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Synthesis of DNA conjugates by solid-phase fragment condensation via aldehyde–nucleophile coupling

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Abstract—Oligodeoxyribonucleotides were synthesized that contain a novel nucleoside, 2′-*O*-(2,3-dihydroxypropyl)cytidine. Its 2′-diol group was blocked by an allyloxycarbonyl protecting group. Selective deprotection of diol group(s) of the support-immobilized blocked oligodeoxyribonucleotide by Pd(0) followed by periodate oxidation resulted in generation of the 2′-aldehyde group(s) on solid-phase. The modified oligonucleotides were used to prepare a number of conjugates with acridine, biotin and *N*-modified laminin peptides by oxime, hydrazone and hydrazine formation. The method may be applicable to the synthesis of oligonucleotide–peptide conjugates.

Nowadays, oligonucleotide derivatives are widely used in many fields of biotechnology and medicine for diagnostics, for example, by PCR or hybridization assay, and as potential therapeutic agents.[1,2] However, synthesis of modified oligonucleotides and their conjugates is still evolving. Attachment of reporter groups enhances sensitivity of detection of DNA sequences; coupling of peptides, carbohydrates or cholesterol leads to increase in cellular uptake. A variety of methods for conjugation of oligonucleotides with target compounds have been developed so far.[3,4] Oligonucleotide conjugates may be synthesized by use of phosphoramidite derivatives of target compounds, or by post-synthetic conjugation on solid-phase, or in solution by reaction of oligonucleotides with other molecules. Amongst the methods for covalent joining of large biomolecules, aldehyde–nucleophile coupling is gaining popularity in biomolecular chemistry[5–8] and is particularly efficient in the case of difficult conjugation with multifunctional compounds, for example, peptides. The advantages of this approach are in the high yields of conjugates, chemoselectivity of the reaction and use of smaller excess of the nucleophilic component. Synthesis of oligonucleotide–peptide conjugates is well attested.[9–11] Total stepwise solid-phase synthesis is the most direct method for the preparation of oligonucleotide–peptide conjugates. Although it provides generally good yields of the final product, difficulties in finding a combination of compatible protecting groups for both oligonucleotide and peptide moieties and losses due to incomplete coupling at every elongation step limit the nature and length of peptide sequences, especially those suitable for cell delivery. To overcome this problem, solid-phase coupling strategies have been developed for synthesis of DNA–peptide[12–14] and PNA–peptide[15] conjugates. However, joining of protected peptide fragments via acylation reactions proceeds slowly and in moderate yields, due to their limited solubility in aprotic bipolar solvents and adverse steric effects.

Here we report the preparation of blocked 2′-*O*-(2,3-dihydroxypropyl)cytidine phosphoramidite, its incorporation into oligonucleotides, and their subsequent use for chemoselective conjugation. Use of allyloxycarbonyl protecting groups ensures selective unmasking of the diol group from the still protected and immobilized oligonucleotide. Subsequent periodate oxidation on solid-phase leads to the generation of an aliphatic aldehyde group in the oligonucleotide. Then an efficient chemoselective conjugation with a number of compounds, including *N*-modified laminin peptides, was accomplished.

The target phosphoramidite **3** was synthesized by a route similar to that we published previously for the 2′-*O*-(2,3-dihydroxypropyl)uridine[17] (Scheme 1). Initial

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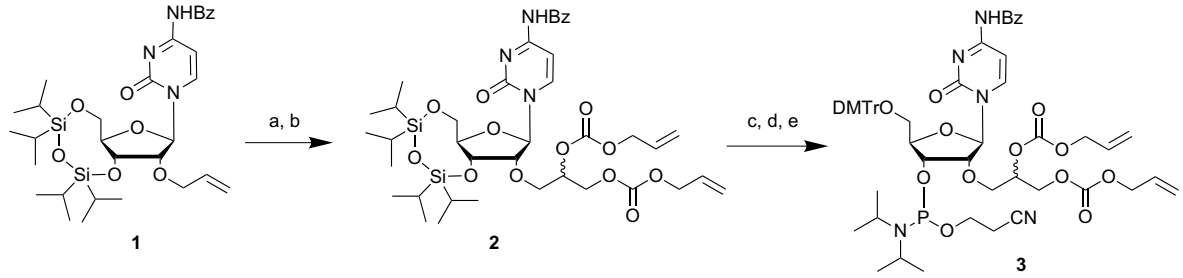

Scheme 1. Preparation of the phosphoramidite **3**. Reagents and conditions: (a) OsO_4 (0.08 equiv), *N*-methylmorpholine *N*-oxide, THF–H_2O (2:1, v/v), 1 h, 92%; (b) AllocCl, Et_3N, CH_2Cl_2, 4 °C, 1 h, 82%; (c) TBAF, THF, 10 min, 91%; (d) DMTrCl, Py, 3 h, 87%; (e) $(^iPr_2N)_2PO(CH_2)_2CN$, diisopropylammonium tetrazolide, CH_2Cl_2, 1 h, 75%.[16]

derivative **1** was obtained as described by Sproat et al.[18] from uridine. Oxidation of the 2′-allyloxy group by osmium tetroxide and *N*-methylmorpholine *N*-oxide resulted in a mixture (60:40) of diastereomeric diols. The portion of osmium tetroxide was increased in comparison to a previously published procedure, since cytosine derivatives are hardly oxidized to the 5,6-diol.[19] An allyloxycarbonyl protecting group (Alloc) was chosen to protect the 2′-diol. Application of allyl and allyloxycarbonyl groups in oligonucleotide synthesis to protect phosphates and heterocyclic bases was pioneered by Hayakawa et al.[20,21] Later Manoharan and co-workers[22] used Alloc for selective generation of an aliphatic amino group in oligonucleotides on solid-phase. Very recently, we have demonstrated successful unmasking of a carboxylic acid group on solid support based on allyl/Pd(0) chemistry.[23,24] Previously, it was shown that side modification of the 3-position of thymine or uracil by allyl chloroformate may take place.[25] The resultant derivative could not be deprotected by the $Pd(0)/Ph_3P$ system under various conditions. This was the underlying reason for selection of cytidine as the nucleoside. The TIPDS protecting group was removed by fluoride ion[26] and the standard transformations[27] of **2** were carried out to provide the building block **3**[28] suitable for automated solid-phase synthesis. The modified oligonucleotides were prepared by the phosphoramidite approach. For the phosphoramidite **3**, the coupling time was increased to 10 min and the coupling was repeated twice. Previously, *tert*-butyl hydroperoxide or 1-(*S*)-(+)-(10-camphorsulfonyl)-oxaziridine had been used for the P(III) oxidation in the case of allyl phosphoramidites or solid supports to prevent possible side-reactions with the standard iodine oxidizer.[20] In our hands, no side-reactions were observed if 0.02 M I_2 solution in THF/Py/H_2O (40:10:1) or 0.5 M 1-(*S*)-(+)-(10-camphorsulfonyl)oxaziridine in MeCN were used as oxidants. The sequences synthesized are shown in Table 1.

Table 1. Sequences and MALDI-TOF data of the 2′-modified oligonucleotides

No.	Oligonucleotide	MALDI-TOF MS calcd/found $[M+H]^+$
I	CT***C***CCAGGCTCA	3656.4/3658.1
II	CT***C***CCAGG***C***UCA	3746.5/3646.9

C—2′-*O*-(2,3-dihydroxypropyl)cytidine.

Deprotection of the 2′-diol group of the modified oligonucleotides was carried out under conditions similar to those reported by Manoharan and co-workers[22] (Scheme 2). However, in our case there was no need to heat the reaction mixture. This may be explained by the lower stability of the allyl carbonate linkage in comparison to allyl carbamate. The allyloxycarbonyl groups were also deprotected during standard ammonia treatment. Thus in effect, synthon **3** is a universal building block for both solid-phase and homogeneous preparation of 2′-aldehyde oligonucleotides, which is followed by their conjugation. Preliminary deprotection of phosphate groups by DBU treatment had no influence on the yields and time of the subsequent oxidation and conjugation, and therefore we avoided this additional step. Generation of the 2′-aldehyde group on solid-phase was carried out by use of sodium periodate in a DMSO—acetate buffer mixture.

Recently Lönnberg and co-workers[29,30] published a method for solid-phase conjugation of various aldehydes to immobilized and protected oligonucleotides bearing a free amino-oxy group. Here we adopted the reverse way of coupling. Initial experiments on conjugation of the immobilized 2′-aldehyde oligonucleotides with a number of hydrazides, hydrazines and amino-oxy compounds led to significant modification of heterocyclic bases (Fig. 1c). Use of 'ultra fast deprotection' 4-*tert*-butylphenoxyacetyl phosphoramidites still afforded about 10–15% of the base-modified oligonucleotide after 1 h treatment with 1000 M equiv of *O*-benzylhydroxylamine followed by standard ammonolysis. However, use of the appropriate salts, for example, hydrochloride, trifluoroacetate or even acetate of the amino component ensured conjugation without any traces of side-products even in the case of standard phosphoramidites. Figure 1b demonstrates that if methoxyamine is used as its hydrochloride, the reaction proceeds exclusively at the 2′-aldehyde group. Addition of triethylamine or pyridine (1000 mol equiv) into the reaction mixture leads to liberation of methoxyamine as its free base and formation of a complex reaction mixture (Fig. 1c). The oxime formed was stable under ammonia treatment for several days at ambient temperature. Under these conditions the 5′-dimethoxytrityl group is partly deprotected during the conjugation step, so a DMTr OFF mode of oligonucleotide assembly was used throughout. The same procedure was also applied for the