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LARGE SCALE BENCH-TOP SYNTHESIS OF A NINETEEN UNIT RIBONUCLEOTIDE ON SILICA GEL.

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Summary: The stepwise synthesis of a nonadecaribonucleotide corresponding to units 9 to 27 of the t·RNA^{fmet} from E.coli is described. The chlorophoshite condensation procedure and a silica gel support were used.

The use of chlorophosphites of deoxynucleosides as highly reactive species for the preparation of oligodeoxynucleotides on silica gel solid supports has been demonstrated (1-3). While oligoribonucleotide synthesis is more difficult than oligodeoxynucleotide synthesis we have previously reported the synthesis of a hexaribonucleotide on a silica gel support using chlorophosphite chemistry (4). In this report we wish to describe the synthesis of a 19-unit oligoribonucleotide (Figure 1) corresponding to units 9 to 27 of the t RNA^{fmet} from E. coli. The synthesis was conducted using nucleoside chlorophosphites and a silica gel support. Reactions were carried out in a bench-top "large-scale" (5g of silica) process.

The polymer support (Vydak TP silica) was derivatized with a guanosine unit as previously described (4). Analysis indicated a loading of 0.08 mmol/g of silica. The nucleosides used in the synthesis were all protected at the 2'-position with the t-butyldimethylsilyl(TBDMS) group and at the 5'-position with a methoxytrityl group (5). Cytidine and Guanosine nucleosides were also protected with the benzoyl group at the N-4 and N-2 amino groups, respectively. The protected ribonucleosides were converted to their 3'-chlorophosphite derivatives using methyl dichlorophosphite. Reactions were carried out using dry THF(1ml/mmole of nucleoside) containing collidine (4mmole) at -78°C in small vessels fitted with rubber septa, a drying tube and flushed with dry nitrogen. Total reaction time was 15 min. and the activated nucleoside was prepared just prior to use.

Condensation reactions on the solid support were carried out at -78°C in

THF(3ml/g of silica). Reaction vessels were flushed with nitrogen and reagents (other than silica) were introduced via rubber septa using syringes. The mixture was stirred throughout the courses of the reaction. At the end of each step the silica was collected by filtration. The steps involved in each cycle are summarized in Table 1. Condensation reactions were carried out for 30 min. and were followed by iodine oxidation of the phosphite to the phosphate. A phenylisocyanate (1% in pyridine) treatment was carried out (60 min.) to remove water from the silica. No reaction with unprotected adenosine can be detected under these conditions. The methoxytrityl group was removed using 1% benzenesulfonic acid in acetonitrile (20 min.). These conditions do not cleave the glycoside bond in riboadenosine which is ~1000 times more stable than deoxyadenosine. The polymer was washed thoroughly and the cycle repeated. The total time for one cycle was ~2 h.

While the trityl color produced at the end of each cycle indicates a successful condensation, nucleotides were removed from a portion of the silica (100 mg) at the hexamer, decamer and nonadecamer stages and were fully characterized. Ammonium hydroxide (NH_4OH:EtOH, 2:1, 16h, 30°C) was used to cleave the oligonucleotide from the silica and to simultaneously remove the N-benzoyl groups as well as the methyl group from the phosphate. The spent polymer was removed by filtration and was washed thoroughly with ethanol. The combined filtrate and washings were evaporated and the residue was stirred with TBAF (2ml, 0.68M solution in THF) for 2h to remove the TBDMS groups. The solvent was removed and the residue, dissolved in a minimum of water, was passed through a Dowex 50W-X8(Na^+ form) column. The eluant was collected, concentrated and applied to Whatman 1mm paper developed in Solvent F(6).

The hexamer (R_f^F 0.21, Rm^{Tp} 0.77) and decamer (R_f^F 0.12, Rm^{Tp} 0.82) were both obtained as clean bands and eluted with water in the normal fashion. The compounds were homogeneous by anion exchange HPLC and by gel electrophoresis (7) (Figure 1). Both nucleotides were characterized by complete enzymatic degradation by phosphodiesterases to give the expected products in the correct ratio as determined by established procedures (8,9).

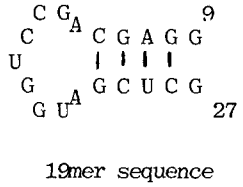
The nonadecamer required continuous chromatography in solvent F for 48h before the product band moved from the origin. The product was eluted with water. Further purification was carried out both by HPLC, on a Brownlee anion exchange column (AX10) using a linear gradient of solvents A and B (6) from 0% to 100%B at a flow rate of 4ml/min., and by gel electrophoresis. The pure 19-mer (Fig. 1) was further characterized by complete enzymatic degradation to the correct ratio of products. The overall yield of the 19-mer, determined by UV absorption of the band eluted from paper chromatography was 33% which would correspond to an average coupling yield of 94%(10).

TABLE 1.

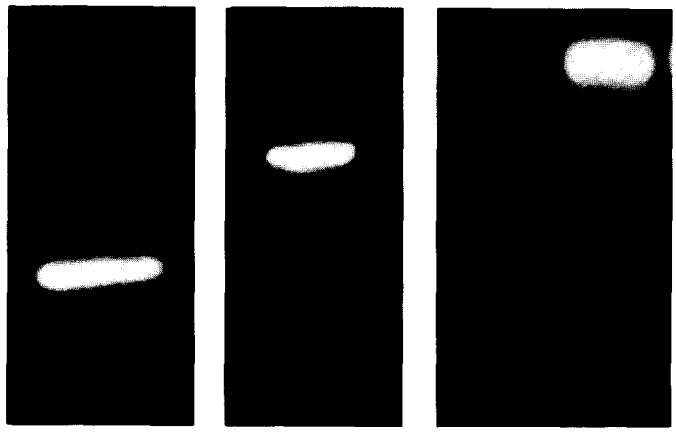
Cycle Steps in Solid Support Synthesis

Step	Reagent or Solvent	Amount	Time (min)
1	Nucleoside Chlorophosphite in THF	3eg in 3ml/g	30
2	4% I_2/THF:H_2O	2ml/g	2
3	CH_2Cl_2 Wash	5ml/g	1
4	Ether Wash	3ml/g	1
5	1% phenylisocyanate in pyridine	5ml/g	60
6	CH_2Cl_2 Wash	5ml/g	1
7	Ether Wash	3ml/g	1
8	1% Benzenesulfonic acid in Acetonitrile	5ml/g	20
9	CH_2Cl_2 Wash	5ml/g	1
10	Ether Wash	3ml/g	1

FIGURE 1



19mer sequence



Sizing Gels:A,AGCUCG
B,UGGUAGCUCG
C,$(T_p)_nT$ (n=9,7,5,3 from top)
D,GGAGCAGCCUGGUAGCUCG

This synthesis demonstrates the potential of the chlorophosphite procedure for large scale ribonucleotide synthesis.

Acknowledgement

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6. Solvent A: 0.2 m triethylammonium acetate (pH=3.5);Solvent B: 0.5M triethylammonium acetate (pH=2.9); Solvent F: n-propanol-NH_4OH-H_2O (55:10:35).
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10. The material eluted from paper chromatography gave a sharp band on gel electrophoresis. Only a trace of material corresponding to a 10-mer was observed along with the 19-mer. We have experienced significant loss of material on HPLC columns. In this case the recovery of pure 19-mer after HPLC corresponded to 60% of the material applied even though the only other UV-absorbing material eluted from the column accounted for less than 4% of the material applied. Thus the yield of high purity 19-mer was 20% corresponding to a total yield of 0.08 mmoles of 19-mer on the 5g of silica used. The yield of single stranded synthetic oligoribonucleotides was determined from the equation mg = $OD_{260}/30$ where $OD_{260}=A_{260}$ x vol.

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