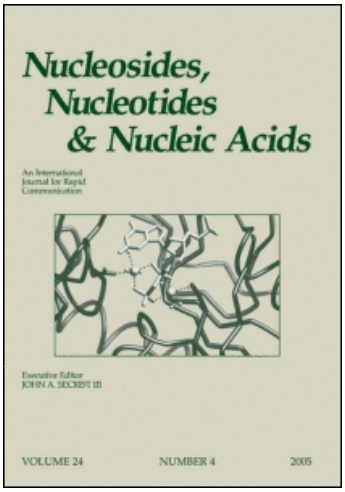

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Automated Solid Phase Synthesis of Oligoribonucleotides. The Synthesis of a tRNA Anticodon Loop from Nucleoside-3'-chlorophosphite Derivatives.

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AUTOMATED SOLID PHASE SYNTHESIS OF OLIGORIBONUCLEOTIDES. THE SYNTHESIS OF A tRNA ANTICODON LOOP FROM NUCLEOSIDE-3'-CHLOROPHOSPHITE DERIVATIVES.

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Abstract: A heptadecaribonucleotide corresponding to positions #28-44 of E. coli's N-formylmethionine initiator tRNA was synthesized on a silica gel support. Methyl dichlorophosphite was used as the coupling reagent.

Introduction

Chemical synthesis has frequently been used to prepare oligoribonucleotides which correspond to portions of tRNA molecules. In particular, special attention has been devoted to the preparation of anticodon loop sequences[1-6]. These syntheses have produced materials with defined sequence (sometimes containing unusual or modified nucleosides) which are valuable for the measurement of physical properties. The anticodon portion (stem and loop) of E. coli's N-formylmethionine tRNA is especially interesting because this molecule initiates peptide chain synthesis. The conformation of this tRNA is also believed to confer an increased stability to the anticodon loop relative to non-initiating tRNA molecules.[7]

Phosphotriester and modified phosphotriester procedures[8-9] have been applied to the synthesis of RNA fragments on a solid phase support. These strategies used some type of arylsulphonyl condensing reagent as the coupling agent. However, these reagents have the disadvantages of causing 5'-sulphonation and modification of uracil and guanine bases[10].

We have found that faster and cleaner reactions can be obtained by using a trivalent dichlorophosphite as the coupling reagent[11]. The chlorophosphite procedure, in conjunction with the use of 2'-t-butyldi-

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methylsilyl protecting groups, has been found to be quite effective for oligoribonucleotide synthesis in solution.[12] A similar methodology can also be used to manually prepare oligoribonucleotides on an insoluble support.[13]

Synthesis on insoluble supports is ideally suited for automation and we have recently described how homogeneous oligoribonucleotide sequences can be prepared in an automated DNA/RNA synthesizer.[14] In this manuscript we demonstrate the application of this procedure to further our long range goal of total tRNA synthesis. The synthesis of octa-, nona-, and heptadecaribonucleotide anticodon loop sequences from E. coli's N-formylmethionine initiator tRNA is described.

Results and Discussion

Derivatization of the Polymer Support

The selection of the appropriate insoluble support is very important in developing an automated synthesis. The substrates most commonly used in either DNA or RNA solid phase synthesis can be divided into rigid and nonrigid categories.

The nonrigid polymers such as crosslinked polystyrene[15-18] or polyacryloylmorpholide[19] resins contain a relatively high number of derivatizable sites throughout their structure. These resins must be used in a swollen condition to permit infusion of reagents.

The rigid polymers, such as silica gel[13-14, 20-25] and controlled pore glass[26-28] are nonswellable and possess derivatizable sites only on their surface. The properties of these supports are strongly influenced by the pore size of the substrate.

We investigated representative polymers from each category during development of our automated procedure. These supports are listed in TABLE 1.

Our general strategy was to attach the 3'-terminal nucleoside of an intended sequence onto the insoluble support by esterification of the 2'- or 3'-hydroxyl position. This required a carboxylic acid function on the support. The Enzacryl K2 and Carboxyl controlled pore glass (CPG) supports were purchased already derivatized in this way. The chloromethyl groups of the Bio Beads resin were derivatized with either

TABLE 1. Insoluble Supports Investigated.

Name	Type	Functional Group	Pore Size (Angstroms)	Particle Size (microns)
Bio Beads SX-1	Polystyrene	$-CH_2Cl$	-----	63-130
Enzacryl K2	Polyacryloyl-morpholide	-COOH	-----	----
Vydac TP	Silica Gel	-----	300	20
Baker Amino-Bonded Phase	Silica Gel	$-NH_2$	60	60
Carboxyl CPG	Controlled Pore Glass	-COOH	500	120-320
Long Chain Alkyl Amine CPG	Controlled Pore Glass	$-NH_2$	500	120-320

succinic or sebacic acid[29]. The Vydac TP silica gel was aminopropylated and then succinylated as previously described[13], while the Baker Amino-Bonded silica gel was only succinylated.

The number of carboxylic acid groups on each polymer was determined by back titration of a mixture of polymer in a known amount of base solution. The observed carboxyl loadings are shown in TABLE 2. However, not all of these carboxyl groups are equally accessible. An alternative procedure[20], which measured the amount of p-nitrophenol coupled to the polymer by DCC, produced much lower values. Lower yields would result if DCC failed to reach the most hindered locations or if DCC converted some carboxyl groups in close proximity into anhydrides.

Attachment via the 2'- or 3'-hydroxyl position of an otherwise fully blocked nucleoside, to the Vydac and Baker silica gels was performed by using DCC and dimethylaminopyridine (DMAP)[13]. However, this procedure was much less effective with the other supports. The DCC/DMAP esterification produced only very small loadings on the carboxyl CPG and the sebacic acid derivatized Bio Beads. No coupling was detected on the Enzacryl K2 and succinic acid derivatized Bio Beads. In these cases, esterification was brought about by first reacting the polymer with excess phosphorous trichloride to form a mixed anhydride[30]. Reaction of a ribonucleoside with the mixed anhydride produced the loadings shown in TABLE 2.

TABLE 2. Attachment of Nucleosides to Derivatized Polymer Supports.

Polymer Support	No. of Sites (Detn. by titration) (umoles/g)	Coupling Procedure	Loading Achieved[b] (umoles/g)
Bio-Beads	590	DCC/DMAP Esterification	10
Bio-Beads	590	$POCl_3$ Mixed Anhydride	30-50
Enzacryl K2	1000[a]	$POCl_3$ Mixed Anhydride	20
Vydac TP	370	DCC/DMAP Esterification	40-60
Baker Amino-Bonded	1750	DCC/DMAP Esterification	120-150
Carboxyl CPG	100[a]	DCC/DMAP Esterification	1
Carboxyl CPG	100[a]	$POCl_3$ Mixed Anhydride	5-10
Long Chain Alkyl Amine CPG	100[a]	Pentachlorophenylsuccinate	30-40

[a]Value supplied by the manufacturer
[b]Determined by trityl colour analysis

The amount of loading on the controlled pore glass support was still low and so a different derivatization strategy was used. This involved reaction of an activated nucleoside-3'-pentachlorophenyl succinate to an amine containing support (long chain alkyl amine CPG). This procedure was similar to the derivatization of 2'-deoxyribonucleosides[26, 31] and gave good results.

A comparison of the nucleoside loadings listed in TABLE 2 with the number of carboxylic acid sites indicates that only a small fraction of the acid sites can be derivatized. The best loadings were obtained with the small pore Baker Amino-Bonded silica gel and decreased as the pore size of the supports increased.

Elongation of the Polymer Supported Nucleoside

Once the first nucleoside has been attached, the support can be used as a substrate for chain extension reactions. To automate this process, samples of polymer (150-200 mg) were placed in stainless steel columns and attached to an automated DNA/RNA synthesizer. The details of this synthesizer have been previously described[14, 32].

TABLE 3. RNA Synthesis Cycle

Operation	Reagent	Step Time (@ 4 ml/min)
1, Detritylation	0.1M Benzenesulfonic Acid	5 min
2, Wash #1	Acetonitrile	1 min
3, Wash #2	2% Collidine/acetonitrile	4 min
4, Nucleotide Add'n	0.03M Nucleoside chlorophosphite	1.25 min
5, Recycle	0.03M Nucleoside chlorophosphite	23.75 min
6, Oxidation	0.1M I_2 water:THF:pyridine 2:78:28	2 min
7, Wash #3	Acetonitrile	1 min
8, Capping/Drying	Acetic anhydride, DMAP, collidine	5 min
9, Wash #4	Acetonitrile	5 min

The synthesis cycle employed (TABLE 3), contained four basic steps:

(1) Acidic treatment to remove 5'-monomethoxytrityl protecting groups

(2) Condensation of the polymer bound nucleoside/tide with a nucleoside-3'-chlorophosphite

(3) Oxidation, using iodine and water, to convert the the phosphite linkages into phosphate linkages

(4) Capping with acetic anhydride and DMAP to block off unreacted sites and remove residual moisture

The most difficult step is the chain extension reaction. Unlike 2'-deoxyribonucleosides, protected ribonucleosides are much more hindered about the 3'- position. This is due to the bulky t-butyldimethylsilyl or triisopropylsilyl protecting group which we used on the 2'- position. Consequently we have found that it is necessary to use higher concentrations (0.03M vs 0.01M) and longer coupling times (25 min vs 7 min), relative to chlorophosphite oligodeoxyribonucleotide synthesis[32], in order to obtain acceptable coupling yields.

Reactive nucleoside-3'-chlorophosphites were prepared fresh each day by the dropwise addition of a suitably protected ribonucleoside (1.0 eq.) into a rapidly stirred solution of methyl dichlorophosphite (0.9 eq.) and collidine (4.5 eq.) in cold (-45°) acetonitrile. After 10

minutes the reaction was warmed to room temperature to produce a clear solution. The nucleoside concentration was 0.03M and 5 ml of solution was used during each coupling cycle.

The excess of nucleoside to phosphite ensured complete utilization of the methyl dichlorophosphite, but produced some 3'-3' linked dimers. However, this was preferable to reactions which used 1.0 or 1.2 equivalents of methyl dichlorophosphite. In these reactions, the nucleoside chlorophosphite solution was coevaporated with toluene, under argon, to remove any unreacted methyl dichlorophosphite. However, coupling reactions using these solutions, particularly those involving guanosine monomers, did not give high yields. This was attributed to the incomplete removal of all of the excess methyl dichlorophosphite.

The synthesis cycle was used with each of the different supports. The two nonrigid polymers, Bio Beads and Enzacryl K2 were found to swell very differently between acetonitrile and THF containing reagents. It was necessary to use only one solvent, acetonitrile, throughout the entire cycle. The oxidizing and capping reagents were therefore prepared in acetonitrile instead of THF. These modified conditions allowed the preparation of an octauridylic acid test sequence on both of the nonrigid supports. Average coupling yields of 93 and 108% were obtained for the Bio Beads and Enzacryl K2 supports, respectively, in this trial sequence. These yields were determined by trityl colour analysis.

The greater than 100% average coupling yield (108%) obtained with the Enzacryl K2 support indicated that this support was unsuitable for use with chlorophosphite reagents because some reaction of the resin's structure must be occurring with each coupling step.

The result with the Bio Beads support was satisfactory but further syntheses were not attempted with this support because it was not as easy to handle as the rigid silica gels and controlled pore glass supports. These latter supports were preferred because they could be used with any solvent and at higher flow rates and operating pressures.

The Baker Amino-Bonded support appeared to be the most promising support because of its high nucleoside loading. However, preliminary experiments with this support indicated that the coupling yields decreased rapidly after the second or third nucleotide addition. For example, in the synthesis of the sequences **GUC**, **GUCG**, **CGUCG** and **UCGUCG** the average yields of the first, second and third coupling reactions

were 85, 63 and 44% respectively. These results were probably due to the relatively small pore size (60 angstroms) of this support which prevented the formation of long nucleotide chains. The same coupling cycle produced much better results on the Vydac and controlled pore glass supports. Both of these supports had large-size pores (300-500 angstroms) and gave similar coupling results in preliminary reactions.[33]

The Vydac silica gel was used in our automated procedure to prepare the two octa- and nonaribonucleotide sequences **UAACCCGA** and **UCGGGCUCA.** These two sequences are the 3'- and 5'- halves of the anticodon loop of the initiator tRNA (FIGURE 1). In addition, we also prepared the entire heptadecamer sequence **UCGGGCUCAUAACCCGA** in one continuous synthesis.

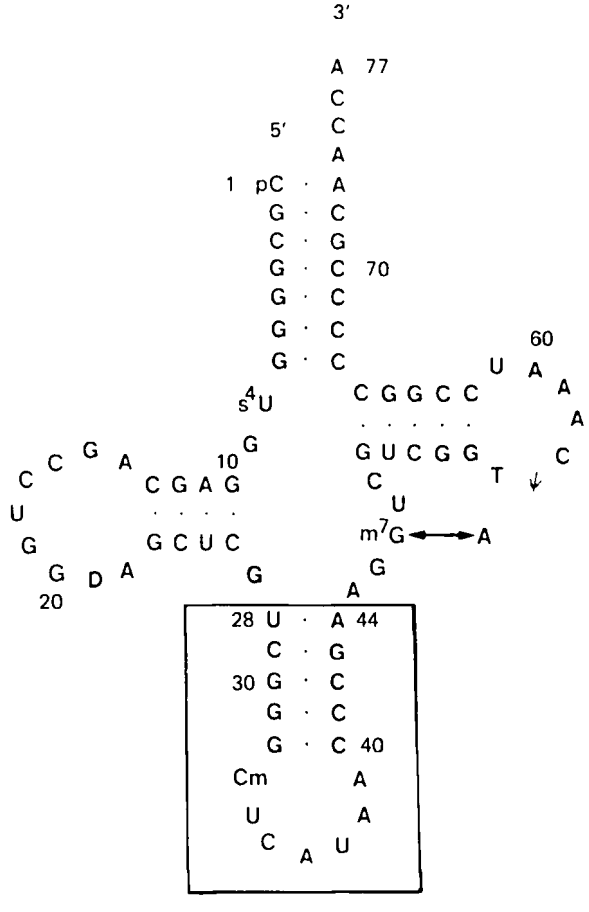

FIGURE 1. E. coli's N-formylmethionine initiator tRNA sequence. The synthesized fragment is shown in the boxed area.

TABLE 4. Coupling Yields in the Synthesis of the Anticodon Loop.

Sequence	Length	Average Coupling Yields	Overall Yield
UAACCCGA	8	87%	38%
UCGGGCUCA	9	91%	47%
UCGGGCUCAUAACCCGA	17	93%	31%

The coupling yields in these syntheses were again determined by quantitation of the amount of monomethoxytrityl cation released during each detritylation step. The results were quite good and the average and overall coupling yields for each synthesis are tabulated in TABLE 4. In each case the desired sequence was assembled in only one or two working days since less than one hour was required for each nucleotide addition.

Deprotection and Isolation of the Final Products

After assembly of the desired sequence by the synthesizer, the final product must be cleaved from the polymer, deprotected and isolated. This procedure is more difficult for oligoribonucleotides than for oligodeoxyribonucleosides for two reasons. First of all, the 2'-protecting group requires the inclusion of an extra deprotection step. Secondly, the deprotected oligoribonucleotides are much more sensitive towards chemical and enzymatic hydrolysis. Precautions need to be taken to prevent any accidental introduction of ribonucleases.

The deprotection began by treatment of the polymer with t-butylamine to remove the methyl protecting groups. Acyl linkages were then hydrolyzed by treatment with ethanolic ammonium hydroxide. The octa- and nonaribonucleotide sequences were left with the 5'- terminal monomethoxytrityl group in place and at this point they were purified by silica gel TLC. The monomethoxytrityl group identified the desired band on each plate. This was removed, desilylated with TBAF solution, desalted and then detritylated. Cellulose TLC was used to purify the completely deprotected sequences.

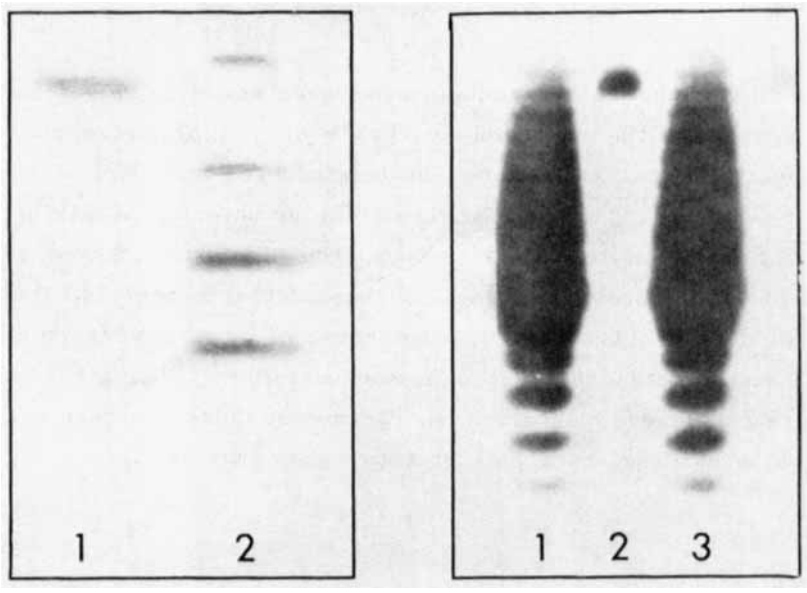

FIGURE 2. Left - Sizing gel of the synthetic nonanucleotide (lane 1) and, in descending order, 8, 10, 12, and 14 unit long oligo-T markers (lane 2).

Right - Sizing gel of the synthetic heptadecaribonucleotide (lane 2) and oligo-T markers (lanes 1 and 3). (A less exposed plate allowed the marker nucleotides to be distinguished.)

The heptadecamer sequence was deblocked slightly differently. In this case the monomethoxytrityl group was removed first. The silica gel purification step was omitted and the crude mixture was desilylated and then desalted. A combination of reversed phase HPLC and preparative polyacrylamide gel electrophoresis was used to purify the heptadecamer sequence.

All three of the above sequences were characterized by kinasing samples and sizing them (against oligothymidylic acid standards) on gel electrophoresis. Sample radiograms of both the nona- and heptadecaribonucleotides are shown in FIGURE 2. The nucleoside composition of each sequence was also determined by enzymatic degradation and HPLC analysis[34].

Conclusion

The chlorophosphite procedure has been shown to be sufficiently reactive to allow the automated synthesis of oligoribonucleotides on silica gel supports. Nucleotides can be assembled at a rate of less than one hour per coupling cycle. The laborious preparation of di- or trinucleotide blocks can be avoided because the high coupling yields allow the use of simple mononucleosides as the starting materials. The preparation of three anticodon loop sequences, of up to seventeen units in length, demonstrates that RNA oligonucleotides of biological significance are now readily obtainable. Further advances in this area will assist us in our long term goal of total tRNA synthesis.

Experimental

Reagents and Materials

Protected nucleosides and nucleoside-3'-chlorophosphites were prepared as previously described[12-14]. The crosslinked chloromethylated polystyrene resin (Bio Beads SX-1) was obtained from Bio Rad (Richmond, CA). The polyacryloylmorpholide resin was obtained from the Aldrich Chemical Co. (Milwaukee, Wis.). The controlled pore glass beads and the Vydac TP silica gel were manufactured respectively by the Pierce Chemical Co. (Rockford, IL) and The Separations Group (Hesperia, CA). The Baker Amino-Bonded silica gel was donated by the J. T. Baker Co. (Phillipsburg, NJ).

All of the glassware and plasticware used in the purification steps was treated with diethyl pyrocarbonate and autoclaved to prevent accidental enzymatic degradation.

Measurement of the Coupling Yields

The coloured washings from the detritylation of the monomethoxytrityl group (0.1M benzenesulphonic acid/anhydrous acetonitrile) were collected. Aliquots were diluted with 0.1M benzenesulphonic acid in anhydrous acetonitrile. The presence of the benzenesulphonic acid was

necessary to stabilize the colours. Absorbance values at 389 and 470 nm were measured on a Cary 17 spectrophotometer. The extinction coefficients for the two peaks were 16,000 and 56,000 l $cm^{-1}mol^{-1}$ respectively. Coupling yields were calculated by comparing successive absorbance values. The uncertainty of the measurement was estimated to be plus or minus 2%.

Deprotection and Purification Procedure for Octa- and Nonanucleotide Sequences

Polymer (30-50 mg) containing the tritylated sequence was stirred in neat t-butylamine (2 ml) at 50° overnight. The t-butylamine was evaporated off and 15M ammonium hydroxide:ethanol 3:1 (2 ml) was added. After heating (50°, 2-4 hours) the polymer was washed (5x2 ml) with more ethanolic ammonia and the washings were dried in a Speed-Vac. The amount of nucleotide recovered was determined by UV spectroscopy. Typically, 80 to 120 A_{260} units were obtained.

The crude mixture was applied to a silica gel TLC plate (0.1 mm x 20 cm x 20 cm) and developed in either 1:1 ether:chloroform (nonanucleotide) or 8:2 ethanol:1M aqueous ammonium acetate (octanucleotide). The monomethoxytrityl containing band was identified with an acid spray (applied to the edge of the plate). This material was removed and the nucleotide eluted off with ethanol and then water.

The material was dried and then dissolved in 1M TBAF/THF solution (1 ml, 2-4 hours). The solution was concentrated, diluted with water (1 ml), and passed down a column of Dowex Na^+ ion exchange resin (20 ml). The eluant was lyophilized to give a white or light brown solid. If an oil or gum was obtained the ion exchange column was repeated.

The residue was desalted on a Sephadex G-25 column (0.8 cm i.d. x 50 cm) by elution with 0.05M ammonium acetate. The nucleotide was detritylated in 80% acetic acid (0.5 ml, 12 hours) at room temperature. The acid was removed in the Speed-Vac and the residue was washed with ether (3x0.2 ml). The aqueous layer was applied to a cellulose TLC plate (0.1 mm x 20 cm x 20 cm) which was developed in n-propanol:15M ammonium hydroxide:water 55:10:35. The product had an Rf in the 0.1 - 0.2 range.

Deprotection and Purification of the Heptadecamer Sequence **UCGGGCUCAUAACCCGA**

Polymer (55 mg) was treated with t-butylamine (2 ml) and ethanolic ammonia as above. The polymer was washed (5x2 ml) with the ethanolic ammonia solution and the combined washings were dried. 280 A_{260} units of material were recovered. The material was dissolved in 1M TBAF solution (2 ml) and left at room temperature (16 h) before being desalted as described above.

The crude nucleotide mixture was applied to a column of DEAE-cellulose (0.8 cm i.d. x 10 cm) and eluted with a stepwise gradient of pH 7.5 TEAB buffer (15 ml @ 0.25M, 0.50M, 0.75M, 1.0M, 1.25M and 40 ml @ 1.5M TEAB). The material eluted off by the 1.5M TEAB was not sufficiently pure (as judged by gel electrophoresis).

A sample of the material (2 A_{260} units) was dissolved in water (0.1 ml) and purified by HPLC on a Whatman ODS-2 C-18 column. The gradient used was 7-20% acetonitrile in ammonium acetate (1% wt/v) over 30 minutes (at 2 ml/min). The peak eluting at 15.85 minutes contained the desired heptadecamer sequence. This was further purified by gel electrophoresis.

The sequence was enzymatically degraded into C:U:G:A (6:3:4:4) by snake venom phosphodiesterase and alkaline phosphatase.

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