Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 16, pp. 2017-2022, 1994
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0960-894X/94 $7.00+0.00

0960-894X(94)00279-7

EFFICIENT SYNTHESIS OF ANTISENSE OLIGODEOXYRIBONUCLEOTIDE PHOSPHOROTHIOATES[1]

Mark Andrade, Anthony Scozzari, Douglas L. Cole, & Vasulinga T. Ravikumar*
Isis Pharmaceuticals
2292, Faraday Ave. Carlsbad, CA 92008

Abstract: Efficient and economical synthesis of antisense oligodeoxyribonucleotide phosphorothioates utilizing 2 equivalents of phosphoramidite synthon is reported.

Modulation of gene expression by antisense oligonucleotides requires the development of modified oligonucleotides which have increased nuclease resistance, bind to complimentary nucleic acid targets effectively, and also are taken up by cells.[2-10] Among the modifications reported to date, uniformly modified oligodeoxyribonucleotide phosphorothioates have been the first class of compounds to reach the clinic. Both animal data[11] and clinical findings[12] demonstrate the therapeutic potential of oligonucleotide drugs. It is thus of prime importance to develop low-cost, scaleable oligodeoxyribonucleotide phosphorothioate manufacturing technologies. Typically in a solid supported synthesis of oligonucleotides utilizing the phosphoramidite approach, several equivalents of monomeric synthon have been used, presumably to drive the reaction to completion. Given that large scale synthesis of oligonucleotides is needed (100 to 500 gm) for the clinical evaluation of these potential therapeutic agents, the waste of expensive monomeric synthon could be a significant development cost. Consequently, the development of a synthesis using fewer equivalents of synthon would be desirable. We report herein our results on efficient and economical large scale synthesis of oligodeoxyribonucleotide phosphorothioates at reduced amidite excess.

Oligodeoxyribonucleotide phosphorothioate S-d(GCG-TTT-GCT-CTT-CTT-CTT-GCG) (1) and S-d(GCC-CAA-GCT-GGC-ATC-CGT-CA) (2) were chosen as examples. The former sequence, targeted to therapy of CMV retinitis is in Phase I/II clinical trials and the latter for suppression of ICAM expression, is in preclinical trials. The syntheses of both sequences were performed using 11 gm batches of controlled-pore glass (CPG; 480 μmole/synthesis) on a Milligen 8800 automated synthesizer using a modified cycle to include sulfurization using

Beaucage reagent.[13,14] Standard commercially available phosphoramidite synthons were used for syntheses. Table 1 shows conditions used for the synthesis of sequences (1) and (2). Table 2 shows the amount of each synthon used and the results of coupling for both sequences. As shown, synthesis using only two equivalents of each amidite has produced excellent average coupling efficiency (ACE) of 99.1 and 99.3 for sequences (1) and (2) respectively. It was observed that better results in terms of full length content were obtained (based on PAGE densitometry analysis of crude product) using double coupling of amidite synthon instead of single coupling with the same two total equivalents of monomer. Also, better results were obtained when detritylation was performed using flow through technique instead of sparging the reactor.

Table 1. Conditions for synthesis of sequences (1) and (2).

Reagent	Condition Utilized
Std. dA phosphoramidite	0.1 M solution in CH_3CN
Std. dC phosphoramidite	0.1 M solution in CH_3CN
Std. dG phosphoramidite	0.1 M solution in CH_3CN
Std. T phosphoramidite	0.1 M solution in CH_3CN
Tetrazole	0.45 M solution in CH_3CN / 20 fold excess
3H-1,2-Benzodithiol-3-one 1,1-dioxide	0.05 M solution in CH_3CN / 6 fold excess
Deblock Solution	2.5% CCl_2CO_2H
Cap A	Ac_2O / THF (1:9 v/v)
Cap B	Py / N-methylimidazole / THF (1:1:8 v/v)

Table 2. Equivalents of amidite synthon used and the yields of oligos.

Sequence	Total Equivalents of Synthon Used				Average Coupling Efficiency*
	dA	dG	dC	T	
(1)	2	2	2	2	99.1 %
(2)	2	2	2	2	99.3 %

*Based on usual spectrophotometric quantitation utilizing double coupling of amidite synthon and detritylation being perfomed using flow through technique.

Polyacrylamide gel electrophoreses analyses of the crude synthesized oligomers are shown in Fig 1.

Fig. 1. PAGE analyses of sequences (1) (lanes 3 & 4)) and (2 (5 to 8)). T_{23} = lanes 1 & 2.

The crude oligomers were purified by reverse-phase and then by strong anion-exchange HPLC since the latter purifcation is considered to be a more accurate and precise quantitation[15] of the monophosphodiester content caused by non-quantitative sulfurization by Beaucage reagent (Fig. 2). The oligomers of interest were characterized using capillary gel electrophoresis[16] as shown in Fig. 3. Homothymidine 23-mer (T_{23}) was used as internal reference in both cases.

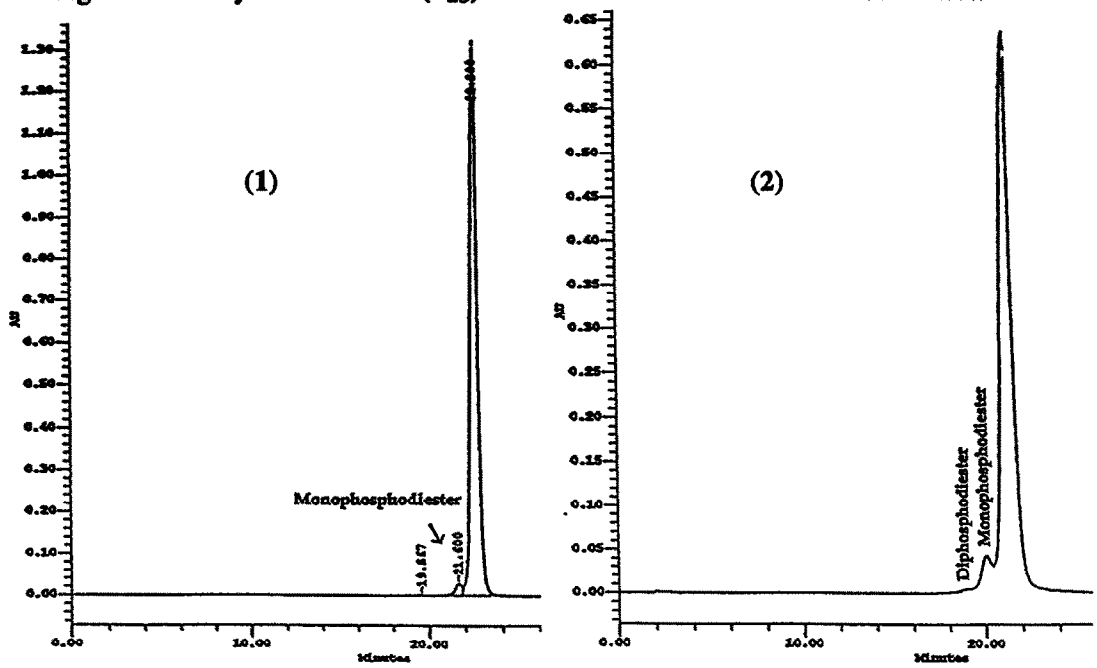
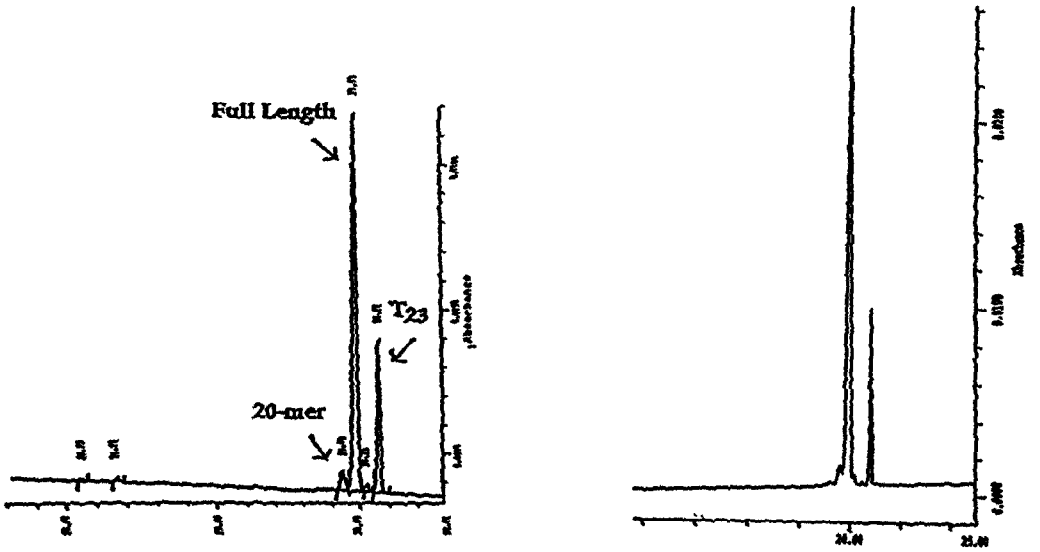


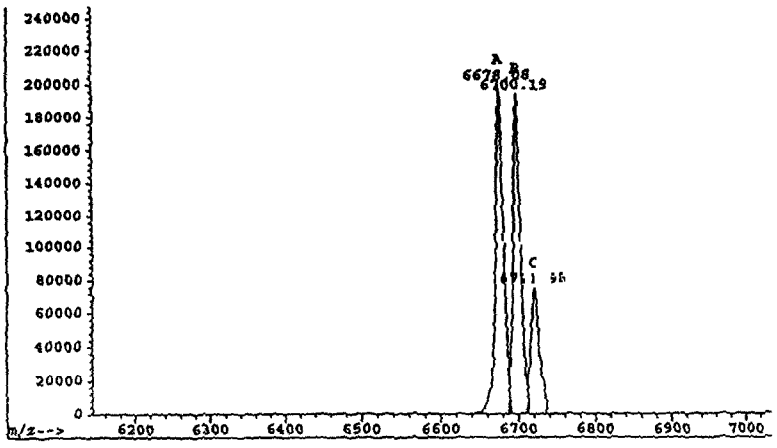
Fig. 2. SAX-HPLC profile of sequences (1) and (2).



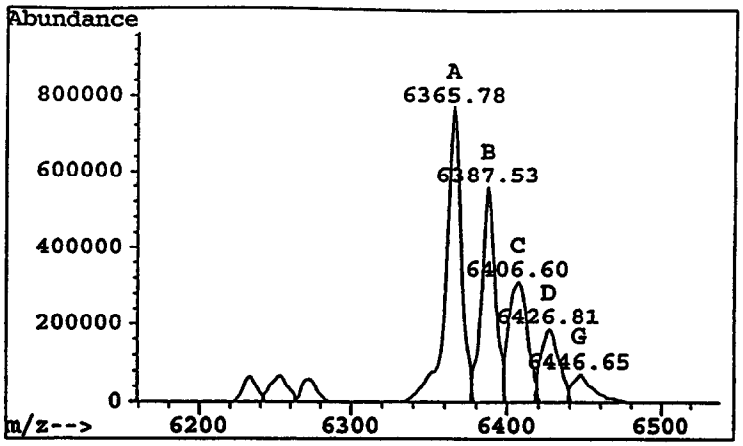
Sequence (1) Sequence (2)

Fig. 3. Capillary gel electrophoresis (CGE) analyses of sequences (1) and (2).

The base composition analyses[17,18] and sequencing of both the oligomers were performed and *shown* to have correct base ratio using only two equivalents of phosphoramidite synthon. Sequences (1) and (2) have been previously[19] determined by MALDI-TOF mass spectrum and were confirmed in this work using deconvoluted electrospray mass spectrum (Fig. 4.). Peaks B, C, etc. in both the spectrum corresponds to mono, bis, etc. sodium salts of the parent peak.



Sequence (1)



Sequence (2)

Fig. 4. Deconvoluted electrospray mass spectrum of sequence (1) and (2).

In conclusion we have demonstrated that for efficient and economical synthesis of oligodeoxyribonucleotide phosphorothioates, two equivalents of phosphoramidites are sufficient.[20] Promising work to further reduce amidite excess and perform synthesis beyond 480 μmol scale are in progress.

Acknowledgments:

The authors wish to thank Mike Bhatt and Patrick Klopchin for their help.

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(*Received in USA* 19 *May* 1994; *accepted* 11 *July* 1994)