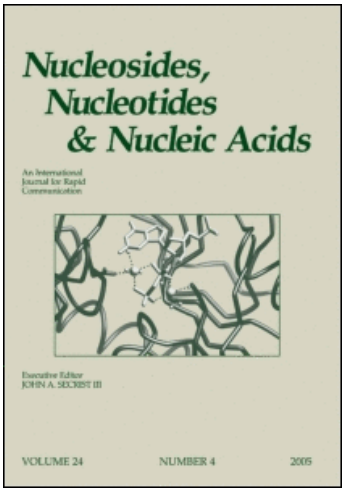

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LARGE-SCALE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDE PHOSPHOROTHIOATE USING CONTROLLED-PORE GLASS AS SUPPORT[1]

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Abstract: An efficient synthesis of oligonucleotide phosphorothioate on a very large-scale using controlled-pore glass as support is described.

The concept that oligonucleotides may have potential therapeutic utility has gained credibility and attention.[2-7] Both animal data[8] and clinical findings[9] demonstrate that this new class of therapeutics works. Since the natural wild type of oligonucleotides are unstable to nucleases, several modifications to the backbone, sugar and nucleobases have been investigated.[3,4,10,11] These modifications have been primarily aimed at i) improving the uptake, permeability, and bioavailability ii) increasing nuclease resistance, iii) activating RNase H, iv) increasing binding affinity and specificity and v) having no serious toxic effects. Among these variations, uniformly modified oligodeoxyribonucleotide phosphorothioates have been the first class of compounds to reach the clinic. It is thus of prime importance to develop low-cost, efficient and scaleable technologies. This necessity becomes even more demanding for treatment of host diseases involving systemic dosing where higher quantities of drug is needed to fully meet the demand.[12] We report here that the above demands can be achieved with success by synthesizing efficient, economical and large scale synthesis of antisense drugs utilizing controlled-pore glass as solid support.[13,14]

Methods and Results:

Automated DNA Synthesis:

The fully modified oligonucleotide phosphorothioate d(GCC-CAA-GCT-GGC-ATC-CGT-CA) (20-mer), which inhibits the expression of intercellular adhesion molecule-1 (ICAM-1) in mice was chosen as a model sequence. Over expression of ICAM-1 is implicated in a wide variety of inflammatory diseases including transplant rejection, psoriasis, rheumatoid arthritis, asthma and inflammatory bowel disease.

The synthesis was performed on an automated synthesizer[15] (Model Milligen 8800; Millipore, Bedford, MA, USA). Although we are exploring the utility of several different supports, we used mainly controlled-pore glass (CPG) as solid support because of its economic and commercial availability. Also we used phosphoramidite chemistry because of its higher coupling efficiency in comparison with H-phosphonate chemistry. We achieved 99% coupling efficiency in our synthesis by introducing several modifications in the automated synthesis program. The cycle was modified to include sulfurization using Beaucage reagent.[16,17] Better results were observed when detritylation was performed using flow through technique instead of sparging the reactor.[18] Also, better results were obtained (based on PAGE densitometry analysis of crude product) using double coupling of phosphoramidite synthon instead of single coupling with the same three total equivalents of monomer. Since detritylation is faster for purine than for pyrimidine nucleosides,[19] the number of deblockings in step 1 of the synthetic cycle have been optimized accordingly. The average coupling efficiency was found to be 99.3% based on usual spectrophotometric quantitation. Table 1 shows the details of synthesis and Table 2 explains the conditions used for the synthesis of the oligomer. Table 3 shows the reaction conditions for individual steps involved during synthesis.

Recovery and Purification:

At the end of synthesis, the CPG was washed four times with dry acetonitrile and the rinse removed by filtration. The CPG resin with the oligomer attached was then dried by purging the entire reaction vessel with argon for 30 min. and then transferred to a pyrex reaction vessel. Concentrated ammonium hydroxide was added, the bottle sealed and incubated at room temperature for approximately 90 min. to facilitate the cleavage of the polymer from the support and to deprotect the phosphorothioate triester. The crude product was then transferred to a pressure flask and sealed. This ammonium hydroxide cleavage step was repeated one more time. The ammonium hydroxide solution containing the crude product was incubated at 55 °C for 18-24 h to complete the deprotection of exocyclic amino protections and then filtered through a 0.2 μm filter. Triethylamine (ca. 1%) was added and then concentrated on a

Table 1. Synthesis of phosphorothioate

Sequence	d(GCC-CAA-GCT-GGC-ATC-CGT-CA) (20-mer)
Scale (μmole)	2350
CPG Loading (μmole/g)	47
Excess phosphoramidite	3 equivalents
Catalyst	1H-Tetrazole
Sulfurization reagent	Beaucage reagent

Table 2. Conditions for synthesis

Reagent	Condition Utilized
dA phosphoramidite	0.1 M solution in CH_3CN
dC phosphoramidite	0.1 M solution in CH_3CN
dG phosphoramidite	0.1 M solution in CH_3CN
T phosphoramidite	0.1 M solution in CH_3CN
Tetrazole	0.45 M solution in CH_3CN / 10 fold excess
Beaucage reagent	0.05 M solution in CH_3CN / 3 fold excess
Deblock Solution	2.5% CCl_2CO_2H
Cap A	10% Ac_2O / THF
Cap B	10% Py / 10% N-methylimidazole / THF
Acetonitrile	less than 100 ppm of water

Table 3. Reaction conditions for individual steps involved during synthesis

Step No.	Step	Volume	Time	Repetition
1	Deblock (2.5% CCl_2CO_2H/ CH_2Cl_2)	75 ml	90 s	5x for dG 6x for dA 8x for dC 8x for T
2	Wash (CH_3CN)	90 ml	90 s	5x
3	Coupling	2 x 21 ml	3 min + 5 min	2x
4	Wash (CH_3CN)	90 ml	90 s	2x
5	Sulfurization	55 ml	120 s	2x
6	Wash (CH_3CN)	90 ml	90 s	3x
7	Cap A + Cap B (1:1)	86 ml	60 s	2x
8	Wash (CH_3CN)	90 ml	90 s	3x

rotoevaporator. The deprotected crude synthetic product was purified by reverse phase HPLC.[20-23]

Chromatographic Purification

Chromatographic purification of the crude 5'-protected product was accomplished by preparative HPLC methodology, first chromatographing the 5'-DMT protected oligomer, followed by detritylation to afford the product. A Millipore HC-C18 HA Bonda-Pak® octadecylsilyl silica (37-55 m, 125 Å) radial compression column was equilibrated with an initial eluent of water/methanol/2.5 M sodium acetate (72:20:8, v/v/v) on a preparative HPLC. The aqueous sample from the previous recovery and deprotection step was applied to the column at this same initial flow (volume) ratio. The solution was filtered through a 0.2 μ filter before loading onto the column. The column was eluted at a flow rate of approximately 20% column volume per minute (column volumn is 520 ml) with an increasing gradient of methanol in approximately 200 mM sodium acetate (ca. pH 7.2). The eluate was fractionated in approximately 100 mL fractions by an automated fraction collector, and the elution profile was monitored by continuous UV absorbtion spectroscopy. Separation and purification of the fractions were determined by polyacrylamide gel electrophoresis (PAGE) or by capillary gel electrophoresis (CGE). Peak fractions containing product were identified and further processed. Each selected (based on PAGE/CGE in-process control analysis) reverse phase HPLC product fraction was transferred to a centrifuge bottle and glacial acetic acid equivalent to approximately 20% v/v was added. Following 30 minutes at room temperature, detritylated oligomer was precipitated by addition of cold ethanol and the suspension was incubated and stored at -20°C. The resulting precipitate was isolated by centrifugation and redissolved in 0.1 N NaOH. Material from multiple fractions was combined at this point and pooled material was subjected to a second detritylation under the same conditions, followed by ethanol precipitation and centrifugation. The precipitate was dissolved in minimal 0.1 N NaOH and material from multiple fractions was combined at this point. The pH was adjusted to approximately 7.5 with 1-2 N sodium hydroxide. A third ethanol precipitation was carried out from ethanol/aqueous sodium acetate on pooled materials and the resulting solid was dissolved in purified water. This solution of detritylated material was filtered on a 0.2 μ filter and stored at 2-8°C until next step.

Membrane Ultrafiltration:

The purified product was filtered through an ultrafiltration membrane with a 10,000 molecular weight cutoff (Amicon YM10). The aqueous solution was passed through the filter by argon gas pressurization (50 psi).

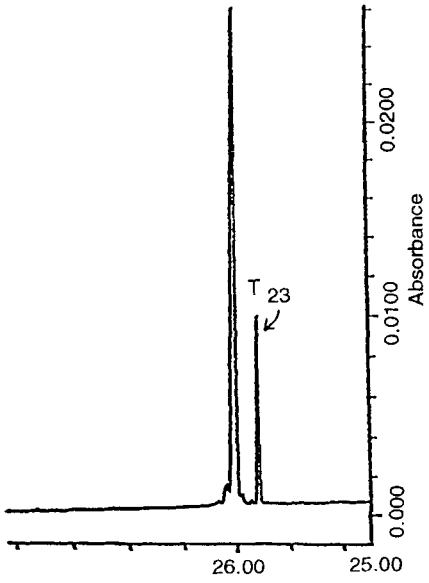

Fig. 1 Capillary Gel Electrophoresis of the synthesized oligomer. T23-mer was used as internal standard.

Desalting/Lyophilization:

Membrane filtered oligonucleotide was desalted and concentrated by dialysis against purified water using 1000 molecular weight cutoff membrane filter (Amicon YM1). The synthesized oligonucleotide in solution was retained by the ultrafiltration membrane. The total volume for dialysis was three to five times the volume obtained in the previous membrane filtration step. Argon gas pressure was used to drive the solution across the membrane (50 psi) and the final product was prepared by freezing the desalted/concentrated solution and lyophilizing in vacuo for 18 h.

Characterization:

The integrity of the purified oligonucleotide was confirmed by capillary gel electrophoresis (Fig. 1) and by deconvoluted electro-spray mass spectroscopy (Fig. 2).[24-29] The oligonucleotide has been further confirmed by base composition analysis[30-31] and by Maxam-Gilbert and MALDI_TOF sequencing.[32-36] Peaks B, C, etc. in the deconvoluted spectrum corresponds to mono, bis, etc. sodium salts of the parent peak. These results indicate the high purity and integrity of oligonucleotide.

Conclusion:

We have developed reaction conditions for the automated synthesis of antisense oligonucleotide phosphorothioates of a typical length of 20 base

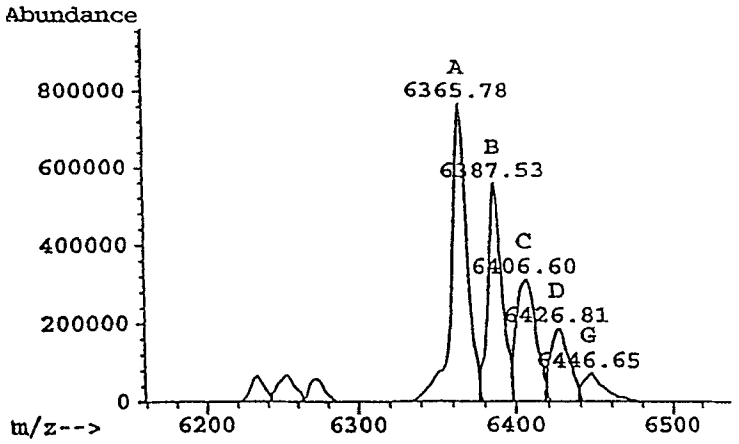

Fig. 2 Deconvoluted electrospray mass spectrum of the synthesized oligomer.

long. As can be seen from above, the synthesis has been made efficient by reducing the equivalents of reagents as well as volume of solvent used. In conclusion we have demonstrated an efficient, economical and large scale synthesis of these first generation of antisense drugs. Compared to the synthesis of oligomers on analytical scale yielding about 1-2 mg of product, we are able to synthesize highly pure oligomers of about 5-6 gm per synthesis using this protocol. Combined with our efforts on the use of Milligen 8800 Plus and/or LSB PCOS-2 automated synthesizers,[37-38] use of high-load CPG and/or highly cross-linked polystyrene solid support which are capable of scaling up oligonucleotides by an order of magnitude and also recent reports on the recovery of excess amidite synthon[39-40] further increase in efficiency, output and continued reduction in the cost[41-43] of these new therapeutic drugs are assured.

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