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Synthesis of oligonucleotides possessing versatile probes for PET labelling and their rapid ligand-free click reaction†

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We synthesised aryl acetylene derivatives as versatile probes for labelling of oligonucleotides. RNA oligomers bearing an aryl acetylene molecule rapidly reacted with benzylazide derivatives under ligand-free click reaction conditions.

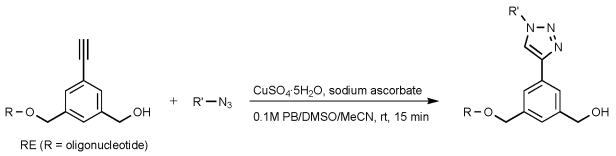
Molecular imaging techniques such as fluorescent molecular imaging, positron emission tomography (PET), single photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI) enable the biological functions of living organisms to be monitored.[1] Although fluorescent molecular imaging is often used because of its ease of implementation, it is affected by the tissue depth to a certain extent. PET is a highly sensitive and quantitative imaging technique for studying molecular interactions *in vivo*, and this imaging technique plays a key role in drug discovery, assessing *in vivo* distribution, pharmacokinetics and pharmacodynamics.

Because nucleic acid medicines such as antisense oligonucleotides and small interfering RNAs (siRNAs) have the capability for sequence-dependent gene silencing, the development of nucleic acid medicines has attracted considerable attention from researchers. For this purpose, it is necessary to label oligonucleotides with PET probes. Several radionuclides including ^{11}C ($t_{1/2}$ = 20 min), ^{15}O ($t_{1/2}$ = 2 min) and ^{18}F ($t_{1/2}$ = 110 min) are known as short-lived positron emitters, among which ^{18}F is the most frequently used in PET probes because of its longer half-life.[2] For instance, *N*-succinimidyl 4-[^{18}F] fluorobenzoate and *N*-[3-(2-[^{18}F]fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide have been developed for ^{18}F-labelling of oligonucleotides.[3] However, no method for labelling oligonucleotides has been widely adopted, owing to the possibility of side reactions or the lack of specificity of the coupling reactions.

The copper-catalyzed azide–alkyne cycloaddition (CuAAC) between an organic azide and a terminal acetylene was developed in the laboratories of Sharpless and Meldal.[4] The CuAAC reaction, a so-called "click reaction", has been applied to functional molecule synthesis in a wide range of fields including the biological and materials sciences, owing to its selectivity, reliability and versatility. Although click reactions involving oligonucleotides have been reported,[5] either tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA)[6] as a Cu(I) ligand or oxygen-free conditions are required for the reaction to progress satisfactorily without degradation of oligonucleotides by Cu(I)-mediated oxidative cleavage.[7] Furthermore, most of the procedures are only applicable to DNA. Ligand-free CuAAC of 2-5A has been reported, but the efficiency and availability are low.[8] Therefore, an efficient ligand-free click reaction for radiolabelling oligonucleotides is needed.

We have recently reported the synthesis and gene silencing activity of modified siRNAs possessing 1,3-benzenedimethanol at the 3′-overhang region.[9] In the present paper, we describe the synthesis of oligonucleotides having an aryl acetylene molecule (**1**),[10] these oligonucleotide derivatives were used in a rapid ligand-free click reaction for PET labelling (Scheme 1). The nucleotides were obtained from a phosphoramidite derivative of 3-[4,4′-dimethoxytrityl (DMTr)]-5-ethynylphenylmethanol (**2**) and a controlled pore glass (CPG) solid support (**3**) linked to **7** by the conventional phosphoramidite method (Scheme 2).

The synthesis of the aryl acetylene molecules is shown in Scheme 2. Sonogashira coupling between dimethyl 5-iodoisophthalate (**5**) prepared from **4**[11] and trimethylsilylacetylene gave 5-trimethylsilylisophthalate (**6**) in 95% yield. Subsequently, **6** was reduced and desilylated with lithium aluminium hydride to give 5-ethynyl-1,3-benzenedimethanol (**1**). One of the two hydroxyl groups of **1** was protected by a 4,4′-dimethoxytrityl (DMTr) group to give a mono-DMTr derivative **7**, which was phosphorylated with 2-cyanoethyl-*N*,*N*-diisopropylamino-chlorophosphite to produce the corresponding phosphoramidite **2**. In order to incorporate **1** at the 2′ or 3′-end of the oligonucleotides, the mono-DMTr derivative **7** was succinated, and the resulting succinate was linked to CPG to create the solid support **3** linked to **7**.



Scheme 1 Rapid ligand-free copper-catalyzed azide–alkyne cycloaddition of oligonucleotide bearing the aryl acetylene moiety.

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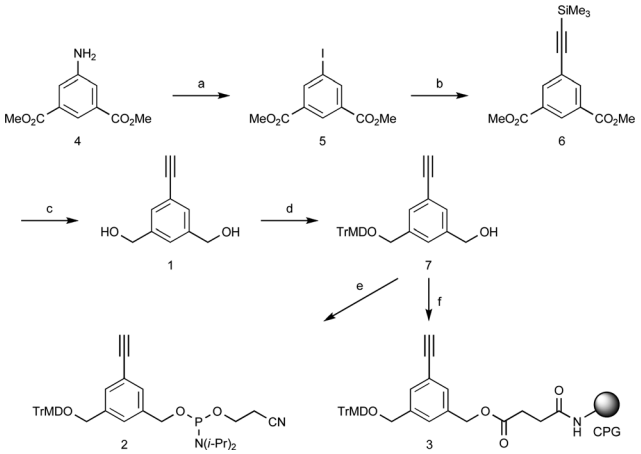
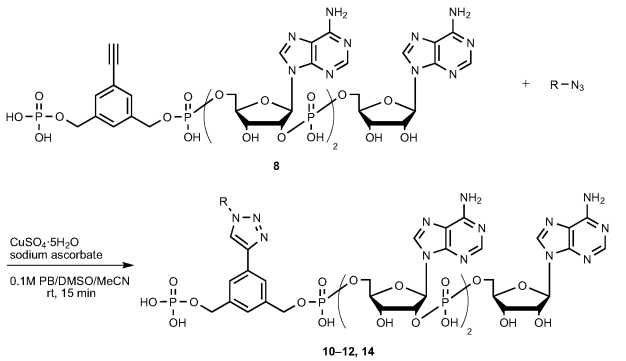


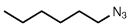
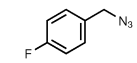
Scheme 2 *Reagents and conditions*: (a) (i) HCl, $NaNO_2$, H_2O, 0 °C; (ii) KI, H_2O, 0 °C → rt, 55% (two steps); (b) trimethylsilylacetylene, $PdCl_2(PPh_3)_2$, CuI, PPh_3, piperidine, THF, rt, 95%; (c) $LiAlH_4$, THF, 60 °C, 70%; (d) DMTrCl, DMAP, pyridine, rt, 49%; (e) $(i\text{-Pr})_2$-NP(Cl)O$(CH_2)_2$CN, i-Pr_2NEt, THF, rt, 38%; (f) (i) succinic anhydride, DMAP, pyridine, rt; (ii) CPG, EDCI, DMF, rt, 48.9 μmol g^{-1} (loading amount).

2′,5′-Oligoadenylate (2-5A) is known to mediate one pathway of interferon action by binding to and activating RNase L,[12,13] which cleaves single-stranded RNA preferentially on the 3′-side of UpN.[14,15] 2-5A is the smallest oligonucleotide with strong bioactivity and ease of handling. We have previously reported the synthesis of a 2-5A analogue bearing ethylene glycol at the 5′-end, which was found to activate human RNase L (only 1.5-fold less potent than the parent 2-5A tetramer).[16] Bearing that previous result in mind, we synthesised 2-5A analogues having 5-ethynyl-1,3-benzenedimethanol at the 5′-end (**8**) and at the 2′-end (**9**). The synthesis of 2-5As bearing the aryl acetylene moiety was carried out on an automated DNA/RNA synthesiser using the phosphoramidite derivative **2** or the solid support **3**. 2-5As could be obtained and purified by reversed phase HPLC. The structures of the 2-5A analogues were supported by MALDI-TOF/MS analysis.

Turning our attention to CuAAC between the 2-5A analogue **8** and benzylazide, we have found that the reaction proceeded smoothly at room temperature. When **8** and benzylazide (1.5 equiv.) were reacted in the presence of copper sulfate pentahydrate (100 equiv.) and sodium ascorbate (100 equiv.) in 0.1 M phosphate buffer (pH 7.0)/DMSO/MeCN, the desired cycloadduct **10** was obtained in quantitative yield within only 15 min (Table 1, entry 1). In the present coupling reaction, addition of TBTA was not effective (Table 1, entry 2). In contrast, phenylazide and hexylazide reacted in low to moderate yields (Table 1, entries 3 and 4), because the reaction did not reach completion. We also investigated the application of the present method to the coupling of the 2-5A analogue **8** with 4-fluorobenzylazide (**13**), considering that the synthesis of 4-[^{18}F]-fluorobenzylazide has been reported by Luxen *et al.*[17] 4-Fluorobenzylazide smoothly reacted with **8** within 15 min, and the desired product **14** was obtained in quantitative yield. These results agree well with previous findings that benzylazide derivatives are highly reactive azide compounds in CuAAC.[18]

Table 1 CuAAC of the 2-5A analogue **8** with various azide[a]



Entry	Azide	Product	Yield[b] (%)
1		**10**	100
2[c]		**10**	66
3		**11**	48
4		**12**	24
5		**14**	100

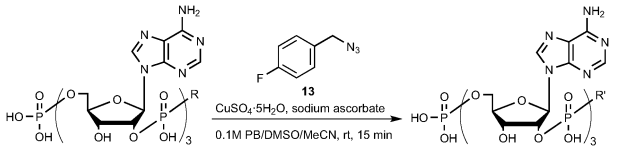
[a] *Reaction conditions*: azide (1.5 equiv.), $CuSO_4 \cdot 5H_2O$ (100 equiv.), sodium ascorbate (100 equiv.), 0.1 M phosphate buffer (pH 7.0)/DMSO/MeCN = 134 : 20 : 22. [b] The yield was determined based on the HPLC analysis. [c] TBTA (100 equiv.) was added as a Cu-ligand.

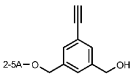
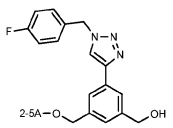
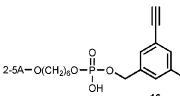
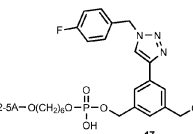
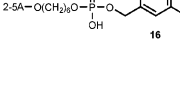
Thus, oligonucleotides bearing **1** could be radiolabelled with 4-[^{18}F]-fluorobenzylazide by using this method. We are now investigating radiolabelling with hot atoms.

Furthermore, the capability of the 2-5A analogues to activate RNase L was evaluated by monitoring the cleavage of synthetic RNA [5′-fluorescein-r($C_{11}U_2C_7$)-3′] by the activated RNase L.[19,20] The enzymatic reactions were analyzed by polyacrylamide gel electrophoresis. The density of fluorescent bands on the gel was measured with a fluorescence gel scanner. The EC_{50} value of the parent 2-5A tetramer was 5.09 nM, whereas that of the analogue **14** was only 36.29 nM.

Next, we investigated the coupling reaction using the 2-5A analogue **9**. The conditions shown in Table 1 could not be easily used for the coupling of **9** with 4-fluorobenzylazide, and the reaction did not reach completion within 15 min even with excess 4-fluorobenzylazide (20 equiv.) (Table 2, entry 1). In the case of **9**, steric hindrance might be the cause of the sluggish cycloaddition reaction. In fact, in the case of the 2-5A analogue **16**, in which a hexanediol spacer is present between **1** and 2-5A at the 2′-terminus, the coupling reaction with 4-fluorobenzylazide (5 equiv.) was remarkably improved and provided **17** in quantitative yield (Table 2, entry 2). The EC_{50} value of cycloadduct analogues **15** and **17** were 11.26 nM and 15.45 nM, respectively. It was found that analogue **17** bearing **1** at the 2′-end was only 1/3 as potent as the parent 2-5A tetramer in terms of RNase L activation ability. In a hot PET-labelled reaction, it is desirable to use a smaller amount of the radiotracer than of the precursor. The reaction of 4-fluorobenzylazide with **16** in the presence of copper sulfate pentahydrate (100 equiv.) and sodium ascorbate (100 equiv.) in 0.1 M phosphate buffer (pH 7.0)/DMSO/MeCN reached completion within 15 min with 99% yield (Table 2, entry 3).

Table 2 CuAAC of various 2-5A analogues with 4-fluorobenzylazide[a]



Entry	Substrate	**13** (equiv.)	Product	Yield[b] (%)
1		2		42
2		5		99
3		0.2		99

[a] *Reaction conditions*: $CuSO_4 \cdot 5H_2O$ (100 equiv.), sodium ascorbate (100 equiv.), 0.1 M phosphate buffer (pH 7.0)/DMSO/MeCN = 134 : 20 : 22. [b] The yield was determined based on the HPLC analysis.

miRNA-143: 5′-r(UGAGAUGAAGCACUGUAGCUCA)-3′

RNA **18**: 5′-r(UGAGAUGAAGCACUGUAGCUCA)dT**E**-3′

RNA **19**: 5′-r(UGAGAUGAAGCACUGUAGCUCA)**E**-3′

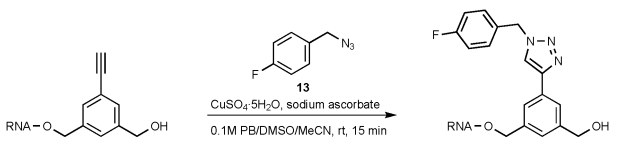
Fig. 1 Sequences of miRNA-143 analogues.

We have also studied the role of anti-oncomirs miRNA-143 in human colorectal tumors.[21] To investigate the generality of the present method, we synthesised short single-strand RNAs **18** and **19** bearing **1** at the 3′-end. Both **18** and **19** include a sequence of miRNA-143 (Fig. 1).

In both cases, the coupling reactions with 4-fluorobenzylazide (**13**) (10.0 equiv.) were completed within 15 min and provided the cycloaddition products **20** and **21** in good yields (Table 3).

In conclusion, we have developed aryl acetylene derivatives and a rapid ligand-free copper-catalyzed azide–alkyne cycloaddition for PET labelling of RNA oligomers. RNA oligomers bearing 5-ethynyl-1,3-benzenedimethanol rapidly reacted with benzylazide compounds to produce the cycloaddition products in good to excellent yield. To the best of our knowledge, this is the first reported method for PET labelling of RNA oligomers.

Table 3 CuAAC of miRNA-143 analogues with 4-fluorobenzylazide[a]



Entry	miRNA-143 analogue	Product	Yield[b] (%)
1	**18**	**20**	83
2	**19**	**21**	82

[a] *Reaction conditions*: 4-fluorobenzylazide (10 equiv.), $CuSO_4 \cdot 5H_2O$ (100 equiv.), sodium ascorbate (100 equiv.), 0.1 M phosphate buffer (pH 7.0)/DMSO/MeCN = 134 : 20 : 22. [b] The yield was determined based on the HPLC analysis.

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