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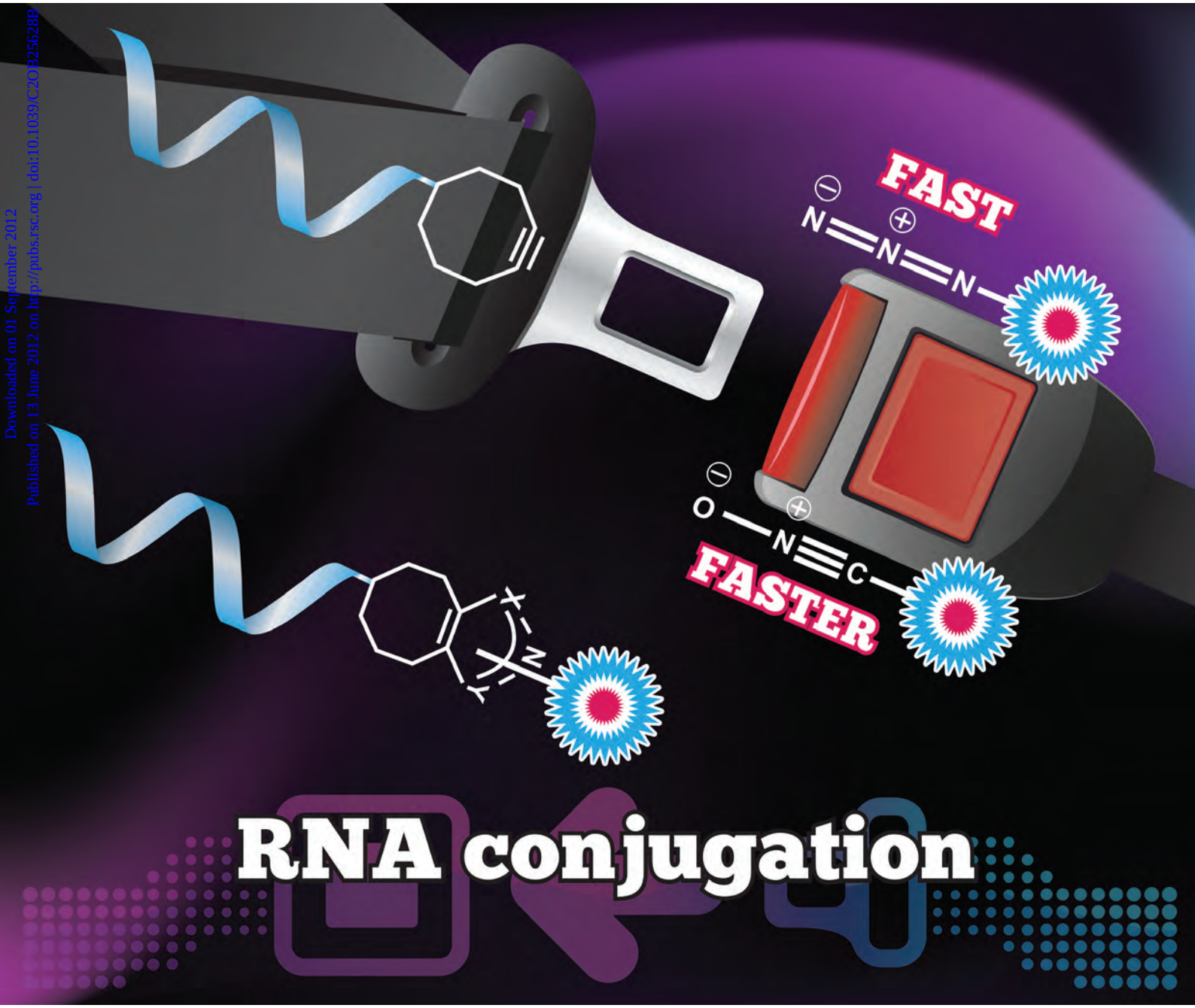

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PAPER

Fast RNA conjugations on solid phase by strain-promoted cycloadditions†

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Strain promoted cycloaddition is presented as a tool for RNA conjugation on the solid phase; RNA-cyclooctyne conjugates are prepared by cycloaddition to both azide (strain-promoted azide–alkyne cycloaddition, SPAAC) and nitrile oxide dipoles (strain-promoted nitrile oxide–alkyne cycloaddition, SPNOAC). The conjugation is compatible with 2′-OMe blocks and with 2′-*O*-TBDMS protection on the ribose moieties of the sugar. Nitrile oxide dipoles are found to be more reactive click partners than azides. The conjugation proceeds within 10 min in aqueous solvents, at room temperature without any metal catalyst and tolerates dipoles of varying steric bulk and electronic demands, including pyrenyl, coumarin and dabcyl derivatives.

Introduction

Currently, chemically-modified oligonucleotides are in high demand due to their utility in diagnostic,[1,2] therapeutic,[3] nanotechnology[4] and materials science applications.[5] The most attractive reactions for the chemical transformation of nucleic acid substrates are those which proceed in aqueous media at ambient temperature and tolerate the presence of oxygen.

Although Cu(I)-catalysed azide–alkyne cycloaddition (CuAAC) reactions are widely employed in synthetic chemistry,[6] materials science,[7] and in chemical biology[8,9] the "click" conditions required are not ideal for nucleic acid modification as copper ions mediate Fenton-type DNA damage, leading to strand breaks.[8,10,11] Although the addition of stabilising ligands can be effective in minimizing this degradation, the toxicity of Cu(I) still remains potentially problematic.[12] To overcome these issues, several metal-free bio-conjugation chemistries have been developed. We and others have reported the application of nitrile oxide derivatives to the modification of nucleosides, oligonucleotides and polymers bearing alkene/alkyne functions.[13–19] Other metal-free strategies include Diels–Alder cycloadditions,[20] photoinduceable cycloadditions of tetrazines or nitrile imines to alkenes[21,22] and strain-promoted azide–alkyne cycloaddition (SPAAC) reactions.

The potential of SPAAC reactions for bio-conjugation was first described by Bertozzi and co-workers in 2004[23] and exploited the intrinsic ring strain of a cyclooctyne moiety. The reactivity of the first generation of monocyclic octynes have subsequently been ameliorated by incorporating a ring heteroatom,[24] electron withdrawing substituents,[20] or introducing further strain by fusing two aromatic rings to the (aza)cyclooctyne core,[25,26] SPAAC reactions have since found applications in bioimaging,[24,27] peptide conjugation,[28] drug delivery[29] and surface and materials science.[30,31]

We have previously reported post-synthetic modification of DNA by strain-promoted cycloaddition of both azide and nitrile oxide dipoles to solid-supported DNA-cyclooctyne substrates.[32,33] Filippov *et al.*,[34] and Manoharan *et al.*,[35] have demonstrated azide-mediated oligonucleotide-dibenzocyclooctyne conjugation in solution phase, using the same methodology as that described for DNA-templated "click"-ligation demonstrated by Brown and El-Sagheer.[36]

As part of an ongoing programme to prepare siRNA conjugates with improved potential for cellular delivery, we became interested in the possibility of RNA functionalization using strain-promoted cycloaddition chemistry. In particular, we were interested in solid-supported RNA substrates bearing monocyclic octynes as handles for conjugation to a variety of ligands/labels. The attractions of solid phase synthesis (SPS) include ease of purification and the possibility to automate the ligation procedure. The choice of a non-substituted cyclooctyne dipolarophile partner for the SPAAC reaction offers advantages in terms of facile synthetic accessibility[33] and reduced steric bulk and lipophilicity leading to enhanced aqueous solubility with a reduced tendency to give non-specific interactions with hydrophobic proteins.[37] We wish to report here solid phase, post-synthetic RNA conjugation by SPAAC and strain-promoted

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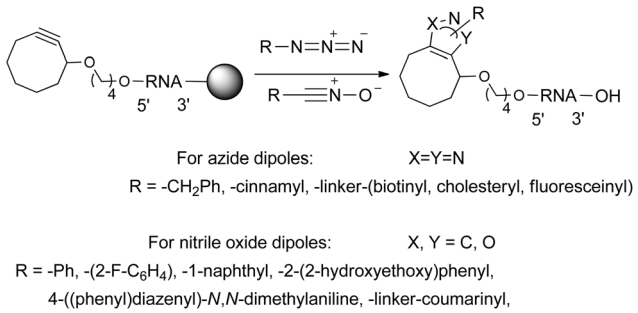


Scheme 1 Solid-supported RNA conjugation by strain-promoted cycloaddition chemistry.

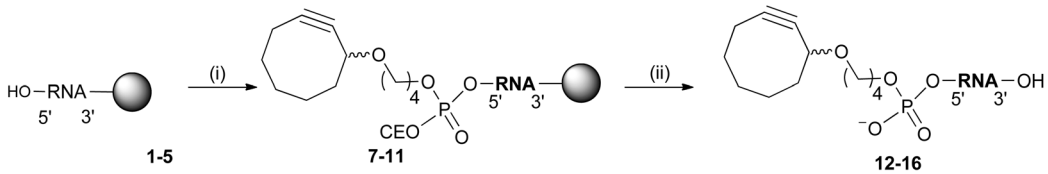
nitrile oxide–alkyne cycloaddition (SPNOAC) reactions as summarised in Scheme 1.

Results and discussion

RNA substrates **1–5** were prepared using standard solid-phase protocols prior to manual coupling of the cyclooctyne phosphoramidite **6** and subsequent oxidation of the terminal phosphite triester (Scheme 2).[33] Near quantitative conversion of **1–5** to the putative solid-bound RNA-cyclooctynes (**7–11**) was indicated by RP-HPLC and MALDI-TOF-MS analysis of the deprotected oligonucleotide-cyclooctyne conjugates **12–16**.

CPG-RNA cyclooctyne **7** and benzyl azide, **17**, were selected as model reactants with which to study solid-supported RNA modification by a SPAAC reaction. The reaction was conducted on a 0.1 μmol scale (supported oligonucleotide) with 20 equivalents of azide in aqueous DMSO (50% v/v). The reaction was complete within 30 min under ambient conditions (as evidenced by RP-HPLC), and the putative support-bound conjugate **22a** was subsequently cleaved from the resin and deprotected to afford the RNA conjugate **23a** (Scheme 3, Table 1). HPLC analysis showed formation of putative regioisomeric cycloadducts; MALDI-TOF-MS data was consistent with the structure of **23a**. Similarly, **7** was conjugated with cinnamyl azide **18**[38] to give regioisomeric products **23b** in high yield (by HPLC).

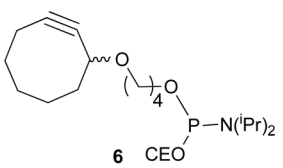
Having established that the solid-supported RNA-cyclooctyne dipolarophile **7** is a suitable substrate for SPAAC with simple azide dipoles, the compatibility of the conditions with labels bearing a range of functionalities was investigated. Thus, **7** was treated with biotinyl-(**19**),[32] cholesteryl-(**20**)[32] or fluoresceinyl-(**21**)[32] azides in both aqueous and non-aqueous media at room temperature. Conjugation to the biotinyl azide (**19**), furnished **23c** after 4 h in 90% (v/v) aqueous DMSO, whilst DCM was the solvent of choice for cholesteryl conjugation. The success of the SPAAC to furnish lipid conjugates is suggestive of a future in



(i) a. **6.**, BMT, CH_3CN, b. I_2, RT; (ii) Cleavage/deprotection

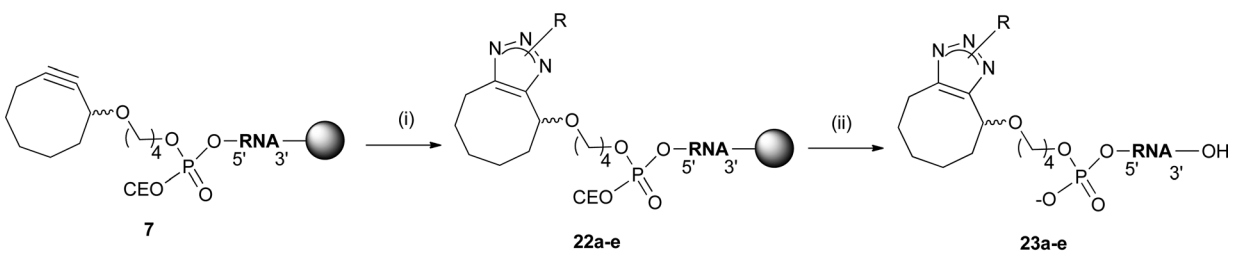
RNA 1, 7, 12 = CAG CCA CAA CGU CUA UAU C
RNA 2, 8, 13 = UUU U
RNA 3, 9, 14 = GUG UGU GCA
RNA 4, 10, 15 = U U U U U U
RNA 5, 11, 16 = G UAC A

Underlined = 2'-OMe nucleotides



CE = $NCCH_2CH_2$-

Scheme 2 Solid-supported synthesis of RNAs bearing cyclooctyne "click" partners.



RNA = CAG CCA CAA CGU CUA UAU C CE = $NCCH_2CH_2$-

(i) RN_3, DMSO/H_2O or DCM, 0.5-20 hr.,RT; (ii) Deprotection/cleavage

Scheme 3 SPAAC of RNA-cyclooctyne **7** to form conjugates **23a–e**.

Table 1 Structures of azides **17–21** and triazole conjugates **23a–e**

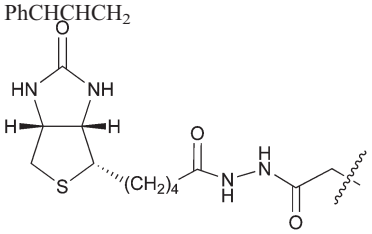
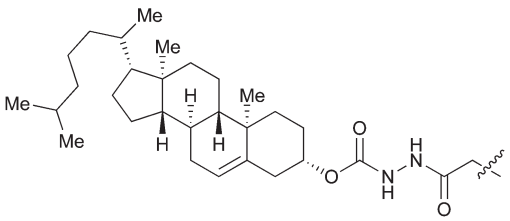
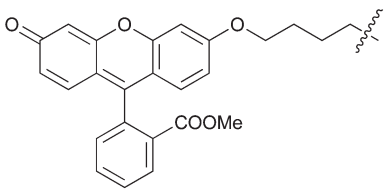
Azide, R-N_3	R	Resin-supported conjugate	Triazole-conjugate
17	$PhCH_2$	**22a**	**23a**
18	$PhCHCHCH_2$	**22b**	**23b**
19		**22c**	**23c**
20		**22d**	**23d**
21		**22e**	**23e**

Table 2 Conditions for SPAAC of azides **17–21** with support-bound RNA-cyclooctyne, **7** and MALDI-TOF MS data

Azide	Solvent	Reaction time	Product	MALDI-TOF MS calculated, found
17	50% aq. DMSO	30 min	**23a**	6624, 6623
18	50% aq. DMSO	30 min	**23b**	6650, 6650
19	90% aq. DMSO	4 h	**23c**	6831, 6833
20	DCM	24 h	**23d**	7018, 7020
21[a]	90% aq. DMF	24 h	**23e**	6931, 6934

[a] Reaction with fluoresceinyl azide **21** was conducted in solution.

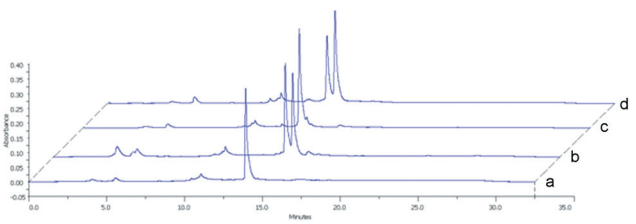
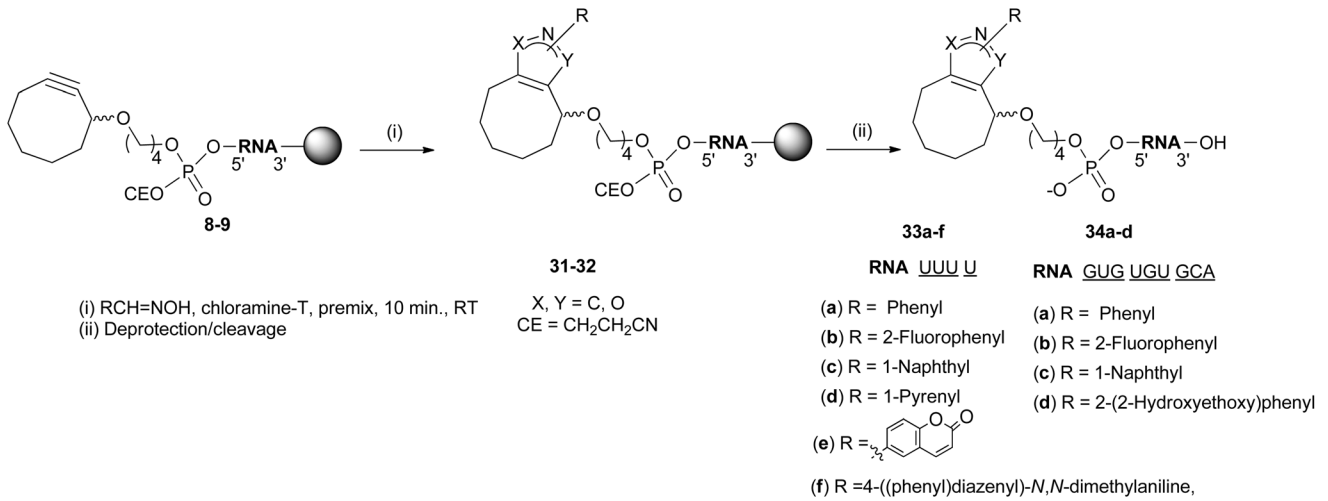


Fig. 1 HPLC traces (recorded at 260 nm) of: (a) crude RNA-cyclooctyne **13**, resulting from deprotection and cleavage from the support **8**; and b–d) the crude reaction products resulting from reaction of the RNA-cyclooctyne **8**; (b) with phenyl nitrile oxide after 10 min showing regioisomeric cycloadducts; (c) with phenyl azide after 10 min showing no reaction; (d) phenyl azide for 18 h showing incomplete consumption of starting RNA-cyclooctyne. HPLC analysis conditions A (for a and b) or C (for c and d).

drug formulation free from the inherent toxicity which plagues cationic delivery vehicles.[39,40] The fluorescein azide **21** was selected to demonstrate the potential of SPAAC for introduction of fluorescent tags. Having previously demonstrated that **21** required post-synthetic conjugation to DNA,[32] the same strategy was adopted for RNA-cyclooctyne **12**. Thus, **12** (0.025 μmol, 1 eq., 200 μM), obtained from **7** following cleavage from the resin and deprotection, was exposed to a solution of **21** (40 eq.) in aqueous DMF. The reaction, as judged by HPLC, progressed cleanly and was complete in 24 h at room temperature. The identity and integrity of the conjugate, which retained the yellow colour characteristic of molecules incorporating a tetracyclic fluorescein skeleton, as **23e** was supported by MALDI-TOF mass analysis (Table 2).

In formation of the conjugates **23a** through **d**, it was observed that as the steric bulk of the azide partner increased, from benzyl through to cholesterol, the rate of conjugation decreased. Commonly reported strategies which enhance the rate of strain-promoted cycloaddition reactions include the use of activated cyclooctynes, *e.g.* dibenzocyclooctynes,[25] and/or dipoles which are more reactive than the azide, *e.g.* nitrile oxides. A flip side of the enhanced reactivity of nitrile oxide dipoles is their potential to partake in side reactions, *e.g.* dimerization, for this reason in exploring the utility of SPNOAC reactions for conjugation to support-bound oligonucleotides the dipoles were generated *in situ* from stable precursor aryl aldoximes. Thus, a suspension of **8** in 50% (v/v) aqueous ethanol was exposed to benzonitrile oxide and reaction progress was followed by RP-HPLC. Conjugation was completed within 10 min at room temperature (Fig. 1b) and the regioisomeric isoxazole-RNA conjugates **33a** were obtained in near quantitative yield following work-up, deprotection and cleavage from the support, (Scheme 4).

The relative rates of the SPAAC and SPNOAC reactions using cycloalkyne **8** were compared using phenyl azide and



Scheme 4 SPNOAC of RNA-cyclooctynes **8** and **9** with a range of nitrile oxides.

benzonitrile oxide, and the success of each reaction judged by HPLC analysis of the crude reaction products (Fig. 1). Whilst reaction with the nitrile oxide was complete in just 10 min at room temperature, under the same conditions no conjugation was observed with phenyl azide. In fact, even after 18 h reaction time with the azide dipole, conjugation was still not complete (Fig. 1d). The failure of HPLC to resolve the expected regioisomeric triazoles **23f** (R = Ph) is not unprecedented.[41]

To demonstrate the scope of SPNOAC reactions, the oximes, **25–30**,[16,33,42,43] (Fig. 2), were selected as nitrile oxide precursors. The analogous dipoles were generated immediately prior to use following 10 min exposure of the parent oxime to a solution of chloramine-T at room temperature. The choice of solvent was dictated by the solubility of the oxime and product nitrile oxide (Table 3). Thus, as with benzaldoxime, **25** **26** and **27** were dissolved in aqueous EtOH and oxidised to the corresponding nitrile oxides. Addition of the nitrile oxide solutions derived from **25** and **26** to the solid-supported oligonucleotide-alkyne **8** and of the solutions derived from **25–27** to the solid-supported cyclic alkyne **9**, followed by agitation of the mixtures at room temperature for 10 min gave near quantitative conversion to the regioisomeric isoxazole-conjugates **33b–c** and **34b–d**, following deprotection. The identity of these compounds was evidenced by MALDI-TOF-MS analysis.

Pyrene-1-nitrile oxide, coumarin-6-nitrile oxide, and 4-(*N*,*N*-dimethylamino)phenylazo-benzonitrile oxide, generated *in situ*, from the corresponding oximes **28–30**, were selected as building blocks with potential to rapidly introduce reporter groups to RNA substrates. DMF/ethanol was selected as the solvent for dipole generation and cycloaddition, and RNA-**8** as the model substrate. In each case, HPLC analysis of the cleaved, deprotected, but unpurified reaction products indicated complete consumption of **8** within 10 min, and formation of regioisomeric mixtures of RNA-conjugates **33d–f** (Scheme 4). MALDI-TOF MS data supported the structural integrity of the conjugates **33d–f**.

No modification to the reaction conditions was required for conjugation to longer oligonucleotides, and the support-bound 19-mer cyclooctyne **7** was converted, almost quantitatively, to

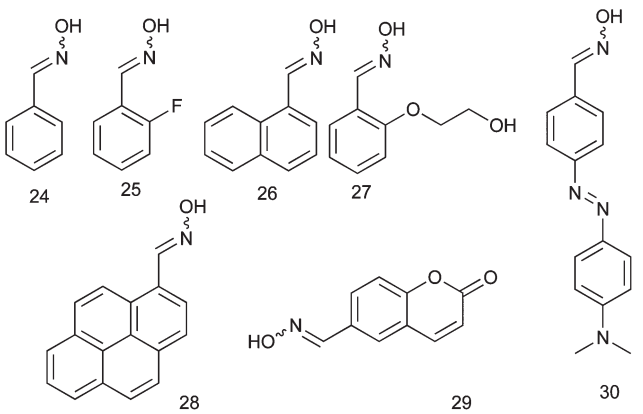
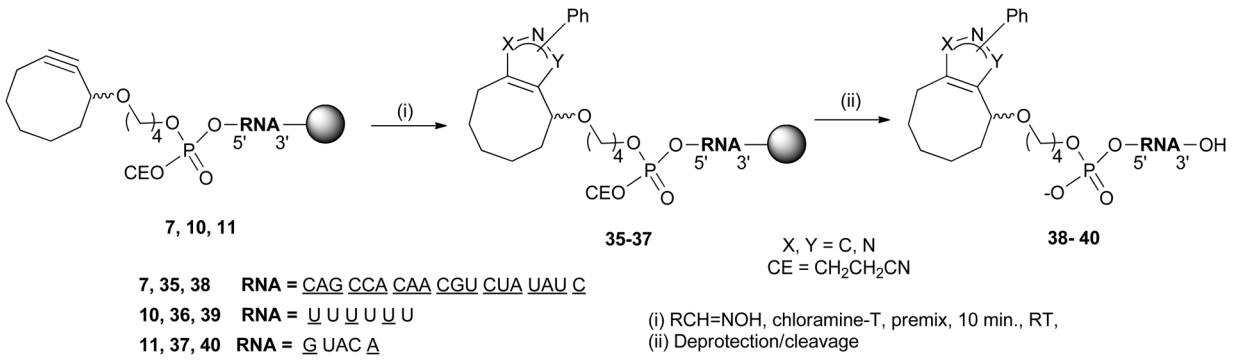


Fig. 2 Oximes employed as nitrile oxide precursors in synthesis of cycloadducts **33–34**, **38–40**.

Table 3 Conditions for SPNOAC cycloaddition to support bound RNA-cyclooctynes **7–11** and MALDI-TOF MS data

Dipole precursor	Reaction solvent	RNA-cyclooctyne	Product (s)	MALDI-TOF MS calculated, found
24	50% aq. EtOH	**8**	**33a**	1595, 1596
		9	**34a**	3374, 3379
		7	**38**	6607, 6611
		10	**39**	2234, 2238
		11	**40**	1958, 1960
25	50% aq. EtOH	**8**	**33b**	1613, 1614
		9	**34b**	3392, 3393
26	50% aq. EtOH	**8**	**33c**	1645, 1647
		9	**34c**	3424, 3428
27	50% aq. EtOH	**9**	**34d**	3434, 3436
28	DMF–EtOH 7 : 3	**8**	**33d**	1719, 1721
29	DMF–EtOH 7 : 3	**8**	**33e**	1688, 1684
30	DMF–EtOH 7 : 3	**8**	**33f**	1744, 1746

the regioisomeric RNA-isoxazole conjugates **38** whose structural assignment was supported by MALDI-TOF-MS (Scheme 5, Table 3). To illustrate the potential of the SPNOAC to deliver "native" RNA conjugates, *i.e.* those bearing a free 2′-hydroxyl group either as an alternate or gapmer, sequences **10** and **11** were



Scheme 5 SPNOAC of RNA-cyclooctynes **7**, **10** and **11** with benzonitrile oxide.

investigated. These sequences have some of the ribose sugars bearing 2′-*O*-TBDMS protecting groups and others bearing 2′-OMe blocks; thus U6-cyclooctyne **10**, has the alternate pattern. The RNA-cyclooctyne **11**, with 2′-OMe blocking groups on the ribose units at each terminus and 2′-*O*-TBDMS protecting groups on the central ribose moieties is defined as a gapmer. Both sequences were tested as substrates in SPNOAC under the standard conditions described above. In each case after 10 min reaction at room temperature with benzonitrile oxide and following work up, including cleavage from the support, and full (base and sugar) deprotection, HPLC analysis revealed complete conversion to the regioisomeric RNA-isoxazole conjugates **39** and **40** (Scheme 5). MALDI-TOF-MS supported the structural integrity of all new conjugates (Table 3).

Conclusions

We have developed fast, strain promoted cycloaddition as a tool for RNA conjugation on the solid phase exploiting the cycloaddition of a series of RNA-cyclooctynes with both azide (SPAAC) and nitrile oxide dipoles (SPNOAC). The reaction is compatible with 2′-OMe blocking as well as with 2′-*O*-TBDMS protection on the ribose moieties of the sugar. Nitrile oxides are found to be more reactive dipole partners than azides. The copper free click conjugations proceed in aqueous solvents, in 10 min at room temperature under atmospheric conditions. The successful ligation to monocyclic alkynes, with reduced steric demands and hydrophobic character relative to diaryl fused analogues, may be of benefit to those applications seeking enhanced aqueous solubility. The SPNOAC reaction, which tolerates dipoles of varying steric bulk and electronic demands, including pyrenyl, coumarinyl and dabcyl provides complementarity to recent reports on copper-promoted and copper free conjugation of, ligase generated, "clickable" RNAs.[44,45]

Experimental section

General experimental

Mass analysis was performed on an Applied Biosystem Voyager with 3-hydroxypicolinic acid or 2′,4′,6′-trihydroxyacetophenone as matrix or recorded by Metabion, Germany. UV analysis was performed on a Jasco V-630BIO spectrophotometer at 25 °C. HPLC was carried out using either using a Gilson instrument equipped with a diode array detector [Nucleosil C18 column (4.6 × 250 mm, 5 μm) or Phenomenex C18 column (4.6 × 250 mm, 5 μm)], or using a Dionex Ultimate 3000 instrument equipped with a Clarity Oligo RP C18 (4.6 × 250 mm, 5 μm) column. RNA monomers were purchased from Link Technologies UK. Desalting of oligonucleotide samples was conducted using illustra NAP-10 Sephadex G-25 DNA grade columns purchased from GE Healthcare.

Synthesis of RNA substrates 1–5

Oligonucleotide syntheses were conducted on an Expedite 8909 DNA/RNA or ABI 394 synthesizer using commercially available 2′-OMe or 2′-*O*-TBDMS phosphoramidites, and followed standard RNA synthesis protocols.

General procedure for preparation of RNA-cyclooctynes 7–11

To manually couple the cyclooctyne phosphoramidite **6**[33] to the oligonucleotide, 500 μL of a 100 mM solution of the phosphoramidite in anhydrous CH_3CN was added to the CPG-supported oligonucleotide (1 μmol) along with 500 μL of a 0.3 M solution of 5-benzylmercaptotetrazole in CH_3CN. The mixture was allowed to react for 15 min at room temperature with mixing between syringes. This procedure was repeated with a second portion of a fresh solution of phosphoramidite and 5-benzylmercaptotetrazole. The CPG was washed with CH_3CN (5 × 2 mL), then exposed to oxidizer (700 μL, 0.1 M iodine solution in THF–pyridine–water; 78 : 20 : 2). Following washing with CH_3CN (2 × 5 mL) the CPG-support was dried under vacuum using a vacuum concentrator. Cleavage and base deprotection of the cyclooctyne modified 2′-OMe blocked oligonucleotides **7–9**, and the alternate or gapmer substrates **10–11**, from the support proceeded by the method described below furnishing cyclooctyne-modified RNAs **12–16**.

General procedure for oligonucleotide cleavage and deprotection

For analytical purposes a portion of each oligonucleotide was cleaved from the CPG and protecting groups removed from the base following incubation either in 40% (w/v) aqueous CH_3NH_2 (500 μL) at 65 °C for 15 min for substrates **12–16** (method i), or in 28% (w/v) aqueous NH_4OH (500 μL) at 25 °C for 30 min for

substrates **33e-f** (method ii). The CH_3NH_2/NH_4OH was evaporated using a concentrator. The CPG was washed with H_2O (4 × 200 μL aliquots), all solutions and washings were combined to afford an aqueous solution of the oligonucleotide products which were concentrated on a vacuum concentrator prior to HPLC analysis.

Full deprotection of alternate and gapmer oligonucleotides 10–11 and 36–37

An aqueous sample of the oligonucleotide, cleaved from the solid support and methylamine-deprotected as described above was cooled on ice. The supernatant was decanted and set aside and the support washed with EtOH–MeCN–H_2O (1 : 1 : 3 (v/v/v) 3 × 150 μL). The supernatant and washings were combined and evaporated to dryness. Removal of the TBDMS group was effected following treatment with a solution of *N*-methylpyrrolidone–triethylamine–triethylamine tris(hydrofluoride) (6 : 3 : 4 (v/v/v), 250 μL) at 65 °C for 1.5 h. The reaction mixture was allowed to cool to room temperature and excess fluoride quenched following addition of isopropyl trimethylsilylether (500 μL) with periodic vigorous shaking over a 10 min period. Diethyl ether (1 mL) was added and the mixture agitated vigorously. Following centrifugation at 5000 rpm, the supernatant was carefully removed and the residual solid pellet re-suspended in triethylammonium acetate buffer (pH 7) (1 mL) before analysis by RP-HPLC.

General methods for HPLC analysis

Cyclooctyne-modified oligonucleotides and click conjugation products were analyzed by reverse-phase HPLC under either conditions A (for products **12–16**, **33a–d**, **34a–d**), conditions B (for products **33e–f**, **38–40**), or conditions C (for products **23a–e**).

Conditions A: 200 μL injection loop. Buffer A: 0.1 M TEAAc (aq), pH 6.5; Buffer B: 0.1 M TEAAc, pH 6.5, 65% (v/v) MeCN (aq). Gradient: 0–3 min, 5% B; 3–23 min, 5 → 95% B. Flow rate: 1.0 mL min^{-1}. Detection at 260 nm. Column: Phenomenex C18 column (4.6 × 250 mm, 5 μm).

Conditions B: 20 μL injection loop. Buffer A: 0.1 M TEAAc, pH 6.5, 5% (v/v) MeCN (aq); Buffer B: 0.1 M TEAAc, pH 6.5, 65% (v/v) MeCN (aq). Gradient; 0–5 min, 5% B; 5–20 min, 5 → 95% B; 20–28 min, 95% B. Flow rate: 1.0 mL min^{-1}. Detection at 260 nm. Column: Phenomenex Clarity Oligo C18 column, 5 μm.

Conditions C: 200 μL injection loop. Buffer A: 0.1 M TEAAc (aq), pH 7.6; Buffer B: 0.1 M TEAAc, pH 7.6, 65% (v/v) MeCN (aq). Gradient: 0–4.3 min, 5% B; 4.3–16.6 min, 5 → 100% B. Flow rate: 1.0 mL min^{-1}. Detection at 260 nm. Column: Phenomenex C18 column (4.6 × 250 mm, 5 μm)

Procedures for click conjugation by SPAAC on the solid phase

Preparation of conjugates 23a–c. To solid-supported CPG-RNA-cyclooctyne **7** (0.12 μmol) in an Eppendorf tube was added a solution of the azide (10 μL of a 240 mM stock solution in DMSO, 2.4 μmol, 20 equivalents) and the volume was adjusted to 20 μL with DMSO and water according to the solubility of the azide (Table 1). The mixture was agitated at room temperature. After completion of the conjugation reaction (Table 1), the CPG was washed firstly with CH_3CN (5 × 300 μL) and then H_2O (1 × 300 μL). In the case of the biotin cycloadduct **23c**, DMSO was used instead of CH_3CN during the work-up. Cleavage from the solid support, deprotection (method i) and HPLC analysis (conditions C) were followed by the procedures described above to give **23a–c**.

Preparation of cholesterol conjugate, 23d. To solid supported CPG-RNA-cyclooctyne **7** (0.12 μmol) in an Eppendorf tube was added a solution of the cholesteryl azide **20** (20 μL of a 240 mM stock solution in DCM, 2.4 μmol, 20 equivalents) and the resulting mixture was agitated at room temperature for 20 h. After completion of the conjugation reaction, the CPG was washed with DCM (5 × 300 μL), CH_3CN (1 × 300 μL) and H_2O (1 × 300 μL). Cleavage from the support, deprotection (method i) and HPLC analysis (conditions C) were followed by the procedures described above to give **23d**.

Preparation of fluorescein conjugate, 23e in solution phase. Following deprotection and cleavage from the solid support (method i), a solution of RNA-cyclooctyne **12** (125 μL, 200 μM, 0.025 μmol) was evaporated to dryness. To this was added a solution of the fluorescein azide **21** (9.0 μL of a 112 mM stock solution in DMF, 1.0 μmol, 40 equivalents) and H_2O (0.5 μL). The resulting solution was agitated for 24 h at room temperature. H_2O (200 μL) was added and this solution was washed with EtOAc (10 × 300 μL) to remove the excess azide. Any remaining EtOAc was removed under vacuum and the resulting aqueous solution was analysed and purified by reversed-phase HPLC (conditions C) to furnish **23e**.

Procedures for click conjugation by SPNOAC on the solid phase

To a suspension of solid supported oligonucleotide-cyclooctynes **7–11** (0.2 μmol) in an Eppendorf tube in 100 μL of the appropriate solvent (Table 2) was added 10 μL of a premixed solution of the oxime (3.3 μmol) and chloramine-T monohydrate (3.3 μmol) in the stated solvent (Table 2). The combined mixture was agitated at room temperature for 10 min. Following settling the supernatant liquid was removed by syringe and the CPG

Table 4 Washing solvents selected for work-up of isoxazole conjugates

Product(s)	Washing solvents
33a, **34a**	CH_3CN (5 × 300 μL), CH_3OH (3 × 200 μL) H_2O (4 × 300 μL).
38, **39**, **40**	
33b, **34b**	CH_3CN (5 × 300 μL), CH_3OH (3 × 200 μL) H_2O (4 × 300 μL).
33c, **34c**	CH_3CN (5 × 300 μL), CH_3OH (3 × 200 μL) H_2O (4 × 300 μL).
34d	CH_3CN (5 × 300 μL), CH_3OH (3 × 200 μL) H_2O (4 × 300 μL).
33d	DMF (5 × 300 μL), CH_3OH (3 × 200 μL) H_2O (4 × 300 μL).
33e	DMF (5 × 300 μL), CH_3OH (3 × 200 μL) H_2O (4 × 300 μL).
33f	DMF (5 × 300 μL), CH_3OH (3 × 200 μL) H_2O (4 × 300 μL).

washed as outlined in the Table 4 below. Cleavage from the solid support, deprotection (method i) and HPLC analysis (conditions A and B) were followed by the procedures described above.

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