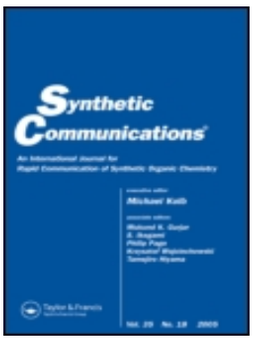

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SOLID PHASE OLIGONUCLEOTIDE SYNTHESIS BY USING β-CE *H*-PHOSPHONATE APPROACH

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ABSTRACT

A new approach to the solid phase synthesis of oligonucleotide is described, which is based on oxidative coupling of nucleoside 3′-β-CE *H*-phosphonates in presence of *N*-Bromosuccinimide (NBS) as a coupling agent.

In the recent years the antisense oligonucleotides have been proposed as a major class of new pharmaceuticals. The need for the production of oligonucleotides has thus led to the investigation of alternative synthetic strategies aimed at increased purity and better yields. Today, most of the antisense oligonucleotides are synthesised either by the phosphoramidite approach[1] or *H*-phosphonate,[2] the condensation and oxidation being the key steps involved in the synthesis.

Despite of the efficiency of the DNA synthesis, impurities inevitably accumulate, the synthesis of a 20 mer oligonucleotide involves more than 100 separate chemical steps, each with a finite possibility of producing

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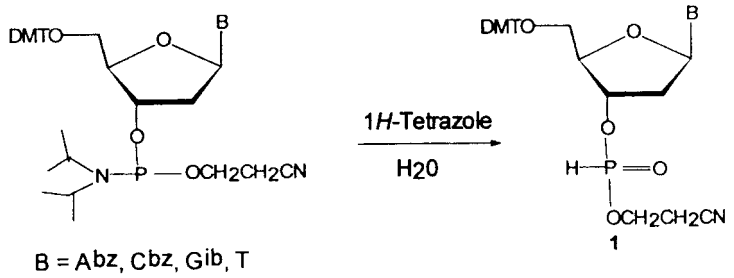
an undesirable side reaction.[3] Thus the development of any alternative strategy which would decrease the number of synthetic steps, without hampering the yields and purity of the synthesis would itself justify the effort.

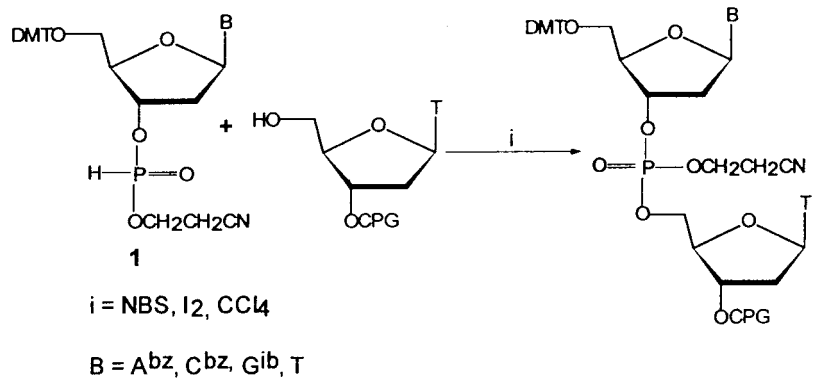
In our previous reports we have described the solution phase oligonucleotide synthesis by using Alkyl *H*-phosphonates.[4] During the synthesis a general drawback was observed that there was about 1% weight reduction of the total amount due to the repeated precipitation and filtration steps, resulted in the low yield of the oligonucleotides. Therefore we have exploited the application of the nucleoside 3′-β-CE *H*-phosphonate **1**, as versatile starting material for the solid phase oligonucleotide synthesis via oxidative coupling method. The nucleoside-β-CE *H*-phosphonates were prepared conveniently from corresponding phosphoramidites (Scheme 1).



Scheme 1.

Although the synthon **1** has been reported to be completely resistant to activation in presence of strong condensing agents,[5] it forms sufficiently reactive intermediate in presence of activating agents such as iodine or NBS and undergoes oxidative coupling with the free hydroxy group of the support bound nucleoside (Scheme 2).

To set up a standard synthetic protocol for solid phase synthesis of oligonucleotides using β-CE-*H*-phosphonates, the effect of different coupling agents, solvent and bases on coupling efficiency were studied by preparing a dimer d(TT), a coupling time of 2 min being given in each case. The results are summarized in Table 1. From all these studies a standard synthetic protocol for solid phase synthesis of oligonucleotide, as shown in Table 2, was set up. To study the effect of type of nucleoside base on coupling efficiency all the four dimers d(AT), d(GT), d(CT) and d(TT) were prepared using 0.4 M NBS in CH_3CN/TEA (4 : 1, v/v) as coupling agent, contact time with the support being 2 min. The results are compiled in Table 3.



Scheme 2.

Table 1. Effect of Type of Coupling Agent, Solvent, and Base Used for the Solid Phase Synthesis of d(TT)

Sl. No.	Coupling System	% Coupling Efficiency
1	CCl_4/Py (4 : 1 v/v)	68
2	CCl_4/CH_3CN/Py (1 : 4 : 1 v/v)	70
3	CCl_4/CH_3CN/TEA (1 : 4 : 1 v/v)	70
4	I_2, 0.4 M in CH_2Cl_2/Py (4 : 1 v/v)	78
5	I_2, 0.4 M in CH_3CN/Py (4 : 1 v/v)	82
6	I_2, 0.4 M in CH_3CN/Py (4 : 1 v/v)	89
7	NBS, 0.4 M in CH_2Cl_2/Py (4 : 1 v/v)	91
8	NBS, 0.4 M in CH_3CN/Py (4 : 1 v/v)	96
9	NBS, 0.4 M in CH_3CN/TEA (4 : 1 v/v)	98

The UV, ^{1}H-NMR and HPLC analysis of these product indicated that the heterocyclic moiety and all protecting group (DMTr, –COR and $–CH_2CH_2CN$) remains unaffected during the synthetic cycle. Additionally, the RP HPLC profile of the product matches with that of the respective authentic sample prepared by the solid phase phosphoramidite method.

The overall procedure for oligonucleotide assembly is represented by the synthesis of d(AATTTTCAGAATTG), starting from dG-lcaa-CPG-500A (0.5 μmol scale, loading 40 μmol/gm). The trityl assay confirmed an average coupling efficiency of 98%. Finally conventional deprotection, (conc. aq NH_3, 55°C, 16 h) cleavage and purification by using a NAP-10

Table 2. Oligonucleotide Synthetic Cycle

Sl. No.	Operation	Reagent	Time in Min
1	Washing	CH_3CN	0.5
2	Detritylation	3% $Cl_2CHCOOH$-CH_2Cl_2	1.8
3	Coupling	0.1 M β CE-*H*-phosphonate-CH_3CN + 0.4 M NBS-CH_3CN/TEA (4 : 1, v/v)	2.0
4	Washing	CH_3CN	0.2
5	Capping	Ac_2O/2,6-lutidine/THF (1 : 1 : 2) + 6.5% DMAP-THF	0.4
6	Washing	CH_3CN	0.5

Table 3. Effect of the Nucleoside Bases on Coupling Efficiency

Sl. No.	Dimer	% Coupling Efficiency
1	d(AT)	98.2
2	d(CT)	98.0
3	d(GT)	98.5
4	d(TT)	98.6

column, afforded the oligo 92% homogeneous by HPLC. The overall yield of the synthesis was 77%. The above synthesised oligonucleotide was characterised by comparing the PAGE and RP HPLC retention time with that of authentic sample having the same sequence synthesised by the phosphoramidite approach. The enzyme artifact (snake venom phosphodiesterase and calf intestine alkaline phosphatase) showed no base modification or wrong linkages, as the oligomer afforded dA, dC, dG and dT in the predicted amounts.

The results outline a method whereby nucleoside β-CE *H*-Phosphonate can be used as synthons for the solid phase synthesis of oligonucleotides. The synthons are easily prepared via a readily scaleable procedure[5] and are stable towards normal laboratory conditions, a particularly useful feature being the activation with NBS or I_2 to directly yields the dinucleoside phosphate as the only detectable product (^{31}P NMR δ −0.157 ppm).

Although the oxidative coupling of nucleoside synthons has been carried out previously,[6] the described approach is unique and adds to the

arsenal for the preparation of oligonucleotides and oligonucleotide analogs. In conclusion, the described oxidative coupling reaction in the presence of NBS as the activating agent is fast, with good yield of coupling as well as purity in the solid phase synthesis of oligonucleotide. Moreover, the method does not require a separate oxidation step, hence less time consuming. The use of an inexpensive activating agent like NBS as compared to the sublimed 1*H*-tetrazole used in phosphoramidite method is an additional advantage.

EXPERIMENTAL

All the ^{31}P nuclear magnetic resonance spectra were recorded on Bruker AMX 500 instrument. ^{31}P NMR chemical shifts quoted are downfield from 85% H_3PO_4. The HPLC analysis was carried out on RP C-18 column using Perkin Elmer, LC pump 250 system in combination with Perkin Elmer UV/Vis LC290 spectrophotometric detector, the solvent system being triethyl ammonium acetate (TEAA) (pH = 7.0) and acetonitrile gradient of 1% min^{-1}, starting from 0%; flow rate 1 ml min^{-1}. The UV analysis was carried out on a Shimadzu 260 A spectrophotometer using water as blank. Analytical TLC were conducted on precoated plates of E-Merck kieselgel 60-F_{254} (0.2 mm).

Acetonitrile was distilled from CaH_2 and was stored over activated 3°A molecular sieves, 4-(dimethylamino)pyridine (DMAP) and 1-*H* Tetrazole were purchased from Aldrich. *N*-bromosuccinimide was purchased from Spectrochem, India and was used after recrystallisation. All the nucleoside phosphoramidites were purchased from Glen Research, U.S.A.

General Procedure for the Synthesis of 5′-*O*-(*p*,*p*′-Dimethoxytrityl)-2′-deoxynucleoside 3′-(2 Cyanoethyl) *H*-Phosphonate (1)

To a solution of 5′-*O*-(*p*,*p*′-dimethoxytrityl)2′-deoxynucleoside 3′-(2 cyanoethyl) *N*,*N*′-diisopropylphosphoramidite (1 mmol) in anhydrous acetonitrile (10 ml) was added 0.30 ml of H_2O and 0.10 gm of tetrazole. The mixture was stirred at ambient temperature and the reaction was followed by TLC [solvent system:-ethylacetate : petroleum ether (60/40 b.p) (8 : 2, v/v)]. After completion of the reaction, the solution was concentrated and the residue was partitioned between chloroform and water. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to yield **1**. The product obtained were dried with co-evaporation with anhydrous CH_3CN and were characterised by ^1H and ^{31}P-NMR spectra.

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