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A New Route to Oligodeoxynucleoside Phosphoramidates (P-NH_2)

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Abstract: We describe a new strategy to introduce phosphoramidate (P-NH_2) internucleosidic linkages in oligonucleoside phosphodiesters using dimer building blocks. The P-NH_2 function is generated under photolysis of the N-2-nitrobenzylphosphoramidate linkage (P-NHR). We achieved the synthesis of N-2-nitrobenzylphosphoramidate β and α-anomeric nucleotides dimers *via* either phosphoramidite or H-phosphonate chemistry. α-dimer (P-NHR) was incorporated at defined positions into α-oligonucleoside phosphodiesters and the P-NH_2 linkage was liberated by U.V irradiation.

Recently, our group reported the first synthesis of acid- and base-sensitive oligonucleoside phosphoramidates (P-NH_2)[1]. This synthesis was achieved with a labile oxalyl anchored solid support in combination with the use of *N-t*-butylphenoxyacetyl protected synthons and of mild deprotection conditions. At the same time Iyer and al. described the synthesis of dimers, trimers and mixed backbone oligos containing (P-NH_2) linkages in using *N*-pent-4-enoyl protected nucleoside H-phosphonates[2,3]. These non-ionic oligonucleotide analogs are strongly nuclease-resistant and are able to form hybrids with complementary DNA and RNA strands although the resulting duplexes are significantly less stable than those of the wild type species, especially with RNA targets. In contrast, we showed that the unnatural α-anomeric dodecathymidine P-NH_2 exhibited a higher affinity for both poly dA and poly rA targets than its β-anomeric homolog and others backbone-modified α and β-oligonucleotides[4]. These attractive data prompted us to extend this work to α-oligonucleoside phosphoramidates containing the four nucleotides and to develop a new strategy for the preparation of these base-sensitive oligonucleotides.

In our current strategy, we prefered to use synthons protected by common nucleobase *N*-acyl groups specially for α-synthons which are easily available following described procedure[5]. The P-NH_2 function is generated under photolysis of the N-2-nitrobenzylphosphoramidate linkage (P-NHR) which is expected to be stable in acidic and basic conditions applied during the synthesis and deprotection of oligonucleotides[6]. Synthesis of N-2-nitrobenzylphosphoramidate β- and α-anomeric dimers was performed by using either phosphoramidite or H-phosphonate chemistry. To make this synthetic methodology applicable for oligonucleoside phosphodiesters containing some phosphoramidate linkages and in order to ascertain that the P-NH_2 linkage is generated under photolysis, we incorporated N-2-nitrobenzyl phosphoramidate α-dinucleotide at defined positions into an α-anomeric oligonucleoside phosphodiester.

Synthesis of N-2-nitrobenzyl-phosphoramidate β-anomeric dinucleoside (β-dA*pn-NBz*dC) **1** (Scheme 1) was undertaken to settle the deprotection conditions of amidate function. The fully protected dimer **2** was obtained with 90% yield by coupling the 3'-methylphosphoramidite **3** and 5'-OH synthon **4** in the presence of tetrazole, followed by oxidation of the intermediate methyl phosphite triester with iodine in the presence of 2-nitrobenzylamine[7,8]. Cleavage of the TBDMS group by treatment with 1.1M tetrabutylammmonium fluoride (TBAF) afforded the dimer **5**. Exocyclic amino functions of the nucleobase were deprotected by saturated

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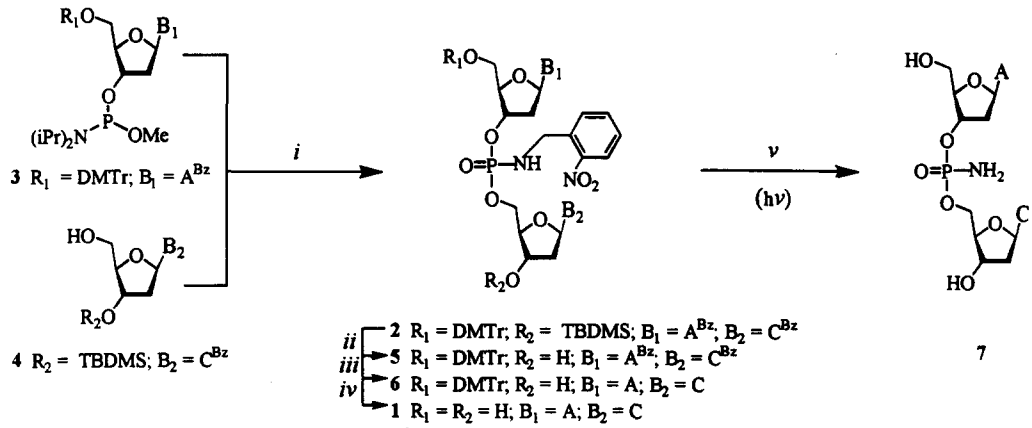
methanolic ammonia to give the tritylated dimer **6**. Finally the DMTr group was removed by a 80% aqueous acetic acid treatment to yield the β-dimer **1**[9](58% overall yield).



Reagents and conditions: *i*: a) tetrazole/CH_3CN, 30 min. b) I_2/ 2-nitrobenzylamine/THF, 5 min. *ii*: 1.1 M TBAF/THF, 1h. *iii*: conc. NH_3 in MeOH, 16 h. *iv*: AcOH-H_2O (80:20 v/v), 30 min. *v*: 1 mM **1** in 0.1M TEAAc (pH7) / CH_3CN (70/30) and 10 mM dithiothreitol, Pyrex filtered U.V irradiation for 10 min. at 20°C.

Scheme 1

The photolysis of **1** (1mM) was carried out in a stirred solution of 0.1M triethylammonium acetate buffer (pH 7) and acetonitrile (70/30, v/v) in the presence of 10 mM dithiothreitol (DTT)[10] which is required to minimize the formation of phosphodiester linkage. In the absence of DTT we noted up to 24% of phosphodiester. The solution purged with argon[11] was irradiated with the Pyrex filtered output of a 125W high-pressure Hg lamp for 10 minutes at room temperature. Several extractions of the crude mixture with diethyl ether and ethyl acetate were necessary to remove side products. Analysis of the crude material by HPLC (Figure 1) indicated a total transformation of the dimer **1** in the desired (P-NH_2) dimer **7**[12] with two diastereoisomers integrating to more than 90% and the presence of the corresponding dinucleoside diester (βApC) integrating to less than 3%.

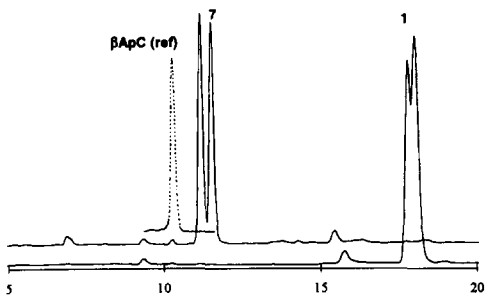
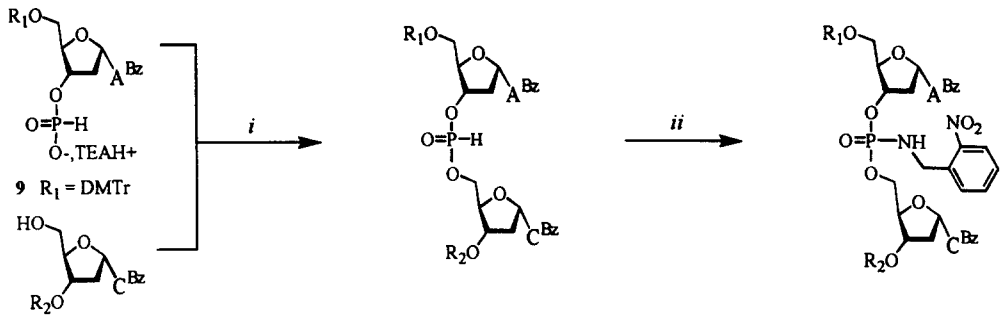


Figure 1: HPLC analysis of dimer **1** with N-2-nitrobenzyl-phosphoramidate linkage (lower profile) and dimer **7** with PNH_2 linkage (upper profile). The dashed profile corresponds to βApC dimer with POO^- linkage.

Alternatively to phosphoramidite chemistry, H-phosphonate methodology can be used to prepare oligonucleoside phosphoramidates [6]. Synthesis of N-2-nitrobenzylphosphoramidate α-anomeric dinucleoside was achieved with this method. Precursor dimer H-phosphonate **8** was synthesized by coupling requisite 3'-H-phosphonate[13] **9** and synthon **10** in the presence of pivaloyl chloride[14](50% yield) (Scheme 2). The amidate linkage of dimer **11**[15] was generated by oxidation with CCl_4 in the presence of 2-nitrobenzylamine (80% yield). After removal of TBDMS group with TBAF, the dimer **12** was converted to the corresponding phosphoramidite derivative **13**[16] with bis-(diisopropylamino)-2-cyanoethoxy-phosphine.



Reagents and conditions: *i*: Pivaloyl chloride/pyridine, 30 min. *ii*: 2-nitrobenzylamine hydrochloride/triethylamine/CCl_4/pyridine. 3 h. *iii*: TBAF/THF, 30 min. *iv*: $P[O(CH_2)_2CN][(i\text{-}Pr)_2N]_2$/diisopropylammonium tetrazolide/CH_2Cl_2, 20h.

Scheme 2

The αdA*pn-NBz*dC dimer 3'-phosphoramidite **13** was incorporated once (oligo **14**) or twice (oligo **15**) in the oligonucleotide sequence $^{5'}$α-(TCTTAACCCACA) complementary to the splice acceptor site of mRNA coding for HIV-1 *tat* protein via the standard phosphoramidite chemistry (1μmole scale). Although the coupling step of **13** was increased to 3 minutes and repeated 3 times, low coupling efficiency (76%) was obtained as indicated by the DMTr cation dosage. As described in literature[17], this poor yield can be explained by water content of the sample. Both oligos were deprotected in standard conditions (aqueous 30% ammmonia 5 hr, 55°C). HPLC profiles (Figure 2) revealed no cleavage of the photolabile group on the phosphoramidate linkage during ammonia treatment. The oligos were then purified by RP-HPLC to remove failure sequences. HPLC analysis indicated two peaks corresponding to the diastereoisomers for oligo **14** (95% purity).

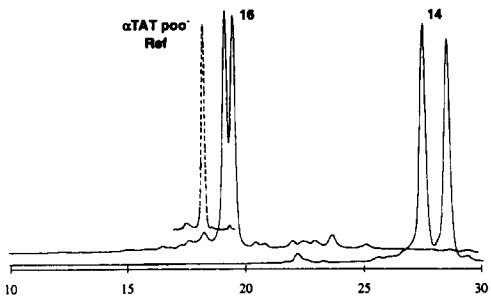


Figure 2: HPLC profile before photolysis (oligo **14** with one αdA*pn-NBz*dC linkage) and after photolysis (oligo **16** with one PNH_2 linkage) The dashed profile corresponds to α-*tat* oligonucleoside phosphodiester.

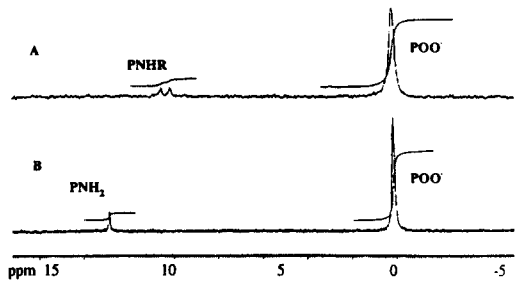


Figure 3: ^{31}P-NMR spectrum of **14** (A) before photolysis and after photolysis (**16**) (B).

The ^{31}P-NMR spectrum of oligo **14** in Figure 3A showed two signals at 10.1 and 10.5 ppm for internucleotide 2-nitrobenzylphosphoramidate linkage (P-NHR) and one signal at 0 ppm (phosphodiester groups POO^-) and the ratio of the areas for POO^- / PNHR was 10/1. The oligo **14** was subjected to photolysis following the same procedure as described above to yield the expected α-phosphodiester oligo **16** (α-5'-TCTTA<u>AC</u>CCACA) containing one P-NH_2 linkage. HPLC analysis of the crude material **16** (Figure 2) showed two peaks with retention time lower than those of oligo **14**. Oligo **16** was purified by HPLC and characterized

by ^{31}P-NMR (one signal at 12.9 ppm for the P-NH_2 linkage and one signal at 0 ppm for POO^- linkages in the expected ratio 1 to 10)(Figure 3B) and by mass spectrometry[18]. As for compound **14**, oligonucleotide **15** containing two αd*A*p*n*-*NBz*dC linkages was irradiated to give the desired oligo **17** (α-5'-TCTTA**AC**CC**AC**A) with two P-NH_2 linkages. This oligo **17** was characterized (data not shown) in the same manner as **16**.

In conclusion, we developped a new strategy to prepare oligonucleoside phosphodiesters containing some phosphoramidate linkages introduced with dimer blocks. The phosphoramidate P-NH_2 function was generated after photocleavage of the 2-nitrobenzyl group which is completely compatible with synthesis and standard deprotection conditions of oligonucleotides. Synthesis of fully or partially modified α-oligonucleoside phosphoramidates by using H-phosphonate chemistry is currently under way in order to study their hybridization properties with their targets.

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16. Analytical data for **13** (4 diastereoisomers): ^{31}P-NMR ($CDCl_3$) δ: 9.5 and 9.72 ppm (phosphoramidate groups) and 150.26, 150.38, 150.85 and 151 ppm (phosphoramidite groups). The ratio for the integrated areas for phosphoramidite/phosphoramidate was 1/1. MS (FAB+/NBA): m/z 1385 MH^+
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