather than the usual 258 nm. For this reason, UV melting was carried out at a wavelength adjusted to the particular sequence. The UV melting temperatures (T_m) of the cyclic constructs are much higher than the corresponding hairpins (Table 2) and all duplexes showed an increase in T_m of more than 20°C on cyclisation. In many cases the addition of the denaturant formamide was necessary to lower the (T_m) sufficiently to observe melting.

Interactions with DNA-binding drugs revealed the sequence-dependence that would be predicted from data on unmodified intermolecular duplexes; this confirms the "normal" behaviour of these constructs. In addition, the cyclic oligomers behaved similarly to the corresponding hairpins. The cyclic heptamer C-7, which contained the preferred AAT

binding site for distamycin A (Lah and Vesnaver Biochemistry (2000) 39, 9317-9326), interacted strongly with this drug. This led to an increase in T_m of 10°C and the cyclic GC dinucleotide C-2 with the preferred GpC binding site for 7-aminoactinomycin D was greatly stabilised by the intercalator. The above studies indicate that the cyclic duplexes
5 are very stable and can partake in stacking interactions expected from the accumulated knowledge of normal DNA. In this study non-intrusive linkers were chosen, consisting of functionalities with low molecular mass, and without aromatic moieties that might interfere with normal DNA base stacking and complicate the study of base pairing by UV melting, CD and NMR. This precludes the use of normal nucleotides in the terminal
10 loops.

Within these constraints the length of the linker has an influence on duplex stability. This was apparent for the CGC hairpin trimer H-3b* which has a single hexaethylene glycol linker (HEG) compared to H-3* with the same base sequence and 2 x HEG (ΔT_m= + 17°
15 C). Similarly the length of the triazole linker greatly influenced the stability of cyclic duplexes. The GC dimer C'-2 with a 20-atom linker is much more stable than C-2 which has a slightly longer 24-atom spacer. Indeed, C-2 with linkers of 19 and 20 atoms formed an extremely stable di-nucleotide duplex, the T_m of which was above the measurable range in aqueous buffer in the absence of a denaturant.
20

Direct evidence for H-bond-mediated base pairing was obtained by ^{1}H NMR studies in H_2O. The appearance of GN1 and TN3-imino protons between 12.5 and 14 ppm and cross-strand NOEs to CN4-amino protons in the range 8 to 9 ppm is indicative of inter base-pair hydrogen bonds. GN1 protons are normally in faster exchange than CN4
25 protons and are therefore sensitive indicators of base pair fraying. In all the structures examined, the hairpin duplexes exhibited fraying at the "open" end, i.e. at the base pair furthest from the stabilizing hexaethylene glycol linker. Although hairpin duplexes are significantly more stable than intermolecular duplexes, their end base pair behaved like the terminal base pair of an intermolecular duplex and was difficult to observe by NMR.
30 In contrast, all base pairs in all cyclic duplexes except C-1 were kinetically stable. This was most striking for the C-2 and C-2 dimers, the shortest duplexes in which base stacking is possible. All exchangeable H-bonded base protons were present at 20^0C, even those of GN1 H. The high thermal stability of the inter-base H-bonds parallels the observed elevated melting temperature of these cyclic mini-duplexes. Hydrogen bonding
35 in the equivalent hairpin H'-2 is much weaker, with no GN1H resonances above 5^0C.

In conclusion, the short cyclic oligonucleotides described here can be prepared in high yield on multi-micromole scale. They give rise to very stable duplexes and can be developed as useful tools in biophysical and biological studies. The click chemistry method of DNA strand crosslinking is also potentially useful in nanotechnology to
5 stabilize DNA nanoarrays.

Example 8: Study of thermal and serum stability of cyclic ssDNA and end sealed duplexes

10 Chemical ligation was carried out of two single strand oligonucleotides (ODN-Ia and ODN-2a; see Table 3) to form very stable double stranded-DNA with a triazole linker at each end (end-sealed duplex 1, EsD-1) as shown in Figure 17. Each single strand had a 5'-terminal alkyne and 3'-azide. The copper-catalysed alkyne-azide cycloaddition reaction (CuAAC) was used to link the two strands by covalently joining the 5'- and the
15 3'-ends.

Synthesis of oligonucleotides

The alkyne based on 6-propargylamidohexane (see above and Kumar et al. J. Amer. Chem Soc (2007) 129, 6859-6864.) was incorporated into the oligonucleotide as
20 phosphoramidite during solid phase synthesis. 4-Azideobutyrate was added as a succinimidyl ester, to a 3'-aminoalkyl group after oligonucleotide synthesis and deprotection. Oligonucleotides were synthesized on an ABI-394 DNA synthesizer as previously using standard phosphormidite cycles. The alkyne phosphoramidites were coupled for 10 mins and 4-azidobutyrate NHS ester was then added post-synthetically
25 in 120 µl of DMSO/0.5M (Na_2CO_3Z$NaHCO_3$ buffer) (1:2) at pH 8.75 (4 hrs at room temperature). The fully labelled oligonucleotides were purified by reverse-phase HPLC.

Synthesis and purification of end sealed duplexes and cyclic oligonucleotide

ODN-Ia (10 nmole) and ODN-2a (10 nmole) were mixed and heated at 80 ^{0}C for 5 min,
30 cooled down slowly such that the click reaction proceeded to give the end sealed duplex EsD-1 . End sealed duplexes and cyclic oligonucleotide ODN-5 was carried out by reversed-phase HPLC. After HPLC purification oligonucleotides were desalted using NAP-10 Sephadex columns (GE Healthcare).

35 The purified products were analysed by polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE) and mass spectrometry. The stability of end-sealed

duplex and its unsealed double stranded precursor were investigated by fluorescence and UV melting. The UV melting showed that the Tm of end-sealed duplex was higher by 30°C than the unsealed duplex and this was confirmed by fluorescence melting. The Tm of the end-sealed duplex was very high and formamide (DNA denaturant) has to be
5 added for it to be measurable. Formation of the end-sealed duplex with two triazole linker in both ends was proved by PAGE, CE and MALDI-TOF mass spectrometry of each single strand and the end-sealed double strands. Extra proof of the existence of the end-sealed duplex was obtained by enzymatic digestion of the unsealed and the end-sealed duplexes using the restriction enzyme EcoRI. The CE (capillary
10 electrophoresis) showed four peaks from cutting the unsealed duplex and two peaks, as expected, from cutting the end-sealed duplex as shown in the figure below. In addition to the two peaks of the resultant digestion fragments, the CE of the end-sealed duplex shows a peak for the starting undigested end-sealed duplex, indicating the slight resistance of this duplex towards the enzymatic degradation compared with the
15 unsealed duplex which was completely digested. The data was confirmed by PAGE (polyacrylamide gel electrophoresis).

Serum stability of ssDNA, cyclic ssDNA and end-sealed dsDNA

To determine the stability of end-sealed duplex in a biological context, the stability of the
20 duplex was investigated in fetal bovine serum. One of the single strands (ODN-3a) was labelled with 5-fluorescein-labelled dT to produce a fluorescein-labelled end-sealed duplex (EsD-2). The end-sealed duplex was incubated with fetal bovine (50%) serum at 37°C.

25 The protocol for assessing serum stability of DNAs was as follows: A mixture of DNA (0.03 OD) in phosphate buffer (15 µL) (10 mM phosphate, pH 7, 200 mM NaCl) and fetal bovine serum (15 µL) was vortexed and incubated at 37 °C for the desired time. The mixture was stored in the freezer until all the samples were collected. Formamide (15 µL) was added to each sample, and the sample then vortexed, heated at 80 °C for 5 min
30 and cooled in ice. The results were analysed by denaturing gel electrophoresis (20% polyacrylamide gel).

The end sealed duplex showed stability up to 3 days while the single strand (ODN-3a) digested in two hours. Similar results were obtained using 10% and 90% serum.

35

To investigate the stability of cyclic single strand, oligonucleotide (ODN-4a) with three hexaethylene glycol (HEG) units in each side and 5'-alkyne and 3'-azide, was cyclised using the copper-catalysed alkyne-azide cycloaddition reaction (CuAAC). UV melting study showed that cyclisation of this cyclic HEG oligonucleotide did not affect its
5 hybridisation with its complementary oligonucleotides (ODN-6a) with HEG or (ODN-7a) without HEG. The T_m of the cyclic construct (ODN-5a) with its complements (ODN-6a) or (ODN-7) was slightly lower than the T_m of the linear oligonucleptode construct (ODN-4a) with its complements.

10 The linear and cyclic ODN-4a and ODN-5a were incubated in 50% fetal bovine serum as above and the reaction was analysed by gel electrophoresis. The cyclic oligonucleotide (ODN-5a) was stable in serum up to 12 hrs. The single strand oligonucleotide with three HEG units in each end was more stable in serum than the single strand without HEG which digested completely in two hours. This is expected as 3'-modified oligonucleotides
15 have increased stability towards endonucleases.

Table 3

ODN	Sequences (5'-3'), (**K** = alkyne, **Z** = azide, **X** = 1,2,3-triazole linker, **F** = fluorescein dT, H = hexaethylene glyccol)
ODN-1a	**K**GCACCAGAATTCATCACG**Z** (Seq. Id. No.33)
ODN-2a	**K**CGTGATGAATTCTGGTGC**Z** (Seq. Id No. 34)
EsD-1, Cyclic 18mer duplex	-**X**-GCACCAGAATTCATCACG-X-CGTGATGAATTCTGGTGC**-** (Seq. id. No. 35)
ODN-3a	**K**CGTGA**F**GAATTCTGGTGC**Z** (Seq. Id. No. 36)
EsD-2, Cyclic	-**X**-CGTGA**F**GAATTCTGGTGC-X-CGTGATGAATTCTGGTGC- (Seq. Id. No. 37)
ODN-4a	**K**HHHGCACCAGAATTCATCACGHHH**Z** (Seq. Id. No. 38)
ODN-5a, Cyclic	-**X**HHHGCACCAGAATTCATCACGHHH- (Seq. Id. No. 39)
ODN-6a	**K**HHHCGTGATGAATTCTGGTGCHHH**Z** (Seq. Id. No. 40)
ODN-7a	CGTGATGAATTCTGGTGC (Seq. Id. No. 41)

Similarly, cyclic 20 mer duplexes formed from hairpin constructs with an HEG linker as
20 in Example 7 were shown to be more stable in serum then the equivalent haipin parent construct.

Example 9: Synthesis of further oligonucleotide catenanes

Synthesis of alkyne oligonucleotides

5 5-Hexyn-1-ol **(5)** was subjected to conventional phosphitylation conditions by addition of 2-O-cyanoethyl-N,N,diisopropylamino chlorophosphoramidite and DIPEA in CH_2Cl_2 under an argon atmosphere as described above in Example 7 and shown in Figure 18 except the final reaction mixture was left for 0.7 hrs at room temperature. The pure phosphoramidite 6 was isolated in 20% yield.

10

This monomer was then incorporated into ODNs at the 5'-terminus by a standard oligodeoxynucleotide synthesis method, to give ODN-I b and ODN-2b (see Figure 18) which were purified by HPLC and characterised by MALDI-TOF MS [$M+H^+$ calcd, 12875; found, 12884] for ODN-Ib and ES MS [$M+H^+$ calcd, 8861 ; found, 8867] for ODN-2b. All 15 ODN sequences are given in Table 5 below.

Synthesis of azidohexanamide-labelled oligonucleotides

Successful azidation of ethyl-6-bromohexanoate was achieved using sodium azide and potassium bicarbonate at 50 °C. These conditions converted the bromure into azide, in 20 86% yield. Compound 8 was then hydrolysed using a solution of sodium hydroxide in a mixture of water and dioxane in a 91% yield. The following esterification of 9 was carried out with N-hydroxysuccinimide and EDC as an activating ester and afforded 10 in a 52% yield (see synthesis scheme in Figure 19).

25 Azide ODN-3b and 4b were prepared by labelling 3'-amino-modified ODN-I b and 2b with 10 in bicarbonate buffer at pH 8.75. 1.00 µmol of C7-aminoalkyl ODN was incubated for 4 hours in buffer (0.5 M Na_2CO_3/$NaHCO_3$, pH 8.75) (70 µL) with N-succinimidyl 6-azido-hexanoate (3 µL) and DMSO (100 µL). The crude ODN was desalted by NAP-10 gel-filtration. The resultant 3'-azide-labeled ODN was then purified 30 by reversed-phase HPLC and characterized by MALDI-TOF MS [$(M+H)^+$ calcd, 13029 ; found, 13024] for ODN-3b and ES MS for ODN-4b [$(M-H)^-$ calcd, 9000; found, 9006].

Click chemistry

Single strand cyclisation

35 The Cu[I] click catalyst was prepared *in situ* from Cu[II] sulphate and sodium ascorbate. All ligation reactions were carried out in 0.2 M aqueous NaCl in the presence of the

water soluble tris-(hydroxypropyltriazolylmethyl)amine ligand. To a solution of Tris-(hydroxypropyltriazolylmethyl)amine ligand (1.22 mg, 2.80 µmol in 590 µL 0.2 M NaCl) under argon was added 0.4 M NaCl (955 µL), sodium ascorbate (8 µL, 4 µmol) followed by $CuSO_4.H_2O$ (4 µL, 0.40 µmol). The azido-labelled alkyne ODN was added and the
5 solution was stored at rt for 2 hours. The reaction mixture was desalted by NAP-25 gel-filtration and purified by reversed-phase HPLC. The purified ODNs were characterised by MALDI-TOF MS.

Self-circularization reactions of ODN-3b and ODN-4b were carried out at respectively
10 30.8 and 46.9 nM concentrations of oligonucleotide. A quantitative conversion to the cyclic ODN-5b was observed by chromatography electrophoresis (CE). Similar CE analyses were obtained after cyclisation of ODN-4b to ODN6b.

Attempted catenation with the first set of oligonucleotides

15 Formation of the double stranded catenane from the cyclic ODN-5b and the linear ODN-4b was carried out in parallel with cyclic ODN-6b and the linear ODN-3b. The purified cyclic ODNs were mixed with their respective linear complementary strands and the click reaction was carried out at 200 mM aqueous NaCl. The following method gave the best yields.

20

To a solution of tris-(hydroxypropyltriazolylmethyl)amine ligand (1.83 mg, 4.20 µmol in 4 mL 0.2 M NaCl) under argon was added 0.4 M NaCl (18 µL), sodium ascorbate (12 µL, 6.00 µmol) followed by $CuSO_4.H_2O$ (6 µL, 0.60 µmol). A solution of cyclised ssDNA (12 nmol) and its complementary azido-labelled alkyne ODN (12 nmol) in 3314.6 µL of 0.2 M
25 NaCl was prepared and heated to 80 ^{0}C then cooled down slowly to rt for 2 hours, then ODN-13 (24 nmol) (if used) was added and the reaction was stored at rt for 0.5 hours. The solution was added to the above prepared catalyst solution and the reaction was stored at rt for 2 hours. The reaction mixture was desalted by NAP-10 gel-filtration and then lyophilized. Finally, the reaction product was analysed and purified by denaturating
30 20% PAGE electrophoresis.

Denaturating PAGE gel electrophoresis analysis and purification: The ODNs were analysed or purified on 20% polyacrylamide/7M urea gels (up to 20 A_{260} of crude DNA per gel) at a constant power of 20 W, for 3-4 hours, using 0.09M Tris-borate-EDTA
35 buffer (pH 8.0). Gels were visualised on a fluorescent TLC plate and illuminated with a

UV lamp (254 nm) or trans-illuminated with a UV light when a fluorescein dT was present.

No new retarded band appeared on the denaturing 8% and 20% PAGE and no new
5 peak was seen by capillary electrophoresis (CE). However, using a standard melt programme (20-80-20 ^{0}C at 0.5 ^{0}C or 0.25 ^{0}C), UV melting of ODN-6b and ODN-3b and then ODN-5b and ODN-4b showed only a small decrease in thermal stability (ΔTm = -2 or -3 ^{0}C) compared with the duplex of the two linear ODNs ODN-3b and ODN-4b

10 The attempted formation of the catenane duplex at 1 M NaCl was unsuccessful.

The rigidity of the two turns of the double helix may explain why the linear ODN was not able to ligate. For this reason, two new ODNs were synthesised with only 12 matching base pairs (just over one helix turn). A fluorescein label was incorporated into the
15 second one to cyclised, in order to improve the visualisation by gel electrophoresis. A third short ODN was also used to help the second circularisation.

Catenation with the second set of oligonucleotides

ODN-9b and 10b were prepared using standard automated DNA synthesis and labelled
20 with 3'-azide and 5'-alkyne. Self-circularisation reaction of ODN-9b and 10b was then carried out at 6.0 and 0.5 nM concentrations of ODN respectively.

The catenane was prepared by mixing the cyclic ODN-1 1b, the linear ODN-IOb, and the helper ODN-1 3b, then the click reaction was carried out at 200 mM aqueous NaCl. A
25 new retarded band appeared on a denaturing 20% PAGE gel, which was attributed to the formation of the covalently closed linked double stranded catenane. However, the crude mixture of the circularisation of ODN-IOb also showed a faint retarded band at the same distance which could be attributed to the dimmer of ODN-IOb

30 In order to remove any doubts about the nature of the retard band, the strategy was modified so the first ODN to be cyclised contained a fluorescein.

Catenation with the third set of oligonucleotides

ODN-1 6b and 17b were prepared using standard automated DNA synthesis and
35 labelled with 3'-azide and 5'-alkyne. Self-circularisation reaction of ODN-1 6b and 17b were then carried out at 20.0 and 8.0 nM concentrations of ODN respectively. The

catenane (ODN-20) was prepared by mixing the cyclic ODN-18, the linear ODN-17b, and the helper ODN-13b, then the click reaction was carried out at 200 mM aqueous NaCl. The yield of the reaction appeared to be around 50%. The catenane was purified by gel electrophoresis extraction. Enzyme digestion with Eco-R1 showed the formation of digested cyclic ODN-18 and cyclic ODN-19 thereby proving catenane existence.

Table 4

(Sequences 5'-3'; H = HEG, K = alkyne, Z = azide, NH2 = amino C7, T* = fluorescein dT)

ODINM	K-TTTTTTTTTTGCACCAGAATTCATCACGGAGTTTTTTTTTT-N H_2 (Seq. Id. No. 42)
ODN-2	K-HHHCTCCGTGATGAATTCTGGTGCHHH-NH $_2$ (Seq. Id. No.43)
ODN-3	K-TTTTTTTTTTGCACCAGAATTCATCACGGAGTTTTTTTTTT-Z (Seq. Id. No. 44)
ODN-4	K- HHHCTCCGTGATGAATTCTGGTGCHHH-Z (Seq. Id No. 45)
ODN-5	Circular ssDNA of ODN-3
ODN-6	Circular ssDNA of ODN-4
ODN-7	K-TTTTTTTTTTTTTTTCCAGAATTCATCTTTTTTTTTTTTTTT-NH $_2$ (Seq. Id. No. 46)
ODN-8	K-GCGTHHHGATGAATTCTGGHHHTGCG-NH $_2$ (Seq. Id. No. 47)
ODN-9	K-TTTTTTTTTTTTTTTCCAGAATTCATCTTTTTTTTTTTTTTT-Z (Seq. Id. No. 48)
ODN-10	K-GCGTHHHGATGAATTCTGGHHHTGCG-Z (Seq. Id. No. 49)
ODINM 1	Circular ssDNA of ODN-9
ODN-12	Circular ssDNA of ODN-10
ODN-13	CGCCGC (Seq. Id No. 50)
ODN-1 4	K-TTTTTTTTTTTTTTTCCAGAATTCATCTTTTTTTTTTTTTTT-N H_2 (Seq. Id. No. 5 1)
ODN-1 5	K-GCGTHHHGATGAATTCTGGHHHTGCG-NH $_2$ (Seq. Id. No. 52)
ODN-1 6	K-TTTTTTTTTTTTTTTCCAGAATTCATCTTTTTTTTTTTTTTT-Z (Seq. Id. No. 53)
ODN-1 7	K-GCGTHHHGATGAATTCTGGHHHTGCG-Z (Seq. Id. No. 54)
ODN-18	Circular ssDNA of ODN-1 6
ODN-19	Circular ssDNA of ODN-1 7
ODN-20	dsDNA from ODN-1 8 and ODN-1 7

Example 10: DNA Strand cross-linking using alkyne and azide groups attached to deoxyuridine

Studies were carried out to show the utilty of the CuAAC reaction for the linking of DNA sequences between modified uracil bases. For this purpose, a single deoxyuridine (dU) nucleoside modified with a terminal alkyne was incorporated in one DNA strand and an azide-modified dU was inserted in its complement. Two different 5-alkyne-modified deoxyurdines were evaluated. The first, 5-ethynyl-2'-deoxyuridine has the closest possible attachment of the alkyne to the nucleobase, and the other, 5-octa-1 ,7-diynyl)-2'-deoxyuridine, has a flexible linker between the base and the terminal alkyne.

The relevant alkyne was inserted as the fifth or third nucleotide from the 5'- end of a 14mer DNA. The azide was inserted 4 nucleotides from the 3' end of a complementary DNA strand (see Figure 20).

Experimental

The phosphoramidite building blocks 1 and 2 (see Figure 21) were synthesised by published procedures (Seela et al. Helvetica Chimica Acta (2007) 90 (3) 535-552; Seela et al. Chem, & Biodiversity (2006) 3 (5) 509-514; Gierlich et al. Org. Lett. (2006) 8 (17) 3639-3642; Graham et al., J. Chem. Soc.-Perkin Trans. 1 (1998) (6) 1131-1 138) and introduced into the oligonucleotides during solid-phase synthesis as outlined below.

5'-O-(4,4'-Dimethoxytrityl)-5-(5"-trifluoroacetamido-1"-pentynyl)-2'-deoxyuridine (4b):

To a solution of 5'-O-(4,4'-dimethoxytrityl)-5-(5"-phthalimido-1 "-pentynyl)-2'-deoxyuridine (F. W. Hobbs, J. Org. Chem. (1989) 54, (14) 3420-3422)) (519 mg, 0.7 mmol) in a mixture of ethanol (6 ml.) and water (3 ml.) was added hydrazine monohydrate (73 µL, 1.5 mmol) and the solution was stirred at room temperature overnight. Water (6 ml.) was then added and the suspension was washed with DCM (3 x 15 mL). The organic layers were combined and concentrated in vacuo and the crude product was purified by silica-gel flash column chromatography (pre-equilibrated with 1% Et_3N in DCM), eluting with methanol in DCM (0-30% + 1% Et_3N). This yielded the amine intermediate (200 mg, 0.33 mmol) which was dissolved in THF (5 mL). DMAP (40 mg, 0.3 mmol) and ethyl trifluoroacetate (0.66 mmol) were added to the solution and the reaction mixture was stirred overnight at room temperature, then concentrated and purified by silica-gel flash column chromatography (pre-equilibrated with 1% Et_3N in

chloroform) eluting with ethanol in chloroform (0-10%). This yielded the product 4b as a foam (188 mg, 38% over 2 steps). δ_H (400 MHz, DMSOd $_6$): 11.60 (s, 1H, NH), 9.39 (t, 1H, J = 5.3 Hz, $NHCOCF_3$), 7.87 (s, 1H, H-6), 7.42-7.20 (m, 9H, DMT), 6.89-6.87 (m, 4H, DMT), 6.13 (t, 1H, J = 6.6 Hz, H-1'), 5.32-5.31 (d, 1H, J = 4.4 Hz, 3'-OH), 4.29 (m,
5 1H, H-3'), 3.92 (m, 1H, H-4'), 3.74 (s, 6H, OCH_3), 3.26-3.10 (m, 4H, H-5' + H-5"), 2.29-2.17 (m, 4H, H-3" + H-2'), 1.57-1 .50 (m, 2H, H-4"); $_c$ (100.6 MHz, DMSO-d$_6$): 162.15 (uracil C-4), 158.55 (uracil C-2), 156.69 ($COCF_3$), 149.81 , 145.22 (DMT), 142.64 (uracil C-6), 136.03, 135.81 , 130.14, 130.1 1, 128.31 , 128.05, 127.08 (DMT), 116.38 ($COCF_3$), 113.65 (DMT), 99.60 (uracil C-5), 92.76 (C-2"), 86.32 (C-4'), 85.26 (C-T),
10 73.15 (C-1"), 70.90 (C-3'), 64.14 (C-5'), 55.46 (OCH_3), 40.44, 38.92 (C-2', C-5"), 27.75 (C-4"), 16.83 (C-3"); m/z LRMS [ES^+, MeCN] 730 ($M+Na^+$); HRMS ($M+Na^+$) ($C_{37}H_{36}F_3N_3NaO_8$): calcd, 730.2347, found 730.2343.

3´-O-(-2-cyanoethoxy-N,N-diisopropylaminophosphinyl)-5'-O-(4,4´-
15 **dimethoxytrityl)-5-(5"-trifluoroacetamido-1"-pentynyl)-2'-deoxyuridine(4c):**
Compound **4b** (520 mg, 0.74 mmol) was dried in an evacuated heating pistol over KOH at 40 °C overnight then dissolved in DCM (7 ml.) under an argon atmosphere. DIPEA (645 µl_, 3.7 mmol) and 2-cyanoethoxy-N,N-diisopropylaminochloro-phosphine (198 µl_, 0.9 mmol) were added and the reaction mixture was stirred for 1 h under argon
20 atmosphere, diluted with DCM (15 ml.) and washed with saturated aqueous KCl (20 ml_). The organic layer was separated, dried over Na_2SO_4 and the solvent was removed *in vacuo* to give an oil. This was purified by silica gel column chromatography (pre-equilibrated with 1% Et_3N in chloroform) using a gradient of ethylacetate in chloroform (0-100%, v/v) to afford the product as a white form, which was directly dissolved in DCM
25 (10 mL) and precipitated from cold hexane (200 mL) to afford the title product **4c** as a white solid (400 mg, 60%). δ_P (121 .6 MHz, $CDCl_3$): 148.8, 148.4; m/z LRMS [ES^+, MeCN] 930 ($M+Na^+$).

Oligonucleotide synthesis: Standard DNA phosphoramidites, solid supports and
30 additional reagents including the C7-aminoalkyl CPG were purchased from Link Technologies or Applied Biosystems Ltd. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 1.0 µmole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the
35 automated trityl cation conductivity monitoring facility and in all cases were >98.0%. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a

concentration of 0.1 M immediately prior to use. The coupling times were 25 s for normal (A,G,C,T) monomers and 10 min for all alkyne and amino phosphoramidites. Cleavage of oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by
5 heating in a sealed tube for 5 h at 55 °C.

Azide labelling: NHS esters of 4-azidobutyrate 5 (2 mg) or 6-azidohexanoate 6 (2 μL) were added post-synthetically to 1.0 μmole syntheses of the amino-modified oligonucleotides in 120 μl of DMSO:0.5 M Na_2CO_3ZNaHCO_3 buffer 1:2 at pH 8.75 for 4 h
10 at room temperature. The fully-labelled oligonucleotides were desalted using NAP-25 Sephadex columns (GE Healthcare) and purified by RP- HPLC prior to cross-linking.

Interstrand cross-linking of DNA duplexes

For the DNA inter-strand cross-linking, the Cu[I] catalyst was prepared *in situ* from Cu[II]
15 sulphate (200eq relative to DNA) and sodium ascorbate (10 eq relative to Cu[II] sulphate). The water-soluble tris-hydroxypropyltriazolylamine Cu[I]-binding ligand was used in 7-fold excess relative to Cu[II] sulphate. All ligation reactions were carried out in 0.2M aqueous NaCl to ensure complete formation of duplex and a standard reaction time of 2 hrs was used. The reaction was quenched by eluting down down a NAP-25 gel
20 filtration column and the ligated DNA duplexes were purified by RP-HPLC. The efficiency of cross-linking was evaluated by denaturing 8% polyacrylamide gel electrophoresis.

General method for cross-linking:

25 The solution containing alkyne oligonucleotide (25 nmol) and azide oligonucleotide (25 nmol) in the appropriate amount of 0.2 M aqueous NaCl was heated at 80 °C for 5 min, then cooled down slowly to 20 °C, degassed using argon for 5 min and added to the degassed solution of tris-hydroxypropyltriazolylamine ligand (35 μmol), sodium ascorbate (50 μmol) and $CuSO_4.5H_2O$ (5 μmol). Total volume of the reaction mixture
30 was 2.5 imL. The concentrations of aqueous stock solutions were: 100 μg/μL sodium ascorbate and 25 μg/ μL $CuSO_4.5H_2O$. The reaction mixture was kept under argon at room temperature for 2 h and processed as noted above.

The same procedure was also applied using tris-hydroxypropyltriazolylamine ligand (875
35 nmol), sodium ascorbate (1.25 μmol) and $CuSO_4.5H_2O$ (125 nmol). Total volume of the reaction mixture was 1.0 imL.

Monitoring of the cross-linking reaction: The reaction mixture was kept under argon at room temperature and at 5, 15, 30, 60 and 120 min aliquot amounts were collected, each directly loaded onto a disposable NAP-10 gel-filtration column and analyzed by denaturing 8% polyacrylamide gel electrophoresis, MALDI-TOF-MS and CE.

Results

The click reaction with ethynyl dU and azide on the 15 atom side chain was not efficient, probably due to steric hinderance at the alkyne. In contrast, the click reaction with octadiynyl dU and azide with a 15 atom spacer went smoothly to completion in 2hrs.

The minimum time required for completion of the click reaction was investigated under an atmosphere of argon in capped vials as indicated above. It was found that the reaction was essentially complete in 5 minutes. In all cases the cross-linked duplexes were thermally very stable and displayed conformational properties typical of B-DNA.

Click ligation using an alkynyl dU such as 5-(octa-1 ,7-diynyl)-2'-deoxyuridine and azide-modified dU is thus also envisaged as an alternative reaction for cyclisation of oligonucleotides, e.g. end-sealing of a hairpin as above.

Example 11: AAC reaction without catalyst

All the cyclisations in the examples above were carried out using Cu(I) catalyst produced *in situ* in the presence of tris-(hydroxypropyltriazolylmethyl)amine Cu(I)-binding ligand. However, the azide/alkyne coupling reaction has been shown to proceed, albeit much slower, in the absence of catalyst. As previously noted above, this is significant in indicating feasibility of using such reaction to link oligonucleotide sequences intracellular^.

AAC reaction with and without Cu(I) catalysis was compared using hairpin constructs of the type described in Example 7 above with an azide group at one terminus and an alkyne group at the other terminus. Figure 22 shows the denaturing gel analysis of the tested reaction conditions with a hairpin having three base pairs joined by a linker comprising HEG units. Using 10 μM hairpin construct, the reaction to form the triazole linker with Cu(I) catalysis was found to be achievable within 2 hours at room temperature with buffer (1OmM Na phosphate, 0.2M NaCl, pH 7) or just 0.2M NaCl (see

gel lanes 6 and 7 of Figure 22(b). Further analysis showed that this time could be as little as 5 mins. Nevertheless, azide-alkyne coupling was found to be occurring even without the presence of Cu(I) to give some cyclised duplex after 1 day at 37° C in the presence of 0.2 M NaCl (see gel lane 5 of Figure 22(b))

5

Claims:

1. A method of oligonucleotide circularisation comprising reacting at least one azide group with at least one alkyne group, wherein said at least one azide group and said at least one alkyne group are carried by the same or different oligonucleotide strands, such that a circularised oligonucleotide is obtained with one or more strands.

2. A method as claimed in claim 1 which comprises template-free cyclisation of a single-stranded oligonucleotide by reacting an azide group with an alkyne group, wherein the azide group and the alkyne group are each presented by a modified nucleotide of said oligonucleotide or spaced from said oligonucleotide by a linker.

3. A method as claimed in claim 2 wherein a single stranded oligonucleotide is circularised by reacting an azide group at one end with an alkyne group at the other end.

4. A method as claimed in claim 1 wherein reaction of at least one azide group with at least one alkyne group is carried out under templated conditions.

5. A method as claimed in claim 4 wherein the template is a cyclic single-stranded oligonucleotide prepared by a method as defined in claim 3 and a double-stranded helical oligonucleotide catenane is prepared.

6. A method as claimed in claim 5 wherein a cyclic double-stranded oligonucleotide is prepared in which each strand contains a single triazole linkage.

7. A method as claimed in claim 5 wherein the catenane has a pseudohexagonal structure formed as a result of the presence of non-complementary regions in the catenane.

8. A method as claimed in claim 7 wherein the vertices of the pseudohexagonal structure are formed as a result of two consecutive thymidine nucleotides in both strands.

9. A method as claimed in claim 7 wherein the vertices of the pesudohexagonal structure are formed as a result of the presence of oligoethylene glycol moieties in both strands.

10. A method as claimed in claim 4 wherein the reacting is between two pairs of alkyne and azide functional groups wherein:

a. the pairs are located at either terminus of a double-helix formed between two hybridised oligonucleotides;
b. the pairs are located internally within a double-helix formed between two hybridised oligonucleotides; or
c. one pair is located internally within and one pair is located at a terminus of a double-helix formed between two hybridised oligonucleotides

such that the two hybridised oligonucleotides are circularised.

11. A method as claimed in claim 4 wherein a cyclic duplex is formed by reacting an azide group and an alkyne group at the termini of hybridised oligonucleotide strands joined by a non-hybridising loop in a hairpin structure.

12. A method as claimed in any one of the preceding claims wherein said reaction is catalysed by copper (I) prepared *in s/ttv* from copper (II) sulfate and sodium ascorbate in the presence of a water-soluble fr/s-triazolylamine copper(I) binding ligand .

13. An oligonucleotide comprising construct obtained by a method as claimed in any one of claims 1 to 12.

14. A circularised single- or double-stranded oligonucleotide construct in which there are present one or more 1,2,3-triazole-containing linkages obtained by a method a claimed in any one of claims 2 and 3 and 5 to 9.

15. A circularised olignucleotide construct as claimed in claim 13 or claim 14 for use as a therapeutic.

16. An oligonucleotide comprising precursor for use in a method according to any one of claims 1 to 12 which presents two alkynes, two azides or an alkyne and an azide.

17. An oligonucleotide comprising precursor of claim 16 wherein the alkynes, azides or alkyne and azide are displayed from the terminal nucleotides of the oligonucleotide.

18. Use of the reaction between at least one alkyne group and at least one azide group to improve stabilisation of a therapeutic oligonucleotide against in vivo degradation, wherein said at least one azide group and said at least one alkyne group are carried by the same or different oligonucleotide strands.

19. The use according to claim 18 wherein improved serum stability of said oligonucleotide results.

20. The use of claim 18 or claim 19 which comprises a method as defined in any one of claims 1 to 12.

21. Use of the reaction between an azide group and an alkyne group in the absence of a catalyst to join oligonucleotide sequences in a cell.

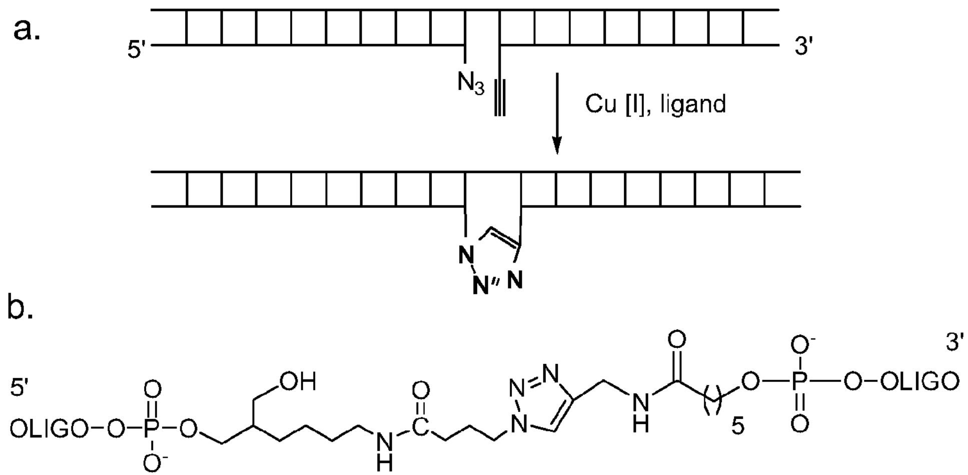


Figure 1

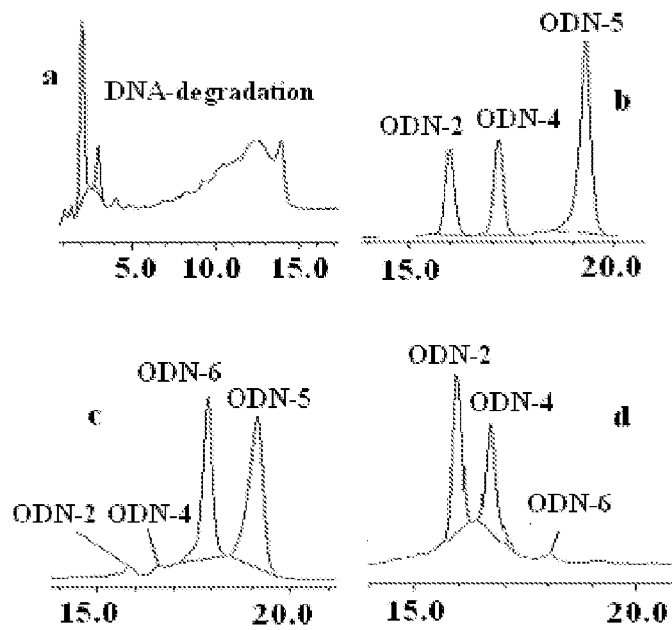
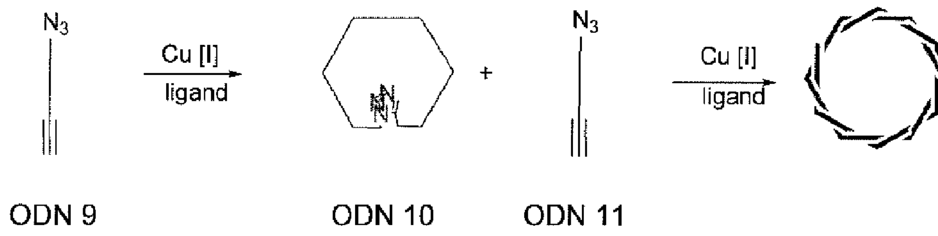


Figure 2



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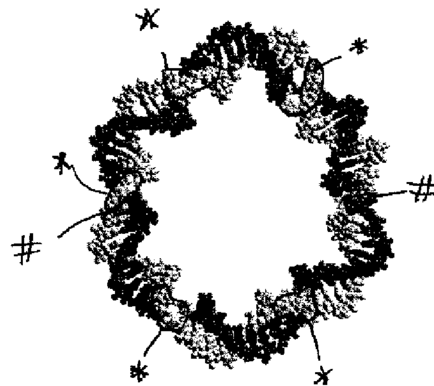
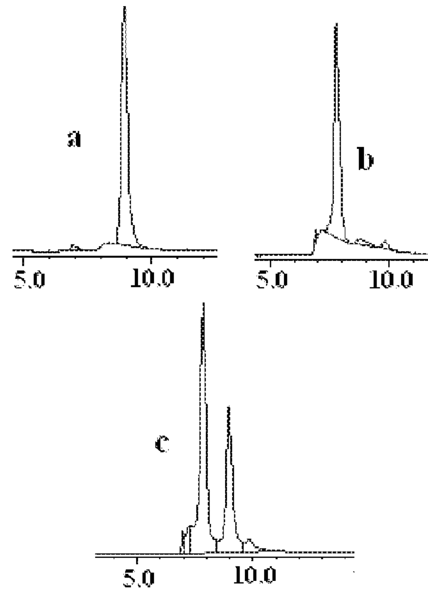


Figure 4

Figure 5



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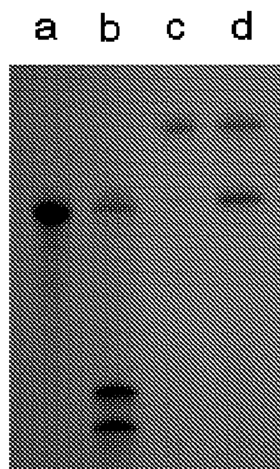
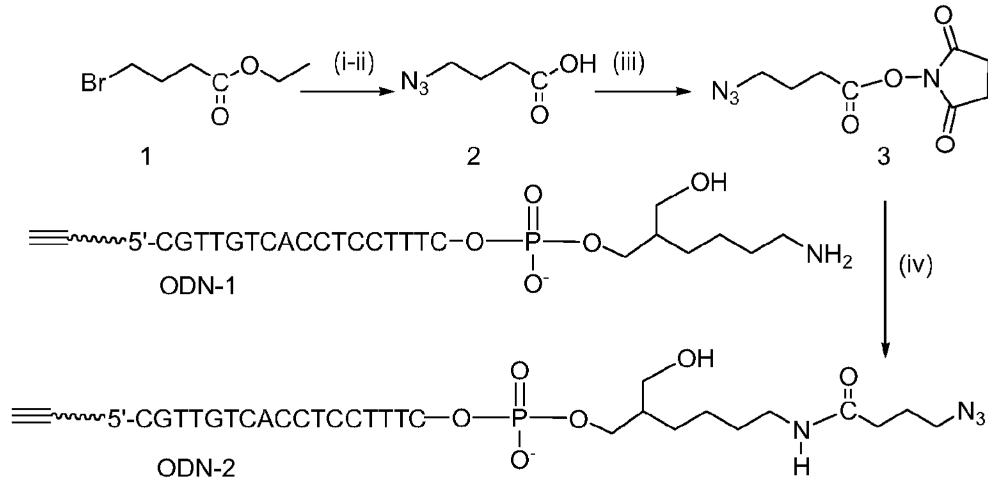


Figure 7

Figure 8



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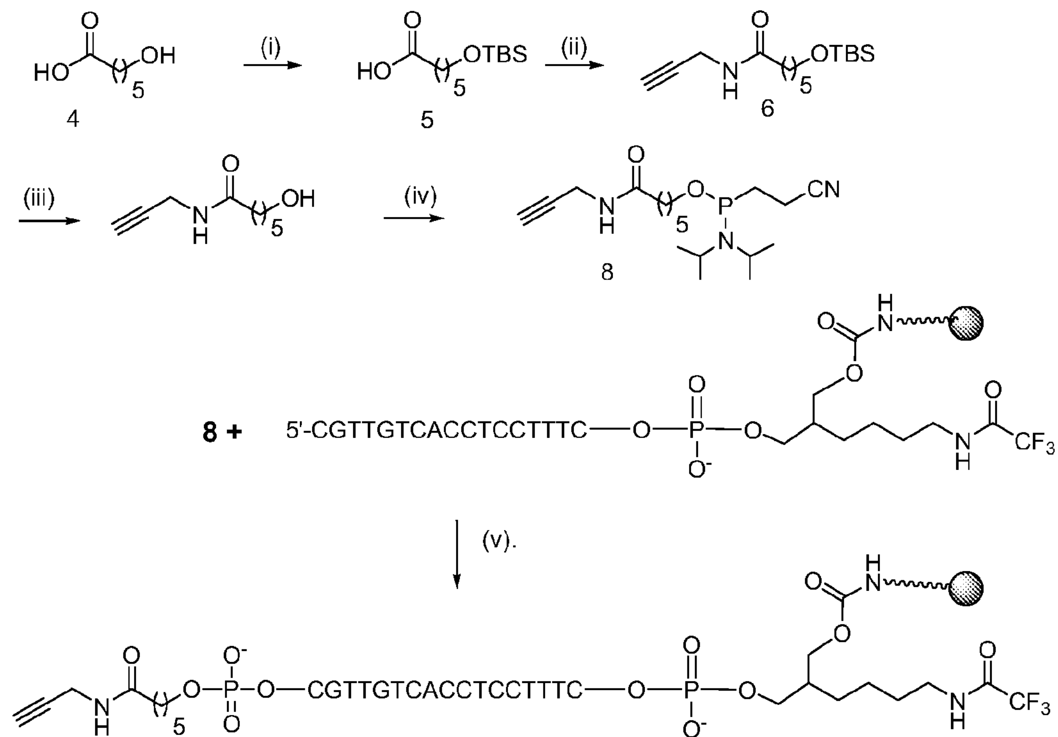


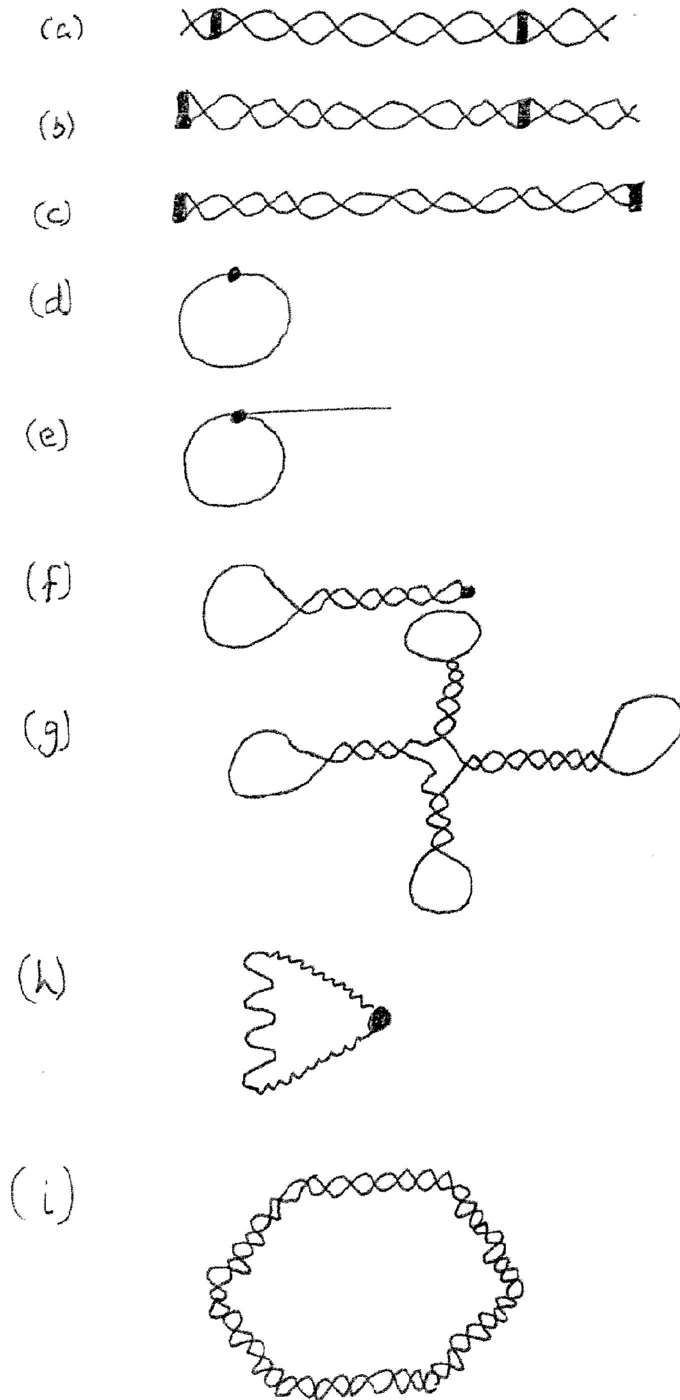
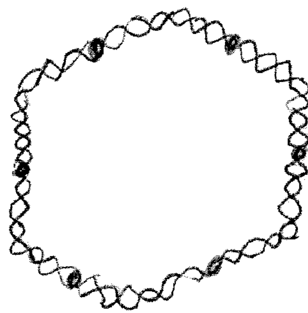


Fig. 10

(a)

(b)



(c)

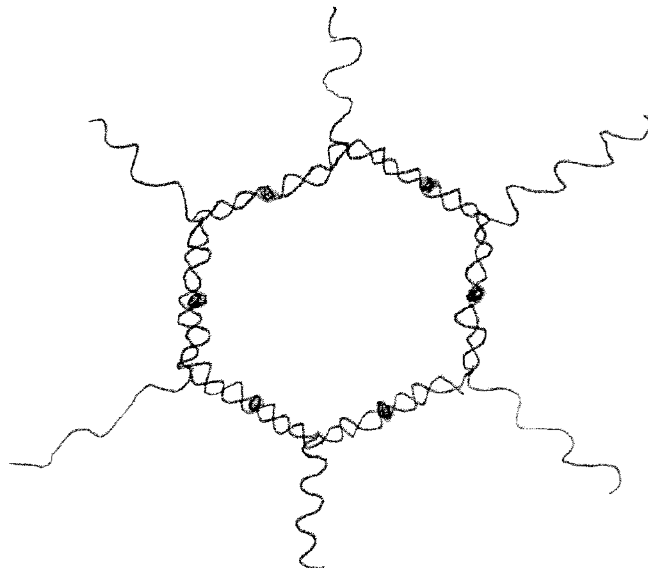
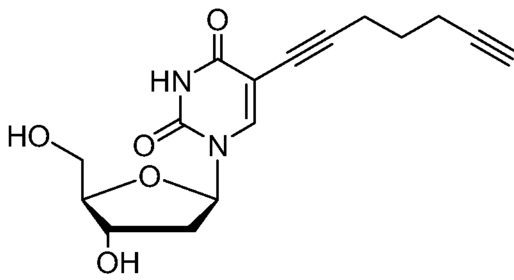
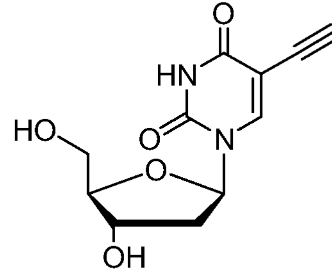
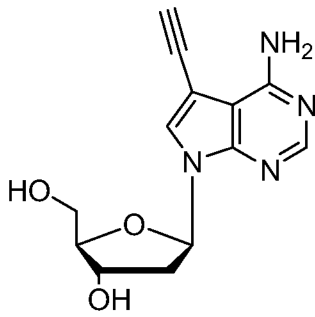
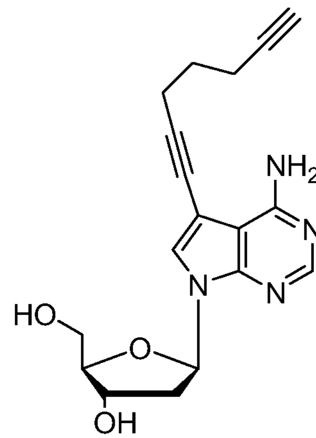


Fig. 11









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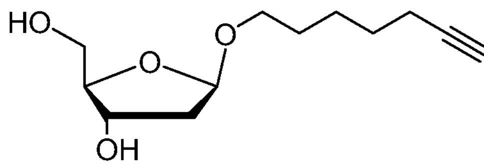
(c)



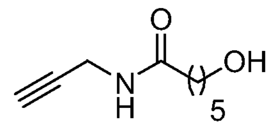
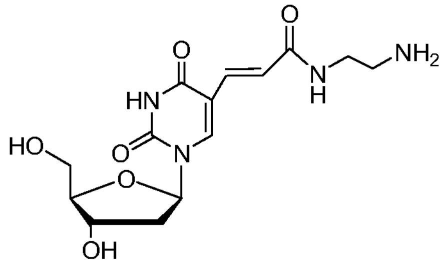
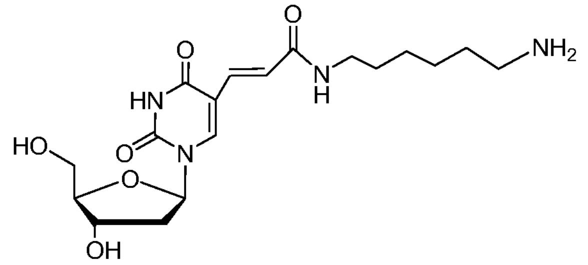
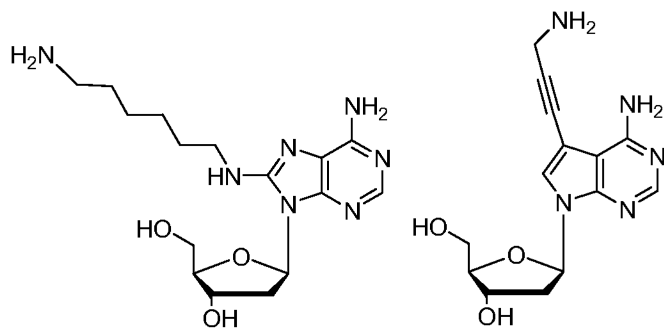


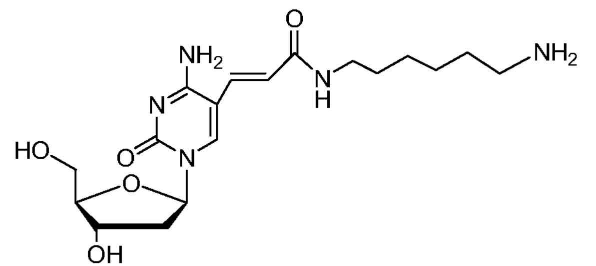
Figure 12

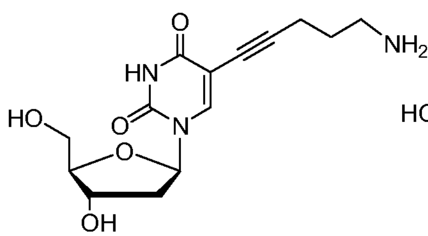
(a)

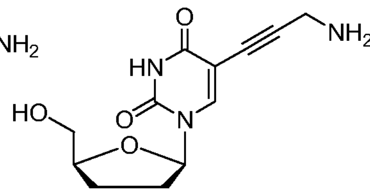


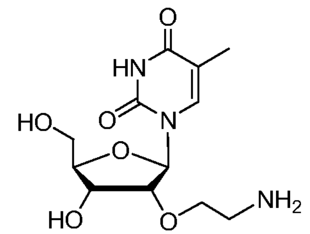












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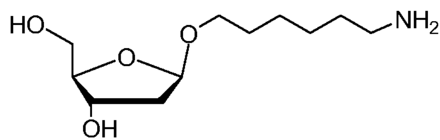

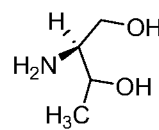

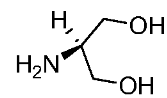

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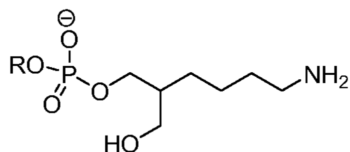


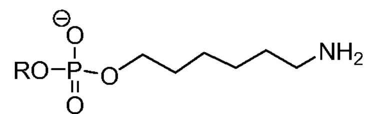


Figure 13

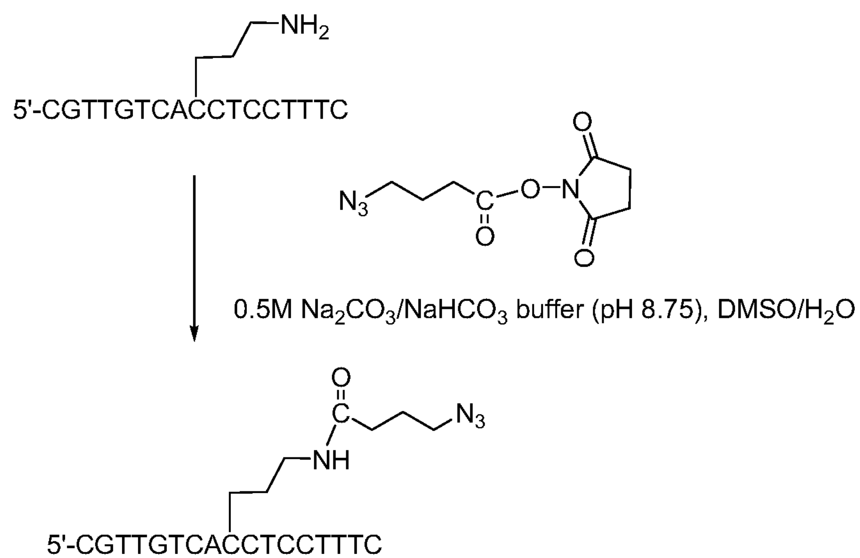


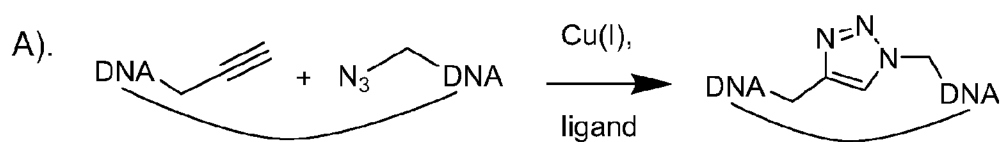
Figure 14

```
          5'-K-CCATACATACTTCCACAGCATCTTGATTAGCGTCTTCGATGGTATCTTGGCTCTACAGTTGAGGAGGATG-N-3'
3'-N-TGCTACCATAGTTCCGAGATGTCTTCTCCTCCTACTTGGTATGTATGTTGGTGTCGTAGTTCTAATCGCAGT-K-5'
```

Figure 15

Figure 16

A).



B).

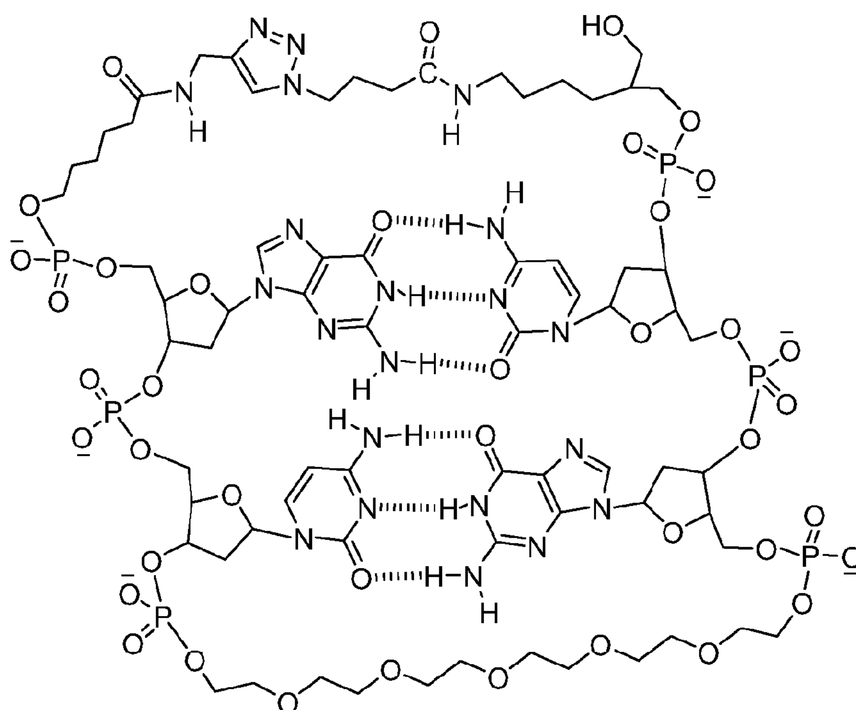


Figure 17

GCACCAGAATTCATCACG
CGTGGTCTTAAGTAGTGC
N_3
N_3
EcoR1
restriction site
Cu(I),
ligand
GCACCAGAATTCATCACG
CGTGGTCTTAAGTAGTGC

Figure 18

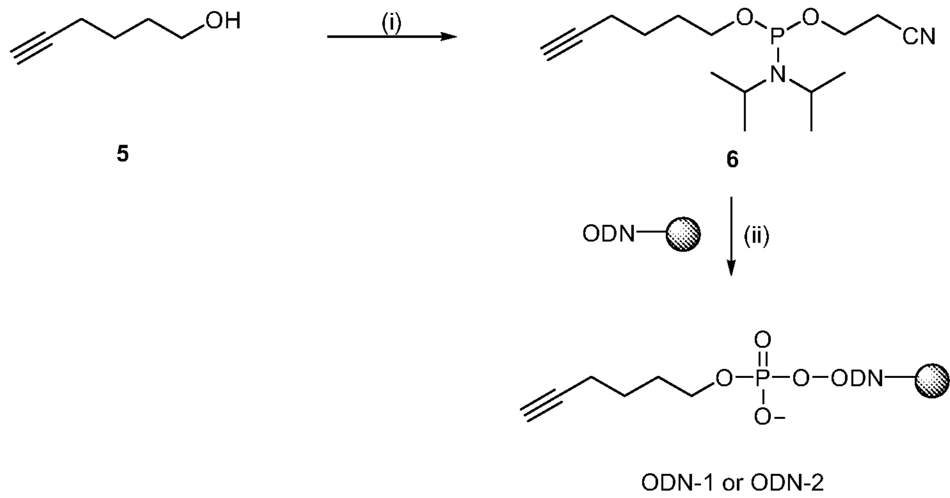


Figure 19

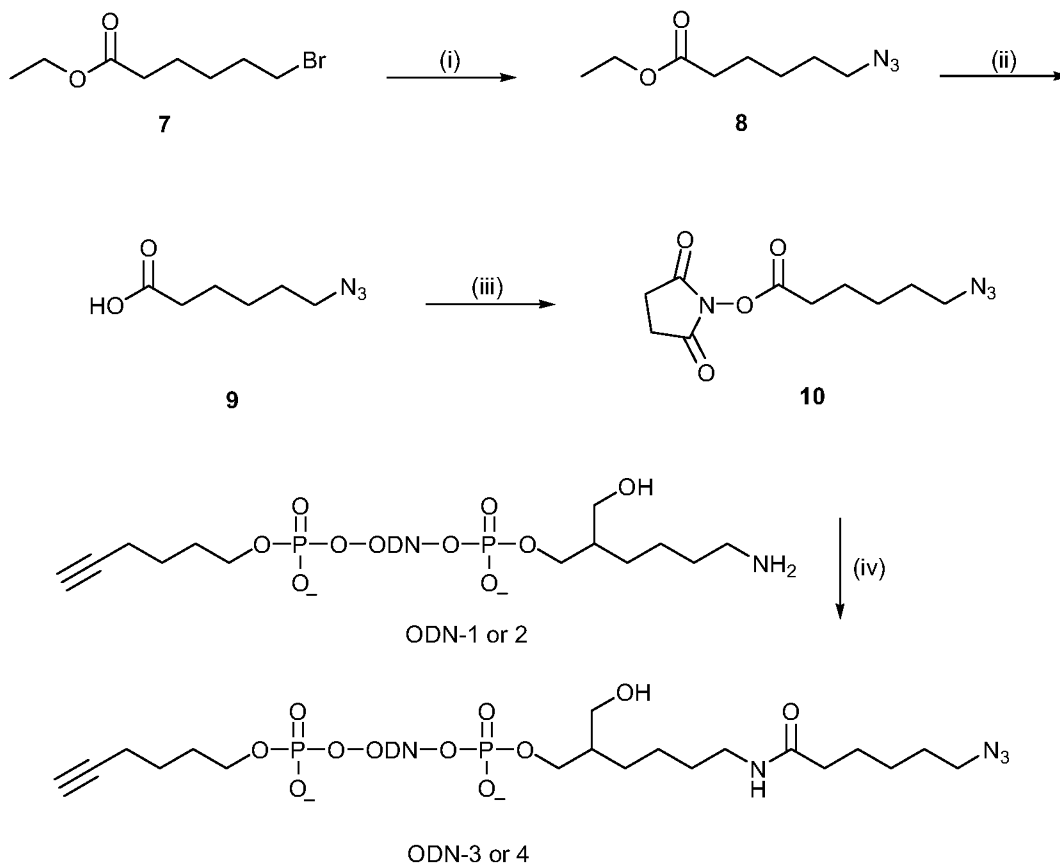


Figure 20

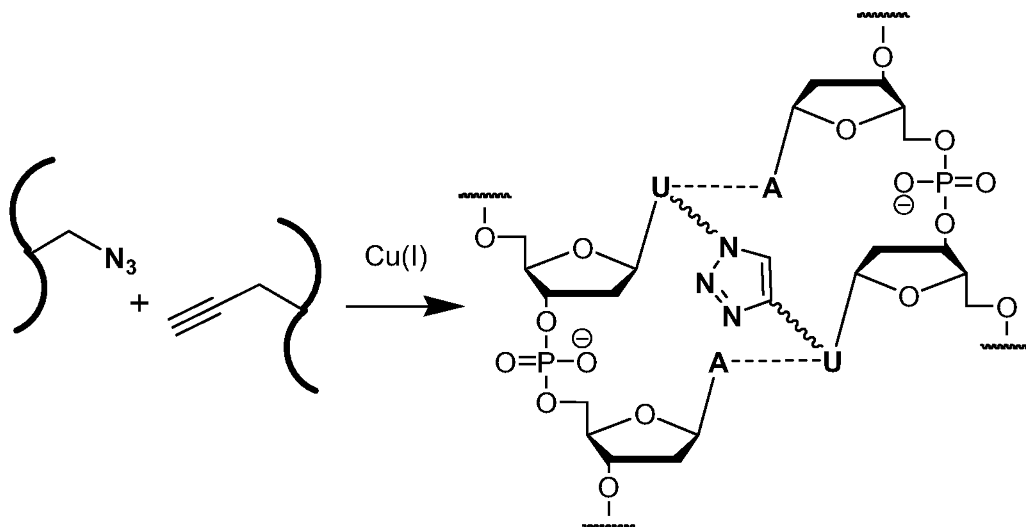
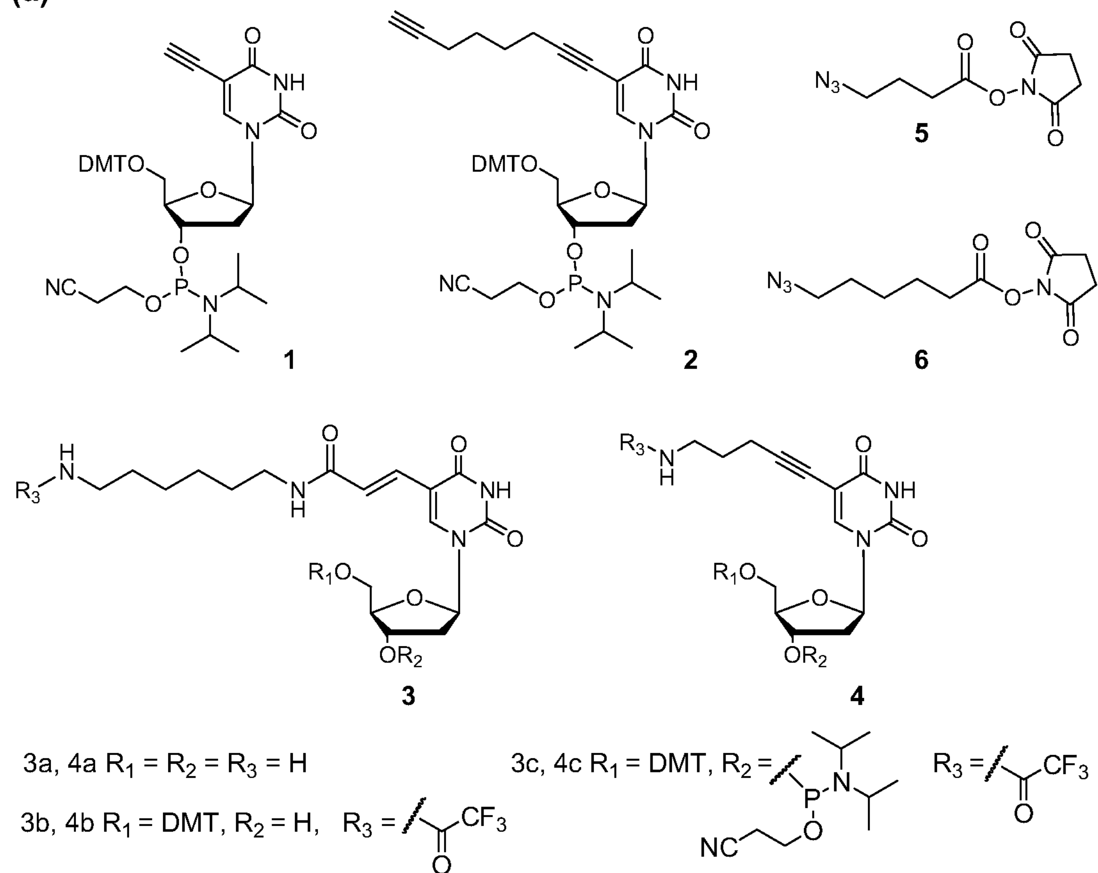


Figure 21

(a)



(b)

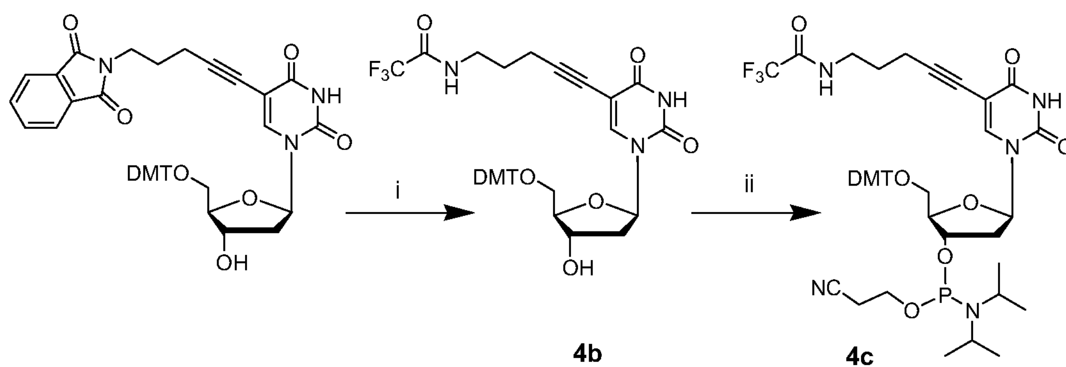
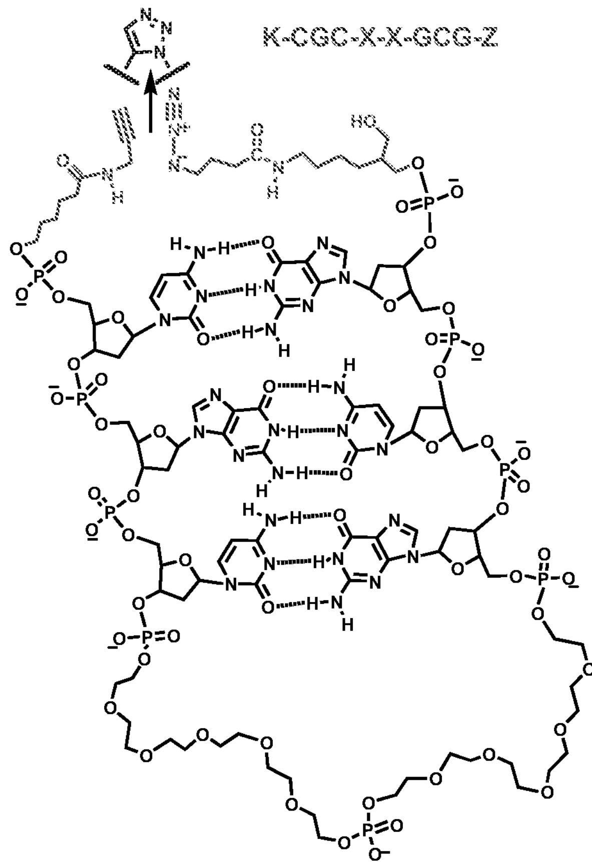


Figure 22

(a)



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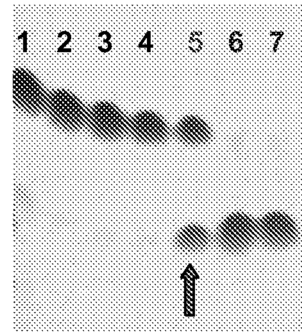

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/050228

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H21/00 B01J19/00

According lo International Palent Classification (IPC) or lo both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07H BOIJ

Documentation searched other than minimum documentation lo the extent thai such documents are included in the fields searched

Electronic data base consulted during the international search (name of dala base and, where practical, search terms used)

EPO-Internal , WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category'	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	FUJIMOTO K ET AL: "Template-directed reversible photocircularization of DNA via 5-vi nyldeoxycyti d ine" TETRAHEDRON LETTERS, ELSEVIER, AMSTERDAM, vol. 41, no. 33, 12 August 2000 (2000-08-12), pages 6451-6454, XP004215085 ISSN: 0040-4039 cited in the application the whole document	1
A	E .T . KOOL: "Recognition of DNA, RNA, and proteins by circular oligonucleotides" ACC. CHEM. RES., vol. 31, 1998, pages 502-510, XP002492395 cited in the application the whole document	1

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[X] Further documents are listed in lhe continuation ol Box C

[X] See patent family annex

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INTERNATIONAL SEARCH REPORT

International application No
PCT/6B2008/050228

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category"	Citation of document with indication where appropriate of the relevant passages	Relevant to claim No
X	WO 2004/055160 A (UNIV COLUMBIA [US]) 1 July 2004 (2004-07-01) cited in the application the whole document -----	18,19,21
X	KANAN M W ET AL: "REACTION DISCOVERY ENABLED BY DNA-TEMPLATED SYNTHESIS AND IN VITRO SELECTION" NATURE, NATURE PUBLISHING GROUP, LONDON, UK, vol. 431, no. 7008, 30 September 2004 (2004-09-30), pages 545-549, XP008042376 ISSN: 0028-0836 cited in the application column 2 , paragraph 4 -----	18,19

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2008/050228

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004055160 A	01-07-2004	AU 2003297859 A1	09-07-2004