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An Efficient and Versatile Solid-Phase Synthesis of 5′- and 3′-Conjugated Oligonucleotides

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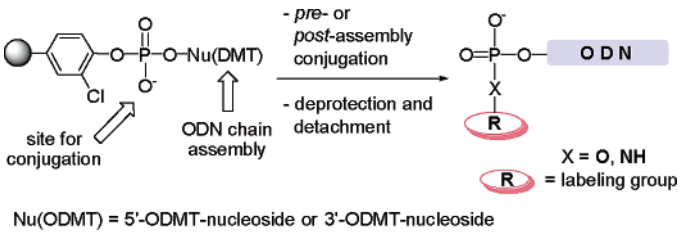
ABSTRACT



An easy and efficient solid-phase strategy to obtain 5′- and 3′-oligonucleotide conjugates in highly pure form has been developed. Ad hoc derivatized solid supports, to which the first nucleoside unit can be attached through a phosphate linkage, have been exploited both in a pre- and post-DNA assembly conjugation approach. A number of 5′- or 3′- oligonucleotide conjugates, incorporating a variety of labels covalently linked through a phosphodiester or a phosphoramidate bond, have been synthesized and characterized.

Modified and/or conjugated oligonucleotides are valuable tools as therapeutic candidates and as mechanistic and diagnostic probes.[1−3] For most in vitro and in vivo experiments, typically synthetic oligonucleotides have to be covalently linked at their 5′ or 3′-end with a variety of molecules, as for example fluorescent tags, intercalators, hydrophobic species, and peptides.

Current methods[4−7] for the synthesis of conjugates include the utilization of prefabricated labels, previously converted into the corresponding phosphoramidite or H-phosphonate derivatives, or of elaborate supports bearing an appropriate linker to incorporate the conjugating residue generally in a post-synthetic modification of the oligonucleotides. In both strategies, dedicated, tedious purifications (in the first approach, for the reactive phosphorylated derivatives of the labels; in the second one, for the preparation of the linker or in the very final step) are typically required to isolate in a pure form the desired conjugated molecule.

Aiming at a general concept for the solid-phase synthesis of oligonucleotide 3′- or 5′-conjugates by a strategy not requiring final HPLC purifications or cumbersome pre-derivatizations of the label to allow its incorporation in the solid support, we devised a straightforward and efficient synthetic protocol to prepare oligonucleotides covalently linked at their termini to a variety of molecules. These labels, in the form of alcohols or amines, can be directly loaded onto a "universal" solid support, obtained through a simple, one-step derivatization of the standard matrices, introducing a stable phosphodiester or phosphoramidate bond, respectively, between the OH-ends of the growing oligonucleotide and the conjugating residue. To demonstrate the feasibility of the here proposed method, both a pre- and post-assembly approach have been explored for the conjugation, adopting

† See Supporting Information

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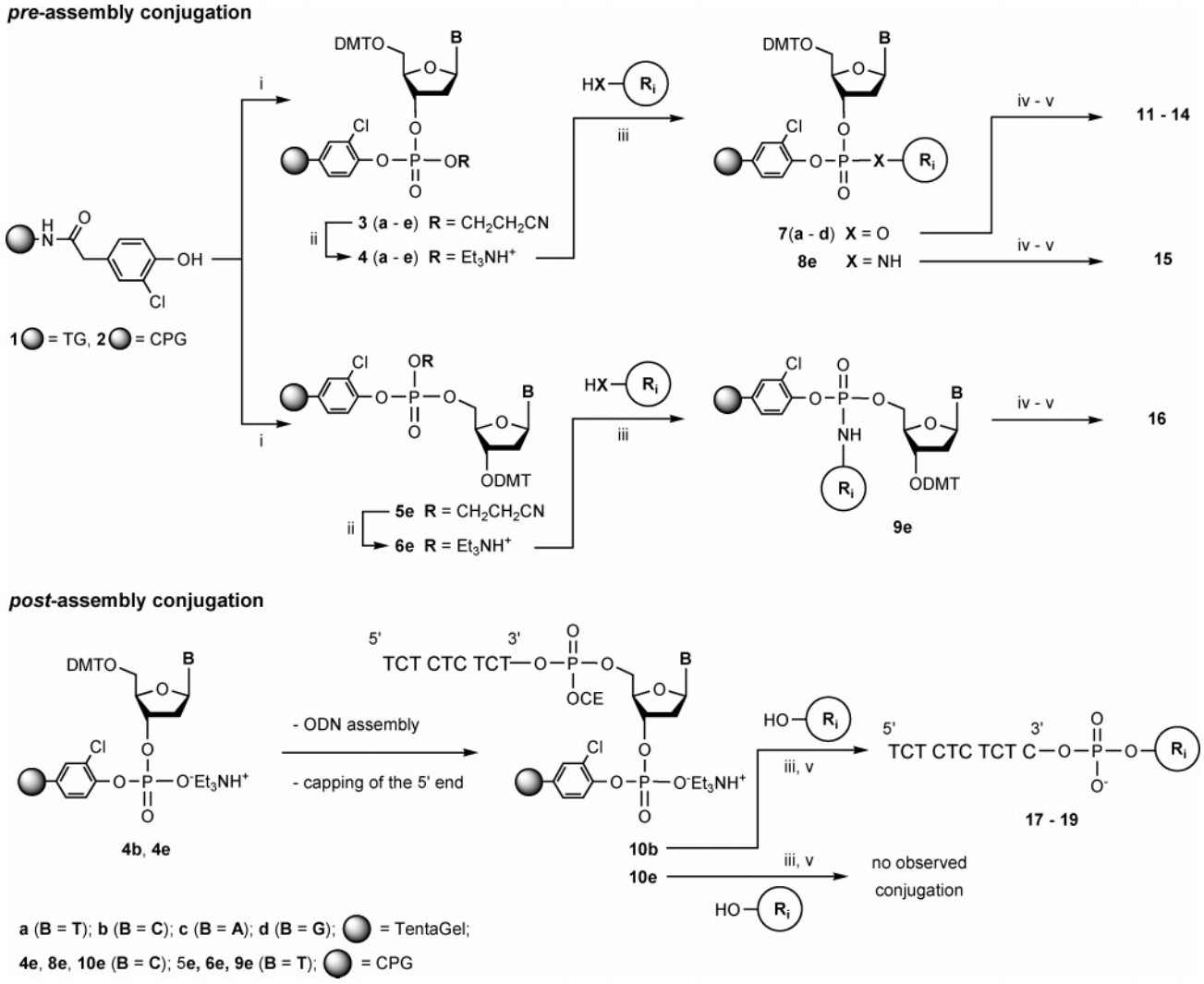
Scheme 1 [a]



[a] (i) Coupling with 3′- or 5′-nucleoside phosphoramidite; (ii) Et_3N/pyridine, 1 h, 50 °C; (iii) coupling by phosphotriester chemistry; (iv) ODN assembly; (v) deprotection and detachment (NH_4OH, 6 h, 55 °C).

both elongation directions (3′-5′ and 5′-3′) for the automated synthesis of the oligonucleotide chains. A list of molecules typically considered for conjugations (intercalators, hydrophobic residues, fluorescent tags, peptides, molecular carriers, etc.) have been efficiently tethered to model sequences. The basic point in our strategy was the derivatization of commonly used solid supports [TentaGel (0.29 mequiv/g) or LCAA-CPG (0.10 mequiv/g) amino supports] with 3-chloro-4-hydroxyphenylacetic acid,[8] leading to **1** or **2**, respectively (Scheme 1). Commercially available 3′- or 5′-phosphoramidite nucleosides were then efficiently loaded onto the functionalized solid supports by exploiting classical phosphoramidite chemistry, following a procedure recently developed for the solid-phase synthesis of 5′-phosphodiester and phosphoramidate monoester nucleoside analogues.[9] The conversion of the phosphite to phosphate triesters, affording supports **3a**−**e** and **5e**, was achieved by a standard oxidizing treatment with I_2 in pyridine/H_2O/THF. The incorporation of the nucleotide, as determined by quantitation of the DMT cation released from weighed amounts of the supports upon acidic treatment, was always in the range 0.19−0.22 mequiv/g starting from the TentaGel amino resin and 0.08−0.10 mequiv/g for the CPG support. Reaction with Et_3N/pyridine (1:1, v/v, 1 h, 50 °C) allowed the removal of the 2-cyanoethyl phosphate protecting group, giving supports **4a**−**e** and **6e**. No loss of the nucleotide loading was observed upon this treatment, as ascertained by control DMT tests on the supports. The diagnostic downfield shift (ca. 1.5 ppm) in the ^{31}P NMR spectra recorded on resins **4a**−**d** confirmed the total conversion of the phosphotriester to phosphodiester functions (see Supporting Information).

So obtained functionalized supports **4a**−**e** and **6e** contain two further reactive functional groups, i.e. the 5′- or 3′-hydroxy moiety on the nucleoside, transiently masked as

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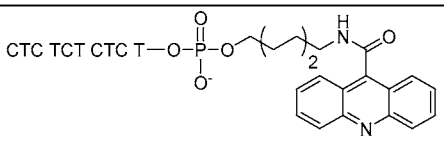
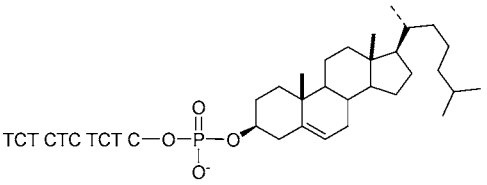
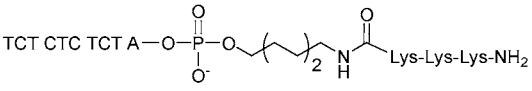
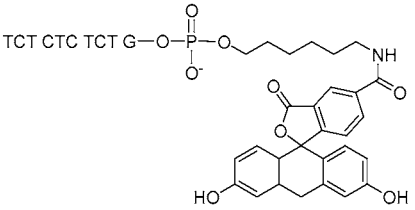
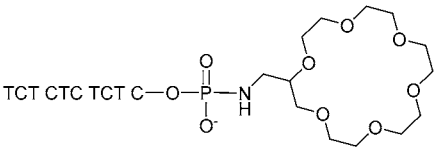
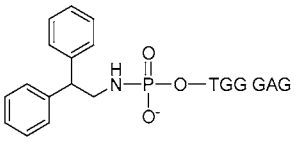
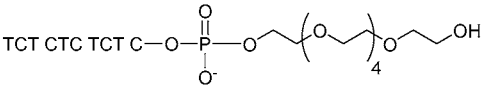
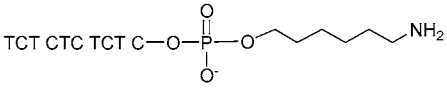
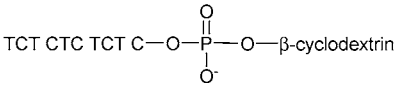
Table 1.

entry	starting support	modified oligonucleotide sequence 5' – 3'	MS obsd/ *calcd*	t_R, (min)	yield,[a] (%)
11	**7a**		3286.96 *3287.63*	18.35	41
12	**7b**		3351.32 *3351.82*	34.10	47
13	**7c**		3484.10 *3490.87*	13.81	31
14	**7d**		3484.44 *3480.63*	22.21	37
15	**8e**		3263.13 *3258.64*	15.83	50
16	**9e**		2133.22 *2130.43*	12.45	38
17	**10b**		3248.69 *3247.63*	18.14	25
18	**10b**		3099.42 *3096.59*	17.35	29
19	**10b**		not detected *4099.83*	18.49	27

[a] Yields calculated on the pure oligonucleotide after gel filtration, with respect to the starting functionalization of the supports.

DMT ether, and the phosphodiester function, correspondingly at the 3′- or 5′-position. These can be exploited, respectively, for the automated DNA chain assembly and for the conjugation with the chosen labels, both in a pre- or post-DNA assembly approach.

In the pre-assembly approach, the functionalized solid supports incorporating the label are prepared by two different methods, both exploiting classical phosphotriester chemistry. In the first strategy, **4a**−**e** were reacted with the chosen alcohol (see Supporting Information) in pyridine in the presence of 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) at room temperature for 12 h. The conjugation efficiency was always in the range 75−80% (0.15−0.18 mequiv/g), except in the case of CPG support **4e**, which gave sensibly lower yields (≤10%). These yields could be indirectly evaluated by DMT tests on weighed samples of the support after ammonia treatment, determining the unconjugated material left onto the solid support. In fact, only the nucleoside linked to the support through a phosphotriester linkage (**7** and **8**) is easily removed upon basic treatment (28% NH_4OH, 50 °C, 5 h), whereas the nucleoside anchored through a phosphodiester bond (**4** and **6**) is not cleaved from the resin under the same conditions. To prepare phosphoramidate supports **8e** and **9e** (Scheme 1), supports **4e** and **6e** were first activated with *p*-tosyl chloride in pyridine for 15 min and, after appropriate washing steps, reacted with

the suitable amine dissolved in pyridine for 15 min.[10] This procedure was iterated three times, and the coupling yields, evaluated by the DMT test on weighed samples of the support after the ammonia treatment, were always in the range 65−80% (0.05−0.08 mequiv/g). Starting from supports **7a**−**d** and **8e**, a 10-mer oligodeoxyribonucleotide (Table 1) was assembled by standard phosphoramidite synthesis (DMT off) in the 3′-5′ direction. Similarly, starting from support **9e**, a 6-mer was synthesized in the 5′-3′ direction. After ammonia cleavage and deprotection (6 h, 50 °C), ion exchange HPLC analyses of the released oligonucleotides showed in all cases a single product corresponding to desired **11**−**16**. This high purity can be obtained because only the oligonucleotide linked to the support through a phosphotriester or phosphoramidate diester bond is cleaved from the resin upon ammonia treatment, whereas the oligonucleotide anchored through a phosphodiester bond, i.e., the unreacted oligonucleotide chain, is not affected under the same conditions. The crude oligonucleotides were then purified by gel filtration chromatography on a Sephadex G25 column eluted with H_2O/EtOH 4:1 (v/v). The purity of the isolated compounds was then checked by HPLC, and their identity was ascertained by MALDI-TOF MS analysis. In a typical experiment, starting from 50 mg of functionalized supports **7a**−**d**, with an average 0.15 mequiv/g incorporation of the conjugating residue, 150−200 OD units of pure oligonucleotides **11**−**14** could be isolated after gel filtration. In the case of supports **8e** and **9e**, bearing an initial nucleotide loading of 0.08 mequiv/g, 80−100 OD units of pure **15** and **16** on average could be recovered starting from 50 mg of functionalized solid support.

In the post-assembly approach, starting from supports **4b** and **4e**, a 10-mer was assembled by standard automated DNA synthesis leading to **10b** and **10e**. The 5′-OH end of the oligonucleotide was then capped by treatment with acetic anhydride in pyridine (1:1, v/v, rt, 30 min). To test the possibility to prepare conjugated oligonucleotides via a post-assembly approach, which can be useful in the case of conjugating residues labile to the treatments classically required for the standard oligonucleotide chain elongation, supports **10b** and **10e** were reacted with DMTO-hexaethyleneglycol-OH or MMTNH-CH_2-$(CH_2)_4$-CH_2-OH, as flexible spacers allowing further terminal derivatizations, in the presence of MSNT in pyridine at room temperature for 12 h. The incorporation yields, as directly determined by DMT or MMT cations tests, were acceptable in the case of **10b** (always above 60%), whereas sensibly lower incorporation yields (≤5%) were observed starting from **10e**. Repeated experiments with different alcohols confirmed that MSNT activation of the phosphodiester functions was not operative on CPG supports. On the contrary, always satisfactory yields for the incorporation of the conjugating residues were observed on Tentagel resins. Further optimization can in principle be achieved by reiterating the coupling procedure with the alcohol and MSNT. After ammonia cleavage and deprotection, HPLC analysis showed also for **17**−**19** a single product corresponding to the desired conjugated oligonucleotides, confirming the high purity of the isolated compounds. Their identity was ascertained by MALDI-TOF mass spectrometry, except in the case of **19**, for which MALDI analysis failed to give the expected peak, presumably because of the incompatibility of cyclodextrins with the matrices and conditions typically used to reveal oligonucleotides. However, the structure of **19** was confirmed by ^{1}H NMR analysis. By incorporating bifunctional amino or hydroxy linkers at the phosphodiester reactive site of **10b**, the potential of the here reported strategy can be significantly expanded, allowing the derivatization of oligonucleotides not only with nucleophiles but also with labels presenting electrophilic groups, such as, for example, carboxylic acids.

In conclusion, we have described an easy and versatile solid-phase method for the preparation of oligonucleotides, tethered to a variety of labels through stable phosphodiester or phosphoramidate linkages. By a one-step, "universal" derivatization of the solid matrices, 3′- or 5′-phosphoramidite nucleosides were efficiently loaded on the supports so that either a pre- or post-DNA assembly conjugation approach, based on standard phosphotriester chemistry, could be successfully exploited. Model conjugated oligonucleotides were synthesized in 3−5 mg amounts and characterized. The method allows the release in solution of the target compounds in a very pure form, requiring simple gel filtration chromatography for the final purification.

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Supporting Information Available: ^{31}P NMR data for **4**(**a**−**d**); HPLC profile for **15**; MALDI specta for **11**−**18**; low field region of the ^{1}H NMR spectrum for **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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