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SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES CONTAINING 5'-AMINOALKYLPHOSPHONATES

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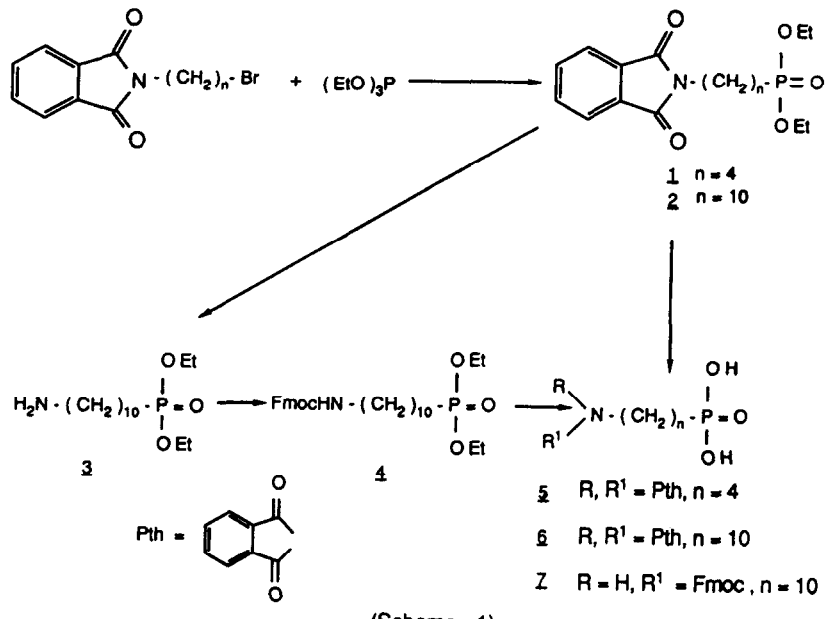
Abstract: A simple synthesis of oligodeoxyribonucleotides possessing 5'-aminoalkylphosphonate both in solution and on solid support is reported. These oligonucleotides have been reacted with the N-hydroxysuccinimide ester of d-biotin and fluoresceine isothiocyanate to give biotinylated and fluoresceinylated oligonucleotides.

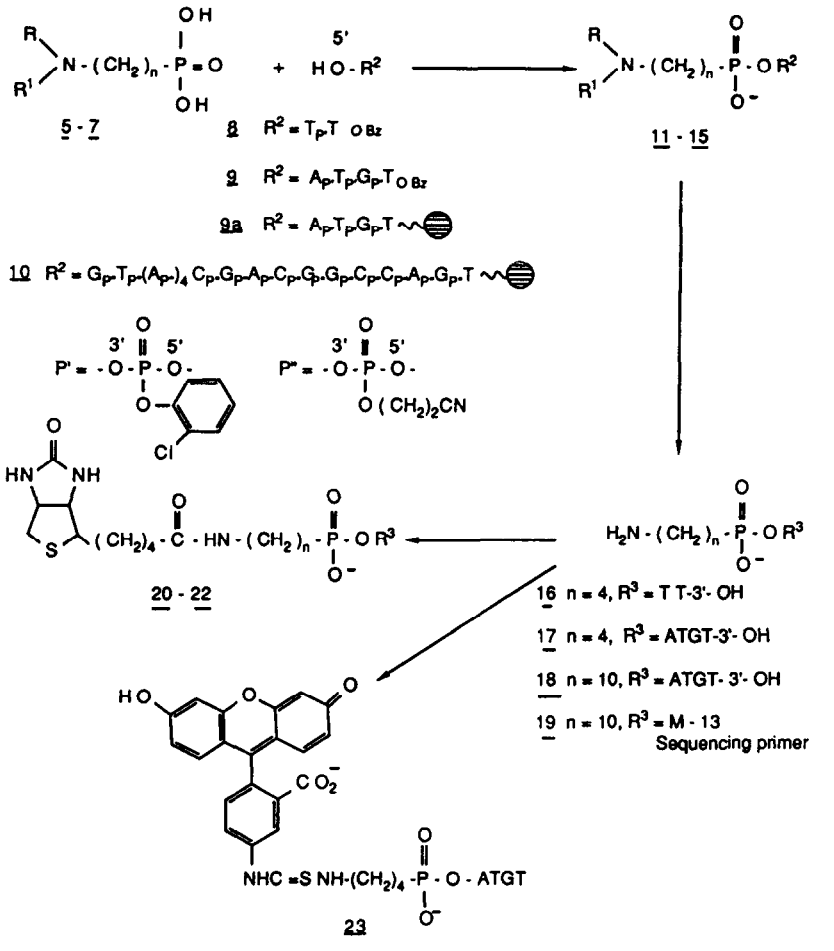
Synthetic aminoalkylated oligodeoxyribonucleotides have prime importance in molecular biology, since they could be transformed to important substrates on reaction with appropriate electrophiles such as activated esters or isothiocyanates to furnish the desired oligodeoxyribonucleotide derivatives. Using these reactions, aminoalkylated oligonucleotides can be covalently attached to d-biotin[1-3], fluoresceine[4], EDTA-Fe^{II} complex[5] or to a solid support[6,7]. These modified oligonucleotides have been used for the detection of nucleic acids[1,2], DNA sequencing analysis[8,9], preparing artificial endonucleases[5], protein isolation[7] and oligonucleotides bearing photoactivatable cross-linking agents[10]. Chemical and enzymatic methods have been developed for the synthesis of these types of oligonucleotides. As a result, appropriately substituted triphosphates have been prepared and enzymatically incorporated in purified DNA[9,11-13]. In another approach, either 5'-amino- 5'- deoxythymidine[8], an appropriately protected aminoethylphosphate group[1,4,14-16] or modified nucleosides[3,10,17,18] have been incorporated during oligonucleotide synthesis to give the desired aminoalkylated oligonucleotides after deprotection.

We report here for the first time, protected aminoalkylphosphonic acids, as a reagent of choice for the synthesis of aminoalkylated oligodeoxyribonucleotides. These aminoalkylphosphonic acids are easy to prepare and stable at 0°C for several months and could be incorporated at the end of the synthesis both in solution and solid phase.

The synthesis of these aminoalkylphosphonic acids was carried out by using an Arbuzov reaction: a mixture of N-(bromoalkyl) phthalimide (1 eq) and triethylphosphite (2.5 eq) was



(Scheme - 1)



(Scheme - 2)

heated at 140°C for 16 hrs to give the corresponding diethylalkylphosphonates 1 (91%) and 2 (84%). Transesterification of 1 and 2 with trimethylsilyliodide (2.5 eq) in CH_2Cl_2 at 0°C followed by methanolysis resulted the desired phosphonic acids 5 (89%) and 6 (90%).Besides the phthalimido group we also used Fmoc as a amino protecting group, since Fmoc could be removed in the same conditions as those used for classical deprotection in oligonucleotide synthesis (Scheme 1).

Treatment of 2 with NH_2NH_2 in ethanol in presence of triethylamine afforded the corresponding diethylaminodecylphosphonate 3 (79%) which on reaction with Fmoc-Cl (9-fluorenylmethyl chloroformate) in presence of N-methylimidazole furnished 4 (74%). Transesterification of 4 with trimethylsilyl iodide (2.5 eq) at -10°C for 2 hrs followed by methanolysis gave the desired Fmoc aminodecyl phosphonic acid 7 (77%)[19] (Scheme 1).

In order to test the efficacy of 5-7 for introducing aminoalkylphosphonate at the 5'-end of oligonucleotides, we first carried out model studies. We therefore reacted 5 with 8 and 9 in pyridine using DCC as a coupling agent in presence of N-methylimidazole at 40°C for 5 hrs to give 11 and 12 respectively in an excellent yield. 8 and 9 were prepared by using standard phosphotriester strategy. The deprotection of 11 and 12 with NH_2NH_2/Et_3N in ethanol followed by treatment with pyridoxime-tetramethylguanidine and then with ammonia resulted in 16 and 17, possessing aminoalkylphosphonates at 5' end. The structures of 16 and 17 were confirmed by their ^{1}HNMR and fast atom bombardment (FAB) spectroscopy[20] (Scheme 2).

After evaluating the efficacy of these reagents in solution, 9 was prepared on solid support and treated with 5-7 in presence of DCC/N-MeI at 30-35° in pyridine for 12 hrs. The solid supports thus obtained were submitted to usual deprotecting conditions to give 17 and 18. However, in the case of phthalimido alkylphosphonate, prior to usual deprotection, solid supports were treated with $NH_2NH_2/Et_3N/EtOH$ at 50°C for 3 hrs.

17 obtained from solid support was found to be identical with an authentic sample prepared by liquid phase synthesis. Furthermore, 17 (1 eq) obtained both by solid and liquid phase synthesis, on reaction with the N-hydroxysuccinimide ester of d-biotin (100 eq) and fluoresceine isothiocyanate (200 eq) in a mixture of DMF and carbonate buffer (pH = 7.8) afforded biotinylated 20 and fluoresceinylated 23 derivatives. Similarly, 18 on reaction with the N-hydroxysuccinimide carboxylic ester of d-biotin yielded 21 in quantitative yield after 2 hrs.

In order to demonstrate the applicability of these reagents at more elaborated model, we synthesized the 5'-detritylated protected M-13 sequencing-primer (GTAAAACGACGGCCAGT) using an Applied Biosystems 380 B DNA synthesizer, which was treated with 7 in presence of DCC/N-MeI at 30°C in pyridine for 12 hrs. The support was washed with a mixture of CH_2Cl_2/MeOH (15%) and subjected to aminolysis to give the M-13 sequencing primer having an aminodecylphosphonate at the 5'-end 19. The M-13 sequencing primer 19 thus obtained was treated with the N-hydroxysuccinimide ester of d-biotin in similar conditions as those described for 17 and 18 to furnish the biotinylated M-13 sequencing primer 22 after 5 hrs in quantitative yield as shown by HPLC.

All the biotinylated oligonucleotide adducts 20-22 reacted positively with the biotin-specific Blugen^R detection kit. It should be pointed out that the detection sensitivity of the biotinylated probes was directly proportional to the chain length of the oligonucleotides.

In conclusion, in this communication, we have demonstrated that aminoalkylphosphonic acids are reagents of choice for preparing aminoalkylated oligonucleotides. The chain length of

the aminoalkyl can be varied by singly choosing the appropriate phtalimidoalkyl bromide. In addition to the synthesis of biotinylated or fluoresceinylated probes, future work will involve the synthesis of other covalently attached derivatives of oligonucleotides and their biological applications.

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19. 7, ^{1}HNMR (200 MHz, DMSO-d_6, in δ ppm): 1.16-1.8 (m, 18H, $(CH_2)_9P$), 3.1 (m, 2H, NH-CH_2), 4.06 (t, 1H, Ar-CH), 4.4 (d, 2H, CH_2-O), 6.9 (t, 1H, NH), 7.4 (m, 4H, Ar-H), 7.73 (d, 2H, J = 9 Hz, Ar-H), 7.85 (d, 2H, J = 9 Hz, Ar-H); MS (CI, M^+ = 459), m/e: 460 $(M + H)^+$.
20. 16, ^{1}HNMR (300 MHz, D_2O, in δ ppm): 1.32-1.60 (m, 6H, $(CH_2)_3P$), 1.7 (d, 3H, J = 1 Hz, CH_3), 1.76 (d, 3H, J = 1 Hz, CH_3), 2.2 (m, 2H, 2',2'-H), 2.35 (dd, 1H, 2'-H), 2.38 (dd, 1H, 2'-H), 2.80 (t, 2H, NH-CH_2), 3.86 (m, 2H, 5',5'-H), 3.95 (m, 3H, 4'-H and 5',5'-H), 4.15 (m, 1H, 4'-H), 4.40 (m, 1H, 3'-H), 4.66 (m, 1H, 3'-H), 6.07 (dd, 1H, J = 5.88 and 5.27, 1'-H), 6.14 (t, 1H, 1'-H), 7.49 (d, 1H, J = 1.3 Hz 6-H), 7.51 (d, 1H, j = 1.3 Hz, 6-H); MS (FAB$^-$, M^+ = 681), m/e: 680 $(M - H)^+$.
    17 ^{1}HNMR (300 MHz, D_2O, in δ ppm): 1.3-1.8 (m, 12H, 3XCH_2 and 2XCH_3), 2.09 (m, 2H, 2',2'-H), 2.59 (m, 6H, 2',2'-H), 2.78 (t, 2H, NH-CH_2), 3.90 (m, 8H, 5',5'-H), 4.06 (bs, 1H, 4'-H), 4.22 (m, 3H, 4'-H), 4.46 (m, 1H, 3'-H), 4.62 (m, 2H, 3'-H), 4.80 (m, 1H, 3'-H), 5.89 (m, 2H, 1'-H), 6.02 (t, 1H, 1'-H), 6.16 (t, 1H, 1'-H), 7.23 (s, 1H), 7.37 (s, 2H), 7.79 (s, 1H), 8.26 (s, 1H); MS (FAB$^+$, M^+ = 1323), m/e: 1324 $(M + H)^+$.

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