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4-CYANO-2-BUTENYL GROUP : A NEW TYPE OF PROTECTING GROUP IN OLIGONUCLEOTIDE SYNTHESIS VIA PHOSPHORAMIDITE APPROACH

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Abstract: 4-Cyano-2-butenyl (CB) is a new type of protecting group for the internucleotidic bonds in the synthesis of oligodeoxyribonucleotides by the phosphoramidite approach. This group is stable to acidic conditions and can be removed under mild conditions by a δ-elimination pathway using aqueous ammonium hydroxide. Copyright © 1996 Elsevier Science Ltd

Oligonucleotides complementary to selected regions of mRNA may inhibit biosynthesis of those proteins encoded or controlled by genetic information included in these particular parts of mRNA.[1] This antisense principle has inspired several groups to extend their work beyond the synthesis of modified oligonucleotides to investigation of the potential of small nucleic acid sequences as therapeutic agents.[2] A major advantage of the antisense strategy lies in its potential specificity of action. In principle, an antisense oligodeoxynucleotide can be designed to target any single gene within the human genome, creating specific therapeutics for any disease for which a causative gene is known. In the design of these new drugs, the natural wild-type DNA is not suitable due to its instability to nucleases. Hence several modifications to the backbone, sugar and nucleobases have been investigated.[3] Of these modifications reported so far, uniformly modified oligodeoxyribonucleoside phosphorothioates have been the first class of compounds to reach the clinic. Recent animal data and clinical findings demonstrate that this new class of therapeutics works.[4] Hence there is a demand for synthesizing large quantities of these drugs.[5]

In order to develop cost-effective synthetic processes, issues related to fast and efficient synthesis, automation, scalability and product purification are being investigated with renewed attention. The introduction of phosphite triester method by Letsinger has greatly aided[6] this effort and several groups have studied in detail the application of different phosphitylating reagents. Important improvements were made[7] by Beaucage and Caruthers who introduced *N,N*-diisopropylaminophosphor amidites. Since the introduction of phosphoramidite synthons, there have been several successful reports on the synthesis of oligonucleotides via the phosphoramidite approach using novel protecting groups.[8] However there still remain some crucial problems of the removal of protecting groups from internucleotidic phosphates. The deprotection should preferably proceed through a mechanism which does not involve nucleophilic attack on the phosphorus center to eliminate the possibility of chain cleavage. In addition, the monomers containing the new protecting groups should be easy to synthesize, cost effective and should involve deprotecting conditions which are scaleable. The problems may be solved in principle by finding more useful protecting groups which can meet the

above demands. Among the currently reported phosphate protecting groups such as 2-cyanoethyl,[9] 2,2,2-trichloro-1,1-dimethylethyl,[10] *p*-nitrophenylethyl,[11] and allyl[12] groups, only the 2-cyanoethyl group meets most of the demands. However, the 2-cyanoethyl protected deoxyribonucleoside phosphoramidite monomers are very expensive even on multi-klogram scales. It is thus of prime importance to develop efficient, low-cost phosphoramidite monomers containing new protecting groups.

The phosphate proctecting groups reported so far can be broadly classified into groups removable by a) β-elimination b) β-fragmentation c) direct displacement d) hydrogenation, and e) other methods. In this report we wish to report a new group removable by δ-elimination and that a) the easily accessible reagent bis[*N*,*N*-diisopropylamino]-4-cyano-2-butenyloxyphosphine (**5**) has excellent phosphitylating properties and b) the cyanobutenyl (CB) group for protecting internucleotide linkage can be removed under mild conditions using aqueous ammonium hydroxide.

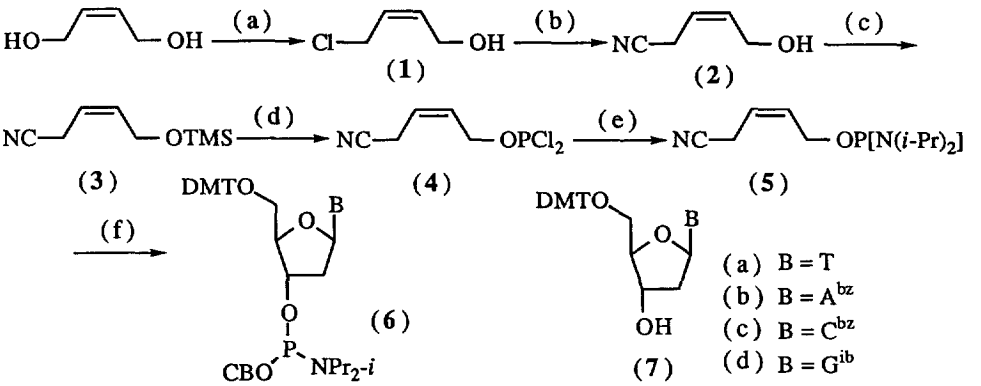
First we examined the preparation of 4-cyano-2-butene-1-ol. Monohalogenation of 2-butene-1,4-diol with thionyl chloride (1.1 equiv.) and pyridine (1.1 equiv.) in anhydrous ether afforded 1-chloro-4-hydroxy-2-butene (**1**)[13] in nearly quantitatively yield. Displacement of the chloro by cyano group was effectively achieved by reacting with potassium cyanide (6 equiv.) and sodium iodide (0.025 equiv.) in anhydrous acetonitrile at room temperature to afford the desired product (**2**) as a colorless liquid (b.p. 89-92°C/2 mm). Treatment of the alcohol (**2**) with chlorotrimethylsilane (1.1 equiv.) and diisopropylethylamine (1.1 equiv.) in anhydrous ether afforded the silyl ether (**3**) in nearly quantitatively yield as a colorless liquid (b.p. 65-68°C/2 mm).

Next we examined the synthesis of phosphoramidites (**6**). To a 1.35 molar excess of phosphorus trichloride in anhydrous ether was added dropwise, during 1 h at -40°C, the silyl compound (**3**) in anhydrous ether. After 2 h, the solution was concentrated[14] to remove excess phosphorus trichloride and redissolved in anhydrous ether. To this solution at -40°C, was added a solution of diisopropylamine in ether. After stirring for 2 h, the reaction mixture was filtered and concentrated under anhydrous conditions to afford the phosphitylating agent (**5**) in almost quantitative yield. The crude phosphine was treated with 5'-*O*-dimethoxytritylthymidine (**7a**) in presence of tetrazole in dichloromethane. After 3 h, at room temperature, the usual aqueous work-up followed by silica gel flash chromatography gave (**6a**) in 72% yield. ^{31}P NMR of (**6a**) showed the characteristic signals (~146 ppm) corresponding to diastereomeric mixtures and no 3'-3' dinucleoside phosphite could be detected. The phosphoramidites (**6b-d**) were prepared by a similar procedure in 60-68% yields.

The applicability of phosphoramidites (**6**) were demonstrated by the synthesis of four hetero-dimers d(TpsC), d(CpsT), d(GpsA) and d(ApsG) (yields > 99%) on solid support. Sulfurization was effected using 3H-1,2-benzodithiole-3-one 1,1-dioxide (Beaucage reagent).[15] The compounds were identified by comparison with the same hetero-dimers independently synthesized using cyanoethyl protection on the phosphate backbone.

While we were evaluating the utility of this new group, we changed our strategy for the synthesis of phosphoramidite monomers in an effort to make it more cost effective. Treatment of 4-

cyano-2-butene-1-ol (2) with bis(diisopropylamino)chlorophosphine[16] in ether at 0°C afforded directly the phosphitylating agent (5) in almost quantitative yield. Subsequent treatment of phosphine (5) without purification with 5'-*O*-DMT nucleosides afforded the phosphoramidites (**6b-d**) in 75-85% yields.



Scheme 1: a) $SOCl_2$, Py, ether, 0°C (95%); b) KCN, NaI, CH_3CN, rt (87%); c) Me_3SiCl, (*i*-Pr)$_2$EtN, ether, 0°C (94%); d) PCl_3, ether, -40°C; e) *i*-Pr$_2$NH, ether, -40°C; f) (7), tetrazole, CH_2Cl_2, rt.

To check the stability of this new protecting group in presence of aqueous ammonium hydroxide, the CB protected phosphorothioate hetero-dimers d(TpsC), d(CpsT), d(GpsA) and d(ApsG) after cleavage from solid support were analysed by ^{31}P NMR. Complete deprotection takes place within 1 h as shown by the shift in values from *ca.* 67 to 56 ppm. This indicates that the removal of the CB group is fast and a selective process as the cleavage of the internucleotide bond was not observed.

Synthesis of homo-thymidine 20-mer: The applicability of CB protection was extended to the synthesis of a homo-thymidine phosphorothioate 20-mer on a solid support. The synthesis was carried out on a 1 μmole scale using an ABI Synthesizer. Standard coupling conditions and concentration of amidite (0.2 M solution in acetonitrile) were used during the synthesis. The overall coupling efficiency was found to be >99% as determined by the usual spectrophotometric quantitation of released *p,p'*-dimethoxytriphenylmethyl cation. After the synthesis of 20-mer, the controlled-pore glass (CPG) was treated with ammonium hydroxide at room temperature for 2 h to afford the deprotected oligonucleotide. The crude oligomer was purified by reversed-phase HPLC and characterized by capillary gel electrophoresis[17] and electrospray mass spectrum.

Next, we synthesized a mixed sequence. Oligodeoxyribonucleoside phosphorothioate S-d(GCC-CAA-GCT-GGC-ATC-CGT-CA) was chosen as an example. This sequence is targeted for suppression of ICAM-1 expression[18] and is in Phase II clinical trials for treatment of renal transplant rejection, rheumatoid arthritis, psoriasis, Crohn's disease and ulcerative colitis. The synthesis was carried out on 1 μmole scale using ABI Synthesizer. The same elongation cycle as used for synthesis

of homo-thymidine 20-mer was utilized. At the end of synthesis, the oligomer was deprotected by treating with aqueous ammonium hydroxide at room temperature for 1 h, and then at 60°C for 20 h. The crude oligomer was purified by reversed-phase HPLC and characterized by capillary gel electrophoresis[17] and electrospray mass spectrum. The compound were identified with the oligomer independently synthesized using cyanoethyl protection on the phosphate backbone.

In summary, the CB is a suitable group for internucleotide phosphate protection.[19] Further applications of the CB group in oligonucleotide synthesis are in progress.

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