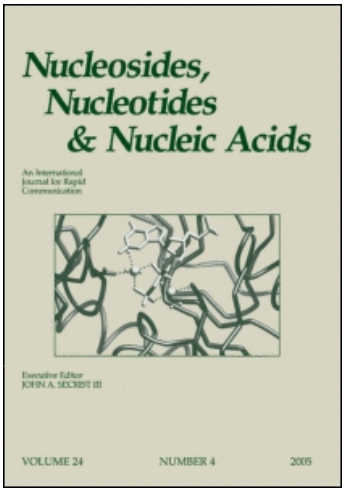

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NEW APPROACH TO THE SOLID PHASE SYNTHESIS OF N3'→P5' PHOSPHORAMIDATE OLIGONUCLEOTIDES

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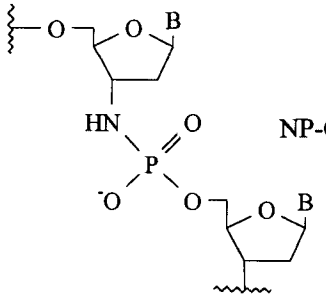
ABSTRACT: LCA-CPG-nucleoside 5'-O-(O-β-cyanoethyl-H-phosphonates) react with 3'-amino-2',3'-dideoxynucleoside in the presence of iodine giving in a high yield N3'→P5' phosphoramidate oligonucleotides.

INTRODUCTION

The chemical synthesis of oligonucleotides bearing modifications at the phosphodiester internucleotide linkages to increase their resistance towards nucleases is a continuous goal of numerous laboratories. In oligonucleotides the substitution of a nonbridging oxygen atom for example by methyl group or a sulfur atom creates, due to stereogenicity of phosphorus atom, new centres of asymmetry, which in consequence results in formation of a mixture of unresolvable diastereomers having variable biophysical, biochemical and biological properties. On the other hand modification at a bridging oxygen atom gives rise to analogs which like natural DNA are achiral at phosphorus. Bridged phosphoramidate analogues of oligonucleotides represents the latter group. A first chemical synthesis of dinucleotide phosphoramidates containing P3'→N5' linkage was reported independently by Letsinger[1] and Hata[2] by coupling 5'-azido-5'-deoxythymidine with thymidine 3'-phosphite intermediates. Also Shabarova reported the synthesis of such modified dinucleotide monophosphates[3] by the Atherton-Todd coupling procedure[4]. In 1988 Bannwarth using amidite method prepared oligonucleotides bearing P3'→N5' phosphoramidate linkages placed into specific position within a given DNA fragment[5]. However, Letsinger in 1992 demonstrated that introduction of a single P3'→N5'linkage in the oligomer backbone has little effect on the hybridization

properties, while phosphoramidates with reversed orientation (i.e. N3'→P5') have enhanced avidity towards complementary DNA or RNA[6].

Recently the synthesis and biophysical characteristics of N3'→P5'phosphoramidate oligonucleotides (NP-ODN, **I**) wherein every 3'-oxygen of 2'-deoxyribose is replaced by a 3'-amino function have been described by Gryaznov.[7-9] These analogues form highly stable, sequence-specific complexes with single-stranded DNA or RNA,[8,9] which makes them attractive candidates for application in the antisense[10] and rybozyme[11] strategies. Moreover, high avidity of NP-ODN towards double-stranded DNA promotes them as promising tools in the antigene strategy.[12] These compounds were prepared by coupling of appropriately protected 3'-amino-2',3'-dideoxynucleosides with nucleosi(ti)de bound to the solid support. The coupling procedure was preceded by *in situ* H-phosphonylation of the 5'-OH function of anchored nucleoside, followed by its activation (CCl_4/triethylamine) according to the Atherton-Todd procedure.[4]



NP-ODN - **I**

Since in the procedure described by Gryaznov the single coupling step is very long (60 min) we have begun search for improved method of oxidative phosphorylation of primary amines. Our attention has been focused on the use of iodine as an activator of that process. In 1980 Ogilvie *et al.* described the iodine-promoted phosphorylation of amines by internucleotide O-alkyl phosphites[13]. Then oligo(nucleoside N-alkylphosphoramidate)s were obtained by Froehler[14] and Jager *et al.*[15] in the reaction of iodine-activated oligo(nucleoside H-phosphonate)s with primary amines. The use of iodine as an activator of H-phosphorothioylation of nucleosides has been also reported by Caruthers *et al.*[16], and high efficiency of the process of amine phosphorylation and P-N bond formation using H-phosphonates activated by iodine is presented in recent work by Stawiński *et al.*[17]

In this paper we describe the solid phase synthesis of N3'→P5' phosphoramidate oligonucleotides based on the use of iodine for activation of dialkyl H-phosphonates and their reaction with protected 3'-amino-2',3'-dideoxynucleosides.

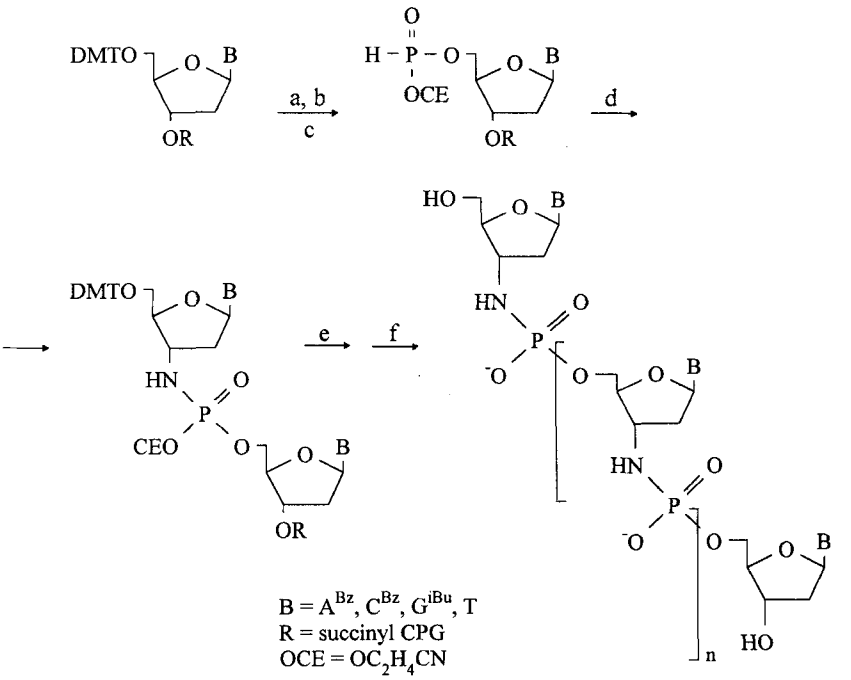
RESULTS AND DISCUSSION

In the presented approach the synthesis of N3'→P5' phosphoramidate oligonucleotides was started by reaction of bis(N,N-diisopropyl)-2-cyanoethyl phosphorodiamidite with 5'-OH group of nucleoside immobilized to the solid support (reaction b, Scheme 1).

Resulting anchored nucleoside 5'-O(O-β-cyanoethyl-N,N-diisopropyl phosphoramidite) was hydrolyzed in the presence of tetrazole (reaction c, Scheme 1) generating support-bound nucleoside 5'-O-(O-β-cyanoethyl H-phosphonate). The coupling step with 5'-O-DMT-3'-amino-2',3'-dideoxynucleoside (reaction d, Scheme 1) was performed in acetonitrile in the presence of iodine, followed by washing with acetonitrile and capping under standard conditions with acetic anhydride/DMAP.

Using the elongation procedure presented in Scheme 1 several tri-, tetra-, octa-, and dodeca(deoxyribonucleotide N3'→P5' phosphoramidate)s were obtained (Table 1) after final detritylation and ammoniacal cleavage from the support followed by nucleobase deprotection. As emphasized by Gryaznov,[7] DMT-ON purification step, typically used for removal of truncated sequences, is not recommended for purification of compounds of type **I**, because ammoniolytic removal of the cyanoethyl protecting groups accompaning cleavage from the support renders NP-ODNs unstable to the acidic detritylation conditions.

Therefore, compounds presented in Table 1 were purified by RP-HPLC on DMT-OFF stage. The identity of compounds **Ia-c** was confirmed by FAB-MS analysis while for **Id-e** ESI-MS analysis were performed [**Id** (triethylammonium salt) m/z 2365 and **Ie** (triethylammonium salt) m/z 3577]. All syntheses were performed manually by syringe-technique. Average coupling yields, calculated from the DMT cation assay, were in the range 92-96% for A,C,T-bases and 87% for G-base. This coupling efficiency was achieved with single delivery of the monomer to the column. We would like to emphasize the advantage of iodine - promoted coupling step, expressed in its essential shortening (30-fold) compared to the procedure described by Gryaznov[7].



SCHEME 1. a) $CHCl_2COOH$; b) $P(N\text{-}iPr_2)_2(OC_2H_4CN)$, *1-H*-Tetrazole; c) *1-H*-Tetrazole, H_2O; d) 5'-O-DMT-3'-NH_2-nucleoside, I_2; e) repetition of steps a-d; f) NH_4OH

TABLE 1. N3'→P5' Phosphoramidate Oligonucleotides Obtained According to Scheme 1.

	Oligonucleotide **I**	OD units	Product purity [%]
a)	$d(G_{PN})_2T$	5.3	65.8
b)	$d(C_{PN})_3C$	9.9	75.1
c)	$d(A_{PN})_3T$	5.8	72.0
d)	$d(T_{PN})_7T$	13.1	77.0
e)	$d(T_{PN})_{11}T$	12.0	50.0

All syntheses were performed on 1 μmol scale, product purity measured by RP-HPLC.

Iodine-activation process most probably involves corresponding phosphoro-iodidates and their reactions with 3'-aminonucleosides. This process is most probably faster than that of phosphorochloridates, suggested as reactive intermediates in the Atherton-Todd phosphorylation.[4] Studies on the improvement of coupling yield of 3'-amino-2',3'-dideoxyguanosine are in progress. However, recently published results may indicate, that H-phosphonate oxidative phosphorylation is less suitable for synthesis of mixed-sequence N3'-P5' oligonucleotides than P^{III}-transamidation process developed by Fearon *et al.*[18]

EXPERIMENTAL

5'-O-DMT-nucleoside-3'-succinyl LCA CPG support,[19] 5'-O-DMT- and base-protected 3'-amino-2',3'-dideoxynucleosides,[7] bis(N,N-diisopropyl)-2-cyanoethyl phosphorodiamidite,[20] were prepared according to published procedures. Acetonitrile and methylene chloride (Baker) were refluxed over CaH_2 and distilled just prior to use. Reverse phase (RP) HPLC was carried out on a Supelco LC-18 (5 μm 2.1 x 250 mm) column with the following eluent systems: A) 0.05 M TEAB pH 7.5; B) 0.05 M TEAB in 40% CH_3CN; gradient 0 to 100% B over 35 min, flow 0.3 ml/min.

Single cycle for the synthesis of NP-ODNs

Reactions were carried out manually by the syringe technique using LCA-CPG support loaded with 1 μmol of nucleoside. A single cycle of chain elongation was as follows:

1) detritylation, 3% dichloroacetic acid in methylene chloride (5 ml)
2) wash, acetonitrile (5 ml)
3) phosphitylation, 0.3 M bis(N,N-diisopropyl)2-cyanoethyl phosphorodiamidite and 0.3 M 1-H tetrazole solution in acetonitrile (20-fold molar excess; 15 min)
4) wash, acetonitrile (5 ml)
5) hydrolysis of nucleoside 5'-O-(O-β-cyanoethyl-N,N-diisopropyl phosphoramidite) with 0.5 M 1-H tetrazole in the mixture acetonitrile: water (9:1, 0.15 ml; 4 min)
6) wash, acetonitrile (5 ml)
7) coupling, 0.2 M acetonitrile solution of 5'-O-DMT-3'-amino-2',3'-dideoxynucleoside (20-fold molar excess) and 0.01 M iodine solution in acetonitrile (2-fold molar excess of iodine; 2 min)

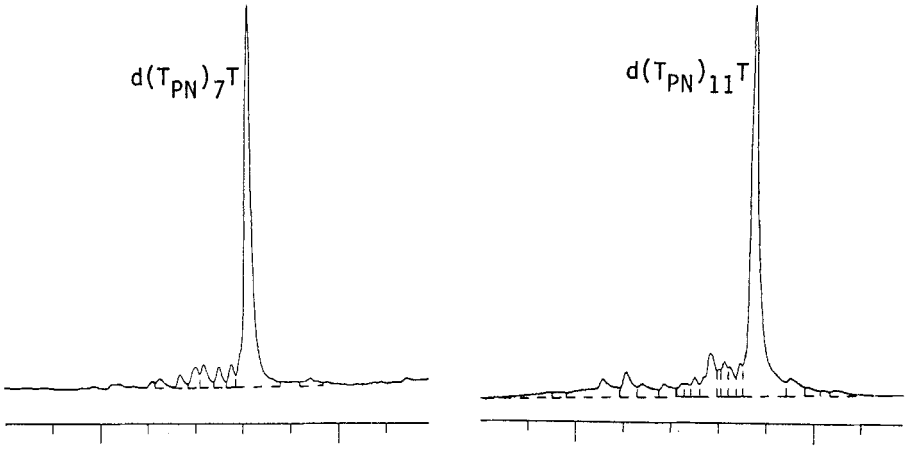


FIGURE 1. RP-HPLC Chromatograms of crude N3'→P5' phosphoramidate oligonucleotides.

8) wash, acetonitrile (2 ml), methylene chloride (2 ml) and acetonitrile (3 ml)

9) capping, acetic anhydride/DMAP/2,6-lutidine/tetrahydrofuran (0.15 ml; 2 min)

10) wash, acetonitrile (5 ml).

The cleavage from the support and nucleobase-deprotection were performed by means of ammonia at 55°C for 8 h.

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