RSC Advances

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Oligo switches: photoresponsive oligonucleotide conjugates by solid-supported click chemistry†‡

Cite this: *RSC Advances*, 2013, **3**, 1652

Received 8th November 2012,
Accepted 20th November 2012

DOI: 10.1039/c2ra22815g

www.rsc.org/advances

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Photoresponsive oligonucleotides (ONs) incorporating isoxazole-linked azobenzene (AB) moieties were prepared by resin-supported nitrile oxide-alkyne cycloaddition (NOAC) chemistry. The thermal and photochromic properties of the modified ONs were significantly influenced by the extent of π-conjugation between the isoxazole and the AB modules.

There is considerable interest in the use of biocompatible wavelengths to elicit reversible control of biomolecular activity.[1] In particular, nucleic acid analogues appended with a diverse range of bistable photoswitches[2] including azobenzene (AB) moieties, have been prepared.[3] The photochromic properties of ABs make them particularly attractive dynamic switches: photoisomerisation of the thermodynamically more stable *E*-form to the less stable *Z*-form is accompanied by significant changes in geometry and dipole moment, and can be reversed by either heating or irradiation with visible light.[4] Successful application of photoresponsive ABs in addressing biological questions relies upon both the synthetic accessibility of appropriately functionalised bioprobes, and on key physicochemical characteristics of the particular switch such as the yield of *E*/*Z*-isomerisation and the lifetime of the *Z*-isomer under physiological conditions.

Current methodologies for the synthesis of AB–ON conjugates typically rely upon unique monomers requiring optmisation of both coupling and deblocking conditions. Although post-synthetic coupling reactions (both in solution and on support) have been described, the accessibility of these approaches remains limited by the use of orthogonal deprotection conditions or labile linkages.[5] Thus, the goal of this work was the development of a general preparative route to AB-derivatised ONs from commercially-available monomers in which the *Z*-AB isomer has relatively high thermal stability.

Bioconjugation protocols centered upon copper-catalysed azide-alkyne cycloaddition (CuAAC) chemistry[6] have many positive attributes and one recent report describes the formation of AB glycoconjugates.[7] However, despite elegant applications to ON derivitisation,[8] this methodology has scarcely been applied to the introduction of photoswitches to nucleic acid substrates.[3b] To-date, both solution-phase and solid-phase catalyst-free nitrile oxide-alkyne cycloaddition (NOAC) chemistry have been applied to the conjugation of dyes incorporating AB-chromophores to nucleic acids.[9] However, the low thermal stability of the *Z*-isomers of these dyes (except in high concentrations of ethanol) renders them unsuitable for photomodulated activity switching.

The known relationships between molecular structure, *E*/*Z*-photoisomerisation characteristics and thermal stabilities of AB moieties have been exploited specifically through the introduction of substituents capable either of conjugation with the diazenyl function,[10] or of exerting steric demands proximal to the AB core.[3d,11] Here we report the site-specific introduction of bistable AB photoswitches into ONs by solid-supported "click" NOAC chemistry and the influence of the ensuing, electron donating, isoxazole-linkers upon their photophysical properties.

The commercial availability of 2′-*O*-propargyl nucleoside phosphoramidite building blocks, and the precedent for "click" type modifications of substrates incorporating this functionality,[12] determined the choice of "click" partners: alkyne-functionalised ON′s and AB bearing nitrile oxides. The regioisomeric aldehydes **2** served as precursors to the oximes **3**, from which the dipoles **4**, were generated as needed (Scheme 1). Lithium aluminium hydride reduction of 4-(phenylazo)benzoic acid provided the alcohol **1a** and the isomeric **1b** was obtained by Mills coupling of *meta*-aminobenzyl alcohol with nitrosobenzene. Dess–Martin periodinane (DMP)-mediated oxidation of the alcohol functional groups afforded the corresponding aldehydes **2a**[13] and **2b** and subsequent oximation (yielding **3a** and **3b**) was readily accomplished under microwave irradiation. Model "click" reactions were performed in solution following chloroamine-T (Ch–T)-mediated oxidation of the oximes to the reactive nitrile oxides **4a** and **4b**, and *in situ*

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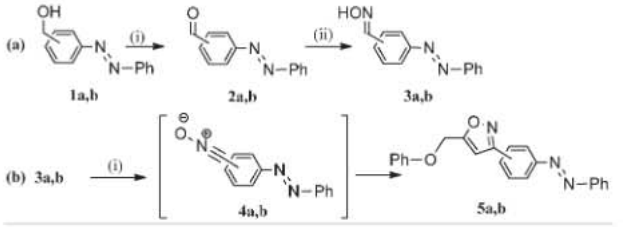
† Electronic Supplementary Information (ESI) available: Experimental procedure, NMR, MALDI-TOF, kinetic and photochemical data. See DOI: 10.1039/c2ra22815g

‡ The manuscript is dedicated to the memory of H. Gobind Khorana.



Scheme 1 (a) Synthesis of azo-oximes **3a** and **3b**. (i) DMP, DCM, 1 h, 97% (**2a**) 71% (**2b**). (ii) $NH_2OH{\cdot}HCl$, pyridine, EtOH, M_W (P_{max} = 300 W, T = 120 °C), 1 h, (**3a**) 94%, (**3b**) 88%. (b) Model NOAC reactions. (i) [*p*-$CH_3C_6H_4SO_2NCl$]Na (Ch–T), EtOH/H_2O (3 : 1), $PhOCH_2CCH$, 40 °C, 1 h, 89% (**5a**) 46% (**5b**).

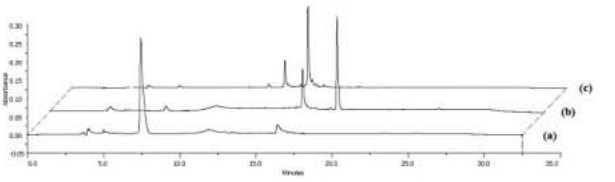


Fig. 1 HPLC traces of (a) 2′-*O*-propargyl uridine and the crude AB-NOAC products (b) **8a** and (c) **10a**.

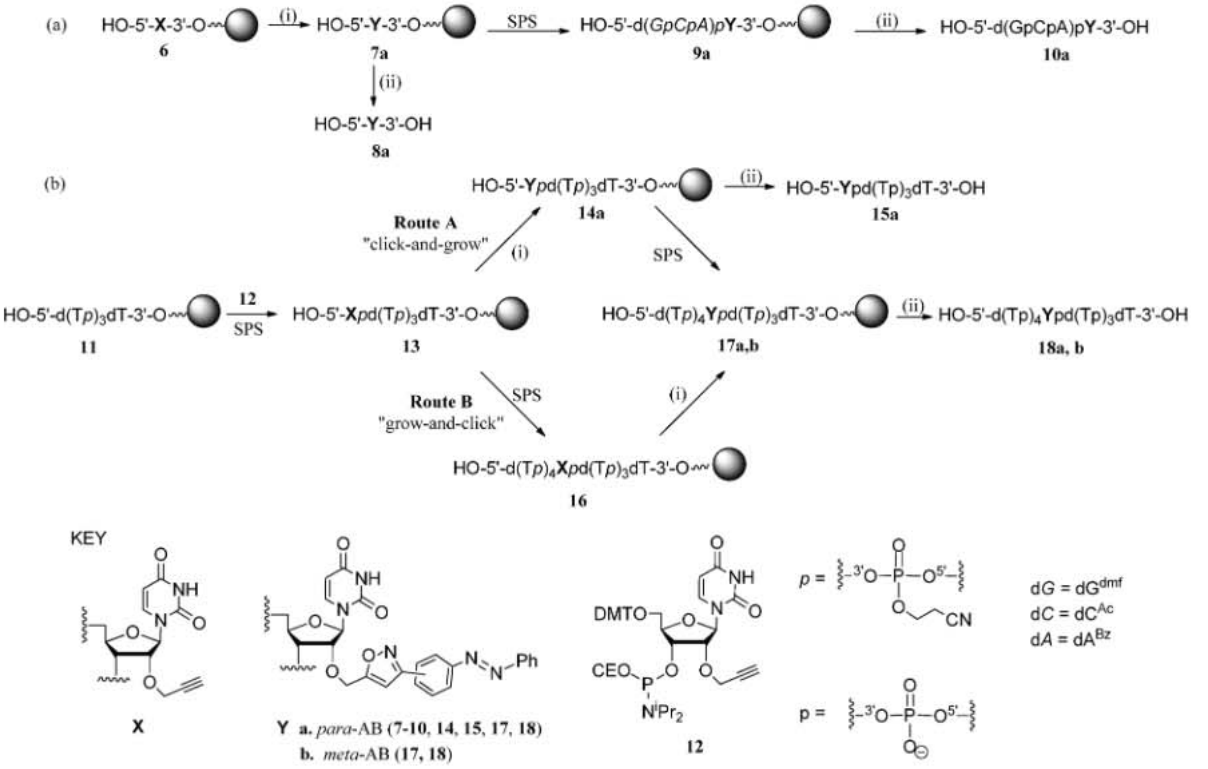
reaction with the dipolarophile phenyl propargyl ether. Regioselective formation of the 3,5-disubstituted isoxazoles **5a** and **5b** was optimal after 1 h stirring in aqueous ethanol (75% v/v) at 40 °C. No degradation products were observed (by ^1H NMR), thus supporting compatibility of the Ch–T protocol with the azo-functionality.

The optimised "click" reaction conditions were subsequently applied to detritylated, CPG-supported 2′-*O*-propargyl uridine (**6**, Scheme 2a); conversion to the putative isoxazole-linked AB-nucleoside **7** followed agitation of a suspension of **6** in aq. ethanol (75% v/v) at rt in the presence of a premixed solution of oxime **3a** and Ch–T (30 eq. each). Cleavage from the resin under standard conditions yielded AB-conjugated uridine **8a** as a ~1 : 3 mixture of *Z* : *E*-isomers as judged by RP-HPLC (Fig. 1b).

To demonstrate compatibility of the isoxazole linkage with standard automated DNA synthesis, three synthetic cycles were performed on the supported isoxazole **7a** to yield the putative, fully-protected tetramer **9a**. MALDI-TOF MS and HPLC (Fig. 1c) characterisation of the product following cleavage and deprotection (**10a**) indicated that the structural integrity of the isoxazole linked-AB was maintained.

ONs modified at the 5′-terminus, or at a predetermined internal position, were constructed employing the 2′-*O*-propargyl uridine phosphoramidite **12** as a building block. The terminally-modified pentamer 5′-X-T_4-CPG (**13**) was an excellent substrate for NOAC "click" chemistry and the crude product **15a** (Scheme 2b) was characterised by HPLC and MALDI-TOF MS.

The introduction of a *para*-AB moiety mid-sequence was investigated using a "click-and-grow" as well as the more frequently demonstrated "grow-and-click"[14] strategy (routes A or B respectively, Scheme 2b). Thus, the "clicked" pentamer **14a** was



Scheme 2 Synthesis of (a) 3′-modified AB–ON conjugate **10a**, (b) 5′-modified AB–ON conjugate **15a** and internally-modified AB–ON conjugates **18a,b** by Routes A and B. (i) **3**, Ch–T, aq. EtOH (75% v/v), rt, 18 h; (ii) 40% (w/w) aqueous CH_3NH_2, 65 °C, 30 min.

Table 1 Photophysical characterisation of **18a** and **18b**

	λ_{max}/nm			χ_E	
	E-isomer		*Z*-isomer		
Compound	π–π*	*n*-π*	*n*-π*	*DA*-PSS	*Irr*-PSS
18a	336	433	433	85%	27%
18b	323	438	428	81%	17%

extended following further rounds of phosphoramidite coupling under standard SPS conditions to give the corresponding nonomer **17a**. Alternatively, following growth of the ON-alkyne **16**, resin-supported click ligation to **4a** (generated *in situ*) also yielded **17a**. Significantly, the efficiency of the "click" reactions appeared to be independent of the alkyne position; conjugation to the 3′- or 5′-termini, or at an internal position (yielding **10a**, **15a** or **18a** respectively) all gave essentially quantitative coupling. Employing the putative nitrile oxide **4b**, the *meta*-AB moiety was also successfully introduced by the "grow-and-click" strategy. RP-HPLC analysis of crude, deprotected oligomers bearing internal modifications (**18a**, **18b**) showed a well-resolved mixture of *E*-(major) and *Z*-(minor) isomers which were desalted and purified to yield individual samples of *E*-**18a** and *E*-**18b**.

Both *para*- and *meta*-isomers of the isoxazole-linked AB chromophore conform to Rau's classification[15] of "AB-like" moieties in that they show large energy gaps between the π–π* and n–π* transitions (Table 1) and thus have relatively high switching efficiencies upon irradiation at 366 nm (to the irradiated photostationary state; *Irr*-PSS) or above 400 nm (to the dark-adapted photostationary state; *DA*-PSS). Monitoring the loss of UV-absorption arising from the E-AB π–π* transition at 320–340 nm, extremely rapid *E*/*Z*-photoisomerisation of both **18a** and **18b** was observed such that switching to the *Irr*-PSS was essentially complete after 10 s using a medium pressure Hg lamp with a band-pass filter (although irradiation for 10 min was used to maintain consistency with related 2′-amido-linked photoswitchable analogues[5b]). Using a cut-off filter to remove incident radiation below 400nm, the *DA*-PSS was achieved within 2 min. The compositions of these photostationary states were analysed using RP-HPLC from which the mole fractions of *E*-isomers (χ_E) were calculated (Table 1).

Subsequently, the thermal stabilities of the *Z*-isomers were determined; solutions of *Irr*-**18a** and **18b** were maintained at temperatures between 60–85 °C and the increase in absorption at 351 nm was monitored. The first order rate constants (k) for the thermal $Z \rightarrow E$-isomerisations derived from these measurements enabled Arrhenius and Eyring parameters to be determined (Table 2). Consistent with previous observations on the relative stability of substituted AB modules[3e,10,11] the *meta*-linked *Z*-**18b** is significantly more stable than its *para*-analogue *Z*-**18a**. The lack of mesomeric interaction between a *meta*-substituent and the azo functionality contributes to the long half-life of *Z*-**18b** (114 h at 37 °C). In contrast, the *para*-relationship between these units in *Z*-**18a** is assumed to have extended π-conjugation, resulting in attenuated activation energy for $Z \rightarrow E$-thermal isomerisation and a reduced half-life of 30.3 h at physiological temperature.

Table 2 Calculated kinetic constants describing the thermal *Z*/*E*-isomerisation of **18a** and **18b**

Compound	E_{act} (kJ mol^{-1})	A (s^{-1})	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1}K^{-1})	$t_{1/2}$ at 37 °C (hr)
18a	97.25	1.549×10^{11}	94.37	−40.22	30.30
18b	112.18	1.065×10^{13}	109.22	−5.30	144

In conclusion, we have demonstrated the concept of solid phase "click" cycloaddition for site-specific incorporation of photoswitches to nucleic acids using ON-alkynes and AB-substrates bearing reactive nitrile oxides *para*- or *meta*-to the azo functionality. In all cases, the "click" reaction was clean and regioselective. The thermal stability of the photoswitch was significantly influenced by the extent of π-conjugation between the isoxazole linker and the photochromic AB moiety. The enhanced thermal stability of *Z*-**18b**, where the AB is ligated to the ON through an isoxazole linker *meta*-to the azo group, suggests that such moieties may be successfully employed in photoswitching applications.

Acknowledgements

The work was supported by the Science Foundation of Ireland (Programme code 05/PICA/B838/NSs) and the Irish Research Council of Science and Engineering (Embark Postgraduate Research Scholarship to CF, Programme code RS/2007/48)

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