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Modification of oligodeoxynucleotides by on-column Suzuki cross-coupling reactions†

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On-column functionalization of oligodeoxynucleotides by base-free Suzuki cross-coupling reactions is reported herein. These cross-couplings were carried out with various boronic acids and either full-length modified oligonucleotides containing one or more 2'-deoxy-5-iodouridine (5IdU) monomer(s) or on oligonucleotide fragments immediately after incorporation of 5IdU. Five different functionalities were coupled to oligonucleotides containing one or three attachment points.

The need for modified DNA and RNA oligonucleotides has increased rapidly due to advances in biotechnology and biomedical research. Multiple methods for modification of oligonucleotides (ONs) have been developed and the modifications are commonly introduced either by automated ON synthesis with modified phosphoramidites[1] or by enzymatic incorporation using modified nucleoside triphosphates.[2] Both methods often require long multistep syntheses for monomer preparations and not all modifications are tolerated or compatible with the required reagents and conditions used for elongation of ONs.[3,4] Some of these disadvantages can be overcome by site-specific postsynthetic modification of ONs. When using this approach, a stable precursor is incorporated into the ON which can subsequently be functionalized employing various conjugation chemistries.[5] Commonly used methods for conjugation is Cu-catalyzed[6] or Cu-free[7] Huisgen cycloaddition, Staudinger ligation,[8] thiol conjugation[9] or amide bond formation.[10] These methods can be efficient but many also form structurally rather complex products upon conjugation (e.g. triazole linkers or phosphine oxides) that might interfere with biological function.

Pd-catalyzed cross-coupling reactions can be used for nucleobase modifications of nucleosides, nucleotides and ONs, most commonly in the 5-position of pyrimidines and the 6- or 8-position of purines.[11-13] The first report of Pd-catalyzed reactions with ONs was a Sonogashira cross-coupling used for on-column postsynthetic modification of a DNA strand singly modified with 2'-deoxy-5-iodouridine (5IdU).[14] The same method was used to introduce 1-pyrenyl[15,16] and a spin-label[17-19] to both purine and pyrimidine bases within an ON. The Sonogashira coupling was later used for on-column reactions with full-length ONs modified with 5IdU to introduce 23 different functionalities,[20] spin-labels[21] and modified Twisted Intercalating Nucleic Acids.[22] More recently, the Stille cross-coupling was used for multiple reactions with full-length RNA strands on-column while also testing different protecting groups for the 2'-hydroxy group.[23] The Stille coupling was performed with both 5'-terminal and intrastrand incorporations of 5-iodouridine and 2-iodoadenosine into either poly-U or poly-A RNA strands.[23] The Stille coupling was also used for on-column modifications in good yields to 5IdU and 2'-deoxy-5-iodocytosine (5IdC) incorporated into DNA strands.[24]

Contrary to the Sonogashira and Stille couplings, reports of Suzuki cross-coupling reactions have been limited to deprotected DNA strands in solution. Suzuki couplings have thus been used to modify 5IdU and 5IdC with a photoswitchable group[25] and various sensitive functional groups.[26] The Suzuki coupling has also been used for arylation of 8-Br-2'-deoxyguanosine thereby synthesizing functionalized DNA strands not accessible by conventional solid-phase synthesis.[27] The mild and versatile nature of Suzuki coupling reactions makes it a good candidate for on-column modifications of ONs. The required excess of reagents for heterogeneous on-column derivatization is easily removed by thorough washings of the column before deprotection from the solid support thus simplifying purification. The Suzuki and Stille couplings generate equivalent C-C bonds, but the Suzuki coupling offers a desirable

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non-toxic alternative to the organotin compounds employed in the Stille couplings. The Suzuki coupling does, however, require base for activation of the organoboron reagent which may lead to a stability issue relating to the base labile succinyl linker between the ON and the support.

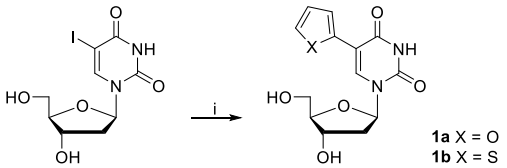
We have sought to modify the 5-position of 5IdU with various aromatic moieties including phenyl, furyl, thienyl, pyrenyl and 3-(dansylamino)phenyl groups as such modified pyrimidines are fluorescent.[28-30] Such nucleosides emit in the visible range and the sensitivity provided by fluorescence-based tools can be used for confirmation of a putative reaction, even if only very low amounts of cross-coupling product are generated.[31,32] Additionally, modifications at the 5-position of pyrimidine bases of an antisense ON offers modulation of target affinity.[33]

Herein we report the introduction of aromatic moieties in the 5-position of uracil bases *via* incorporation of 5IdU into solid-support bound ONs and subsequent on-support Suzuki cross-coupling reactions. Up to five different functionalities were introduced in a single position by cross-coupling with the full-length ON or by individual cross-couplings immediately after incorporation of an 5IdU nucleotide. Both methods were also used to introduce three identical functionalities to the same ON. The latter method was moreover used to introduce three different functionalities to the same ON allowing for easy modification of ONs in a sequential fashion.

Initially, model Suzuki cross-couplings with unprotected 5IdU nucleoside and 2-furylboronic acid or 2-thienylboronic acid were carried out to assess any potential side reactions, and to find the optimal coupling partner for reactions with the corresponding ONs (Scheme 1).

The cross-coupling reactions using conditions adapted from Hopkins *et al*.[34] were successful but proceeded in low yields, giving **1a** and **1b** in 25% and 35% yield (Scheme 1), respectively, while the major product was the dehalogenated 2'-deoxyuridine. 2-Thienylboronic acid gave the higher yield and the least by-products and was therefore chosen as the coupling partner for initial reactions with ONs. No optimization of the conditions was carried out as the local environment for on-column reactions with ONs are very different from reactions in solution, as the former typically require a higher excess of reagents to compensate for the heterogeneous reaction conditions.[14,19]

The first reactions with ONs were carried out with two 5-mer DNA-type strands containing all canonical bases and 5IdU positioned either at the 5'-terminal (**ONa**) or internally (**ONb**) to identify the scope and possible limitations of the approach (Fig. 1a and Table 1). The internal position mimics longer sequences



Scheme 1 Suzuki cross-coupling with unprotected 5IdU. Reagents and conditions: 2-Furylboronic acid or 2-thienylboronic acid, $Pd(OAc)_2$, TPPTS, MQ H_2O/MeCN (3:2 v/v), 80 °C, 16 h.

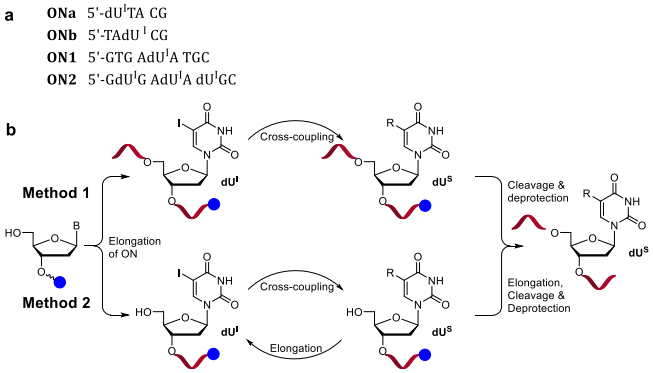


Fig. 1 **Schematic illustration of on-column Suzuki cross-coupling.** Blue spheres represent the CPG solid-support; ONs are drawn in red; dU^I represents the 5IdU nucleotide where dU^S represents a cross-coupling product. (a) ON sequences used for Suzuki cross-coupling reactions; (b) Generel representation of the two methods used for on-column Suzuki cross-coupling reactions. Method 1: Cross-coupling reaction carried out after synthesis of the full-length ON; Method 2: Cross-coupling reaction carried out immediately after incorporation of the 5IdU nucleotide. Reagents and conditions: $Pd(OAc)_2$, TPPTS, boronic acid, Tris buffer (50 mM, pH 8.50)/MeCN (3:2 v/v, 1 mL), 70 °C, 4 h/8 h.

where reactions at the terminal position renders steric factors negligible. Suzuki cross-couplings were carried out with the 5-mer strands retained on the columns whereafter the ONs were cleaved from support, deprotected and subjected to analysis by RP-HPLC and MALDI-MS. However, a significantly lower amount of the test ONs were isolated after cross-coupling than expected from a 0.2 µmol ON synthesis, and the amounts were insufficient for HPLC analysis. The presence of the thiophene-modification was therefore verified by a positive fluorescence signal. The low amount of the ON product after cleavage from support and deprotection was ascribed to cleavage of the base labile linker between the solid support and the ON during cross-coupling. Davis *et al*.[26] reported successful base-free Suzuki cross-couplings with DNA strands in buffer systems which inspired us to optimize the conditions for on-column Suzuki cross-couplings by substituting the medium composed of base and MQ H_2O for a tris(hydroxymethyl)aminomethane (TRIS) buffer. Cross-couplings with Test **ONa** and **ONb** were repeated using these optimized conditions. Full conversion of the two test ONs into cross-coupled products (Table 1) was observed by HPLC analysis with no noticeable formation of by-products (Fig. S17 and S19, ESI†).

Following the initial successful cross-coupling reactions, 9-mer DNA-type ONs with all canonical bases and one or three 5IdU modifications were used for the following cross-coupling reactions. The reactions were carried out by two different methods (Fig.1b): a) Method 1 in which cross-couplings were carried out with the full-length ON, and b) Method 2 in which cross-couplings were carried out immediately after incorporation of 5IdU followed by elongation of the ON. Cross-couplings by Method 2 were thus always carried out with an "end-positioned" nucleotide thereby making the iodinated nucleotide more accessible. The substrate scope was explored by reaction of full-length **ON1** with 2-thienylboronic acid (synthesis of **ON3**(a)), phenylboronic acid (synthesis of **ON4**), 5-methyl-2-thienylboronic acid pinacol ester (synthesis of **ON5**), pyrene-1-boronic acid (synthesis of **ON6**(a)) and

Table 1 On-column modification of a 9-mer DNA strand by Suzuki cross-coupling with one modification site. dU^S represents the modified nucleotide

ON #	Sequence	Modification	Method	Yield (%)[a]
A	5'- dU^{S}TA CG	2-Thienyl	1	-[b]
B	5'- TAdU^S CG	2-Thienyl	1	-[b]
3(a)	5'-GTG AdU^{S}A TGC	2-Thienyl	1	29
3(b)	5'-GTG AdU^{S}A TGC	2-Thienyl	2	38
4	5'-GTG AdU^{S}A TGC	Phenyl	1	29
5	5'-GTG AdU^{S}A TGC	5-Methyl-2-thienyl	1	21
6(a)[c]	5'-GTG AdU^{S}A TGC	Pyrenyl	1	7
6(b)[c]	5'-GTG AdU^{S}A TGC	Pyrenyl	2	27
7(a)	5'-GTG AdU^{S}A TGC	3-(Dansylamino)phenyl	1	28
7(b)	5'-GTG AdU^{S}A TGC	3-(Dansylamino)phenyl	2	48

[a]Isolated yields for full-length product. [b]Product not isolated for determination of yield for test sequences. [c]Tris buffer (50 mM, pH 8.50)/DMF (2:3 v/v, 1 mL) were used to dissolve pyrene-1-boronic acid; the concentration of pyrene-1-boronic acid was also halved due to the solubility issue.

3-(dansylamino)phenyl boronic acid (synthesis of **ON7**(a)) by using Method 1 (Fig. 1a and Table 1). Notably, Suzuki cross-couplings with some boronic acids can generate significant amounts of dehalogenated product, and it has been shown that using the pinacol esters of these boronic acids can push the reaction towards cross-coupling.[26] RP-HPLC spectra for crude ONs (**ON3**(a), **ON4**, **ON5 and ON7**(a)) following reaction and deprotection/cleavage from the solid support showed no or a tiny peak corresponding to the starting **ON1** indicating complete conversion into product ONs for reactions with all four boronic acids (Fig. S22, Fig. S24-S25 and S28, ESI†). Following purification, full-length **ON3**(a), **ON4, ON5** and **ON7**(a) were isolated in a yield of 29%, 29%, 21% and 28%, respectively, in satisfactory purity as shown by HPLC analysis. The yield for **ON5** using the protected boronic acid was lower than for reactions with unprotected boronic acids indicating a slower or more unfavorable reaction likely due to steric factors with the protected reagent (Table 1). The lower yields on isolated **ON6**(a) relative to **ON7**(a) are not surprising, for the mixed reagent solutions in the former quickly turned from light yellow to dark after 0.5 h at 70 °C, indicative of strong side reactions. This may also result partly from the lower concentration of pyrene-1-boronic acid used in the case of **ON6**(a) due to the solubility issue. The isolated yields for **ON3**(a), **ON4**, **ON5** and **ON7**(a) were comparable to the reported yields for Suzuki cross-couplings of ONs in solution by Jäschke[25] but lower than reported by Davis also for in solution cross-couplings.[26] The isolated yields are, however, similar to reports for on-column Sonogashira[14,20] and Stille[24] couplings. We achieved almost complete conversion of dU^I into dU^S also for cross-coupling using Method 2 with 2-thienylboronic acid, pyrene-1-boronic acid and 3-(dansylamino)phenylboronic acid (Fig. S23, Fig. S27 and S29, ESI†). **ON3**(b), **ON6**(b) and **ON7**(b) were isolated in total yields of 38%, 27% and 48%, respectively, which are significantly higher than those isolated of **ON3**(a) **ON6**(a) and **ON7**(a) using Method 1 (Table 1). These results are in agreement with Jäschke's findings[25] showing higher yields for cross-couplings with terminal modifications than with internal modifications. The successful couplings with one modification prompted the expansion of the methodology to multiple incorporations using 9-mer **ON2** with three 5IdU modifications (Fig. 1a and Table 2). This corresponds to requiring a third of the nucleotides in the ON to undergo reaction. Cross-coupling of **ON2** with 2-thienylboronic acid using Method 1 was carried out without increasing the amount of reagents relative to the previous couplings as they were already in great excess. The RP-HPLC spectrum for crude **ON8**(a) showed three major peaks with very similar retention times (< 1.0 min span on IE-HPLC; separation was possible by RP-HPLC and all three peaks were individually isolated (Fig. S30, ESI†)). Common for the three isolated product ONs were successful cross-coupling reactions in two positions combined with either substitution (8%), dehalogenation (7%) or no reaction (11%) in the third position (note that the positional order has not been determined). The isolated yield for ONs where the third cross-coupling was unsuccessful (18%) is twice that of the desired **ON8**(a) with three successful substitutions (8%). Attempted optimization of conditions was performed by extending the reaction time from 4 to 8 h and by employing double cross-couplings. Extension of the reaction time did not lead to isolation of the desired product **ON8**(b) in a higher yield but instead a lower yield of the product in which no reaction had occurred in the third position was observed (from 11% to 5%, Table 2). Employing double cross-couplings led to a significant increase in isolated yield to 18% for **ON8**(c) but a significant amount of dehalogenated ON was still observed (5%). The large increase in yield with double couplings suggests that the catalyst becomes less effective over time, possibly due to influx of oxygen.

Method 2 was also used to obtain triple thienyl-modified **ON8**(d) requiring pausing of the ON synthesis three times in total. Two major peaks were observed in the crude RP-HPLC chromatogram and both were independently isolated (Fig. S33, ESI†). One peak corresponded to the desired **ON8**(d) (22% yield) and another to dehalogenation of the third position (4% yield). By using Method 2, significantly lower amounts of the dehalogenated ON were observed together with disappearance of the peak corresponding to the ON for which no reaction at the third position had occurred (Table 2). This suggests that steric issues likely negatively impact the outcome of three cross-couplings within the same ON. The success of triple modifications when employing Method 2 enables further expansion of the methodology by using a different boronic acid for each sequential cross-coupling reaction.

Thus, an attempt of successively incorporating 2-thienyl (Th), 5-methyl-2-thienyl (MeTh) and phenyl (Ph) afforded one major product ON as observed from RP-HPLC (Fig. S34, ESI†) which was isolated in a yield of 23% (**ON9**). Incorporation of different boronic acids in the same sequence offers great potential for easy but structurally diverse functionalization without the need for synthesis of a series of phosphoramidites. Furthermore, on-column conjugation offers the opportunity for introduction of different functionalities to the same ON which is not possible by the method for Suzuki cross-couplings in solution reported by Jäschke[25] and Davis.[26]

In summary, we demonstrated the first successful use of base-free Pd-catalyzed Suzuki cross-coupling reactions as a convenient strategy for postsynthetic on-column modification of oligodeoxynucleotides. Cross-couplings were accomplished

Table 2 On-column modification of a 9-mer DNA strand by Suzuki cross-coupling with three modification sites. dU^S represents the modified nucleotide

ON #	5′-GdU^{S}G AdU^{S}A dU^{S}GC	Time (h)	Method	Yield (%)[a]	Dehal. (%)[b]	No rx (%)[b]
8(a)	3 × Th	4	1	8	7	11
8(b)	3 × Th	8	1	8	8	5
8(c)	3 × Th	2 × 4	1	18	5	5
8(d)	3 × Th	3 × 4	2	22	4	-
9	1 × Ph, 1 × MeTh, 1 × Th	3 × 4	2	23	-	-

[a]Isolated yield of triply modified ON. [b]Isolated yield for ONs with dehalogenation or no reaction in one position; Th = 2-thienyl, MeTh = 5-methyl-2-thienyl, Ph = phenyl.

with single and triple modifications of 2′-deoxy-5-iodouridine introduced into 9-mers, either by reaction on the full-length oligodeoxynucleotide or immediately after incorporation of each 5IdU monomer. Complete conversion of a singly 2′-deoxy-5-iodouridine modified 9-mer was observed for cross-couplings with five different boronic acids, and with the highest yield observed when cross-coupling reactions were carried out immediately after incorporation of the 2′-deoxy-5-iodouridine monomer. Cross-couplings with on the full-length triply 2′-deoxy-5-iodouridine modified 9-mer were more challenging, but successful cross-couplings were obtained by employing double couplings and extended reaction times. Again, the highest yields were observed for cross-couplings immediately after incorporation of each 2′-deoxy-5-iodouridine monomer, and this method was also used for sequential introduction of different functionalities along the 9-mer oligodeoxynucleotide. We expect that the new protocol for postsynthetic modification of oligodeoxynucleotides described herein will find use during design and synthesis of future oligonucleotides.

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Conflicts of interest

There are no conflicts to declare.

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