CROATICA CHEMICA ACTA CCACAA **74** (4) 779–786 (2001)

ISSN-0011-1643
CCA-2766 *Original Scientific Paper*

Large-scale Solution Synthesis of Phosphorothioate Oligonucleotides: A Comparison of the Phosphoroamidite and Phosphotriester Dimeric Approaches

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Received February 5, 2001; revised June 13, 2001; accepted June 18, 2001

Two different approaches to the solution synthesis of oligonucleotides are compared. Both procedures use dimer synthons as building blocks to save time and to improve the final product yield and quality. These strategies look quite promising due to their easy upscaling, providing an advantageous alternative to the well-established solid-phase methods.

Key words: oligonucleotides, phosphorothioate, scale up, large-scale, synthesis, phosphoramidite, phosphotriester, liquid-phase, polyethylene glycol.

INTRODUCTION

Synthesis of oligonucleotides is turning into a large scale production, since they are increasingly demanded as new powerful drugs, as well as probes and primers in diagnostic kits.[1] Synthesis of large quantities of these nucleic acid derivatives is a prerequisite for their successful commercialization, especially as antisense drugs, which represent their widest field of application.[2]

A simple and cost-efficient method is clearly the critical factor for a profitable production of oligonucleotide-based therapeutics. On the other hand,

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modulation of gene expression by synthetic oligonucleotides is required for specifically modified molecules. In particular, an increased nuclease resistance is required, preserving the complexation capability toward complementary natural nucleic acid targets.[3] Among the modifications reported to date, the phosphorothioate analogues are the first compounds that reached first the clinic, and, very recently, the market.[4,5]

The most diffused approach for the preparation of these biopolymers is the solid supported synthesis based on the phosphoroamidite approach, which requires quite a large amount of expensive syntons to drive each reaction step to completion.[6] Development of processes requiring fewer equivalents of these reagents is obviously most desirable, and was recently investigated.[7] However, despite a successful reduction of reagent consumption, the solid-phase method, based on inert, insoluble supports, suffers from some unavoidable drawbacks as a consequence of the heterogeneity of the reaction. For this reason, it should be desirable to develop alternative processes capable of being performed in homogeneous media. It is reasonable to expect that a large upscaling of solution-based syntheses could be easily and efficiently set up, following the general rules of the classical organic reactions, allowing adequate planning of industrial production.

A possible solution for the disadvantages generated by the Merrifield-like methods is the use of soluble polymeric supports that grant the homogeneity of the synthetic process and assure an easy intermediate purification step through simple precipitation-and-filtration procedures.[8] This approach, named soluble polymer-assisted or liquid-phase synthesis, in analogy to the solid-phase one, is particularly efficient for the oligonucleotide production, even at a large scale. The support of choice is the poly(ethylene glycol) or PEG, which offers unusual, but advantageous, physico-chemical properties.[9] The synthetic method, called HELP or high efficiency liquid phase, adopts the standard phosphoroamidite-based chemistry commonly employed in the solid-phase procedures.[10] Recently, the HELP approach was upscaled for the millimolar-scale synthesis on a bench of phosphorothioate oligonucleotides up to 20 monomers in length.[11] As a further improvement, fully protected dimers were used as synthons to reduce the number of coupling steps and ameliorate the purity profile of the final crude material.[12]

Reese and coworkers have recently proposed an efficient, different solution to the above-mentioned drawbacks.[13] Their approach uses phosphotriesters as building blocks in a classical solution synthesis. Following this block condensation strategy, a quite large scale production of a fully thioate octamer can be accomplished. Interestingly, the same octamer was also the subject of an investigation aimed at the large-scale development of the PEG-based supported synthesis.

On these bases, it appeared worthwhile to compare the two synthetic procedures in order to delineate the advantages and disadvantages of these solution-based methods and highlight their useful properties compared to the well-founded solid-phase procedures.

EXPERIMENTAL

The synthesis of phosphoramidite dimers was performed as described.[12] The HELP protocol was easily transferred to the dimeric synthons following the basic procedure,[10] and details of the experimental procedures are reported.[11] All the experimental procedures required for the solution synthesis of an oligonucleotide *via* phosphothiotriester intermediates are also extensively described.[13] Chromatographic analyses and purifications, as well as spectrophotometric and NMR data, are reported in the references.

RESULTS AND DISCUSSION

The common feature of the two synthetic strategies here examined is the use of pre-built dimeric synthons for the assembling of the same fully thioate sequence, that is d($T_2G_4T_2$).

In the case of phosphoramidite-based units, this procedure derives from the problem commonly experienced in automated synthesis *via* monomeric units including formation of a population of deleted sequences. This phenomenon is mainly due to less-than-quantitative coupling yields, incomplete intermediate reactions as well as defects during the work-up protocol. During the final purification procedure, a rather poor efficiency of the resolution of (*n*-1)mer and *n*mer is observed. Moreover, side reactions during sulfurization steps must also be considered, introduced the presence of unwanted phosphodiester linkages. If all these steps are the main cause of the reduced homogeneity of the final crude product, the blockmer coupling strategy could be a way of decreasing the number of the related side products by reducing the number of the overall intermediate steps required. However, it is obvious that the use of dimers will reduce the overalls steps of the supported synthesis, but it will require a more time-demanding procedure for the preliminary preparation of the synthons needed. In our case, the 5'-DMT-protected, 3'-*O*-2-cyanoethyl-*N*-diisopropylphosphoramidite dimeric units were successfully employed following the standard HELP-based oligouncleotide synthesis, without any problem of solubility or removal of the excess from the reaction mixture. Addition of 3 equivalents of amidites, with regard to the PEG-bound functional groups, in the presence of 1*H*-tetrazole, gave very satisfactory coupling yields, as shown in Table I. Moreover, the reduced number of individual steps increased the amount of the product re-

covered, as shown in Table II. In fact, during the liquid-phase synthesis, precipitation of the PEG-bound product is required after each step, followed by its filtration to remove all soluble by-products and the residual excess of reagents. During these operations, some loss of material as a function of the solubility product of the growing oligonucleotide bound to PEG is clearly present, even if low.

TABLE I

Single step and average yields, deduced from DMT absorption, of 8mer phosphorothioate synthesized on MPEG (M_r = 5,000)

Reaction step	Yield / %
MPEG-OH + d(T) dimer	100
MPEG-2mer + d(G) dimer	99
MPEG-4mer + d(G) dimer	99
MPEG-6mer + d(T) dimer	98
Average step yield value	96.5

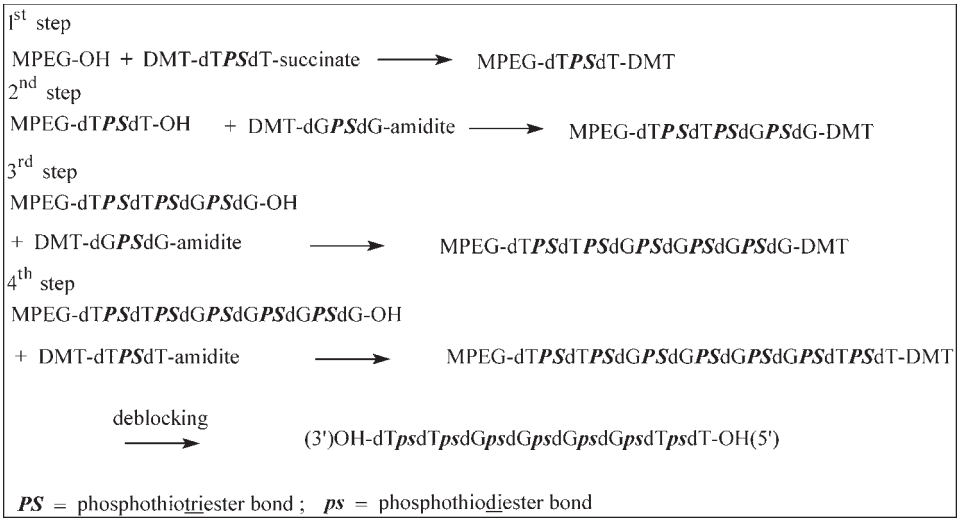
TABLE II

Expected and recovered quantity of MPEG-oligomers, fully protected, starting from 5.0 g of MPEG (M_r = 5,000) [MPEG = monomethoxypoly(ethylene glycol)]

Reaction step	Expected amount / g	Recovered amount / g
MPEG-OH + d(T) dimer	6.00	5.56
MPEG-2mer + d(G) dimer	6.94	6.19
MPEG-4mer + d(G) dimer	7.87	6.73
MPEG-6mer + d(T) dimer	8.62	7.17

The scheme of the overall process is reported in Scheme 1.

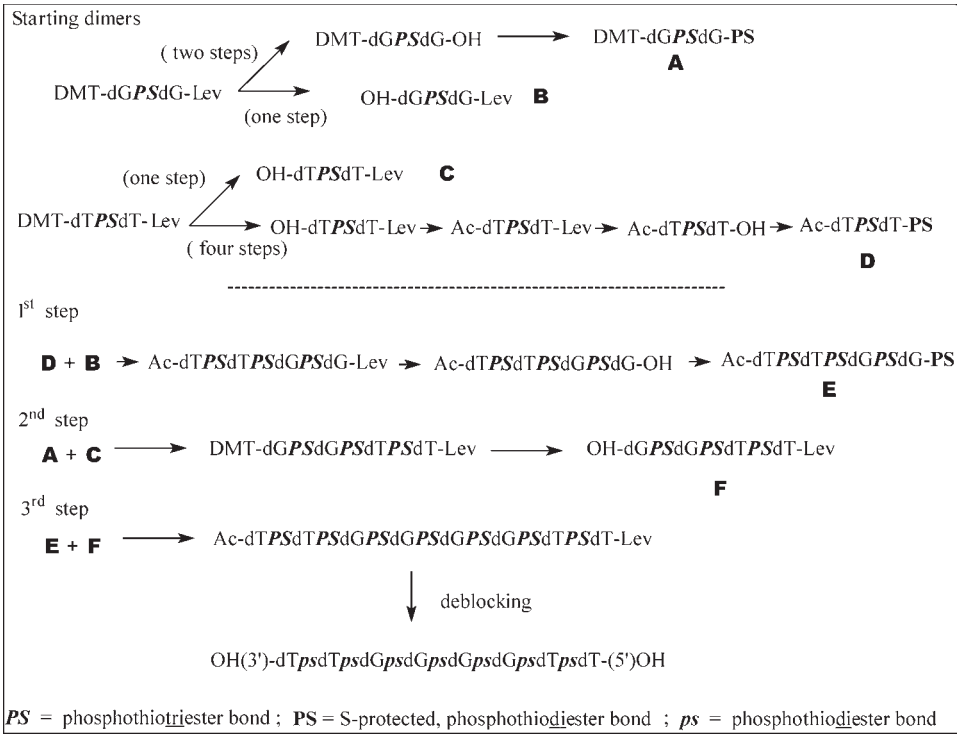
Reese *et al.* proposed a different strategy for the application of a solution procedure based on phosphotriester chemistry. This chemistry is traditionally adopted for the classical solution synthesis of oligonucleotides because of the more advantageous stability and solubility properties of synthons. The key reaction involves coupling between a protected dimer terminating in a 3'-(*S*-2-cyanoethyl)phosphorothioate and a protected dimer terminating in a free 5'-OH function. In this case, the coupling reaction is promoted by an activating mixture (mesitylene-2-sulfonyl chloride and 3-nitro-1,2,4-1*H*-triazole). The main difference in this case is the use of a blockmer of increased size, as the chain is growing, as depicted in Scheme 2. It is quite obvious



Scheme 1. Scheme of the large-scale synthesis of the 8mer phosphorothioate using phosphoramidite dimeric synthons in the liquid-phase procedure.

that when, as for the 8mer under study, a symmetrical sequence is constructed, it is in principle possible to save a further, intermediate coupling step by using the same tetramer synthesized during the process. On the other hand, the general features of the liquid-phase synthesis are common to all the supported procedures and, consequently, all of the intermediate sequences cannot be used as new synthons in the overall synthesis planning. However, in this case, both tetramers are separately synthesized since, as described in Scheme 2, the two final building blocks have the same nucleotide composition, but different 5'- and 3'-protecting groups. In fact, the sequence is self-complementary and consequently the two tetramers have the same order of nucleotides, but a different direction. Moreover, in the phosphotriester method, the two fully protected starting dimers need to be further reacted to remove the terminal protecting groups and introduce the S-protected, phosphothioate moiety. This must be taken into account when the overall yields given by the two procedures are compared. Interestingly, from the IE HPLC profile of the crude oligonucleotide obtained from the liquid-phase method, it can be estimated that at least 35% of the mixture is on charge of the pure product (data not shown). This value is almost coincident with the overall yield obtained from Reese's procedure, as deduced from the data reported in Table III.

The main advantage of phosphotriesters is certainly a highly reduced excess (1.2–1.3 fold) required during each coupling of 3'-phosphoroester.



Scheme 2. Scheme of the large-scale synthesis of the 8mer phosphorothioate using phosphotriester dimeric synthons in the classical solution procedure.

TABLE III

Reaction yields and product quantity from the phosphotriester-based process (calculated)[13]

Dimers (from 1.0 g of fully protected DMT-dimer-Lev)	Yield / %	Amount of purified product / g
A	96	0.77
B	83	0.91
C	98	0.77
D (from 1.0 g of **C**)	85	0.97
Coupling steps		
1st step **D** + **B** = **E**	69	1.03
2nd step **A** + **C** = **F**	80	0.82
3rd step **E** + **F** = 8mer	65	0.87

This will save expensive reagents that can be hardly recovered, but it must be underlined that an increased excess will push yields to higher values and must be avoided since it can hamper all the intermediate purification steps. In fact, the main difference from the classical solution processes and supported syntheses is the requirement, for the former, of laborious two-solvent extractions and chromatographic purifications, instead of handy precipitation-and-filtrations. From the reported data, it can be easily calculated that to obtain 1.0 g of pure 8mer, the same amount of starting dimeric blocks must be utilized, that is, about 4.0 grams.

Another point is the solubility property of the larger oligonucleotide when used as a building block. It is well known that increasing the chain length will reduce the solubility properties of the oligonucleotide inside the reaction media, with a concomitant reduction of the coupling step efficiency.[14] The lipophilicity of the fully protected oligonucleotide can be enhanced through the introduction of proper protecting moieties, however the synthesis of the long oligonucleotide chain, up to 20mers, can be hardly devised in a classical solution procedure. On the other hand, the use of soluble, solubilising supports, as PEG, can eliminate this drawback since the larger polymeric supporting unit will drive the solubility properties of the growing oligonucleotide, if its size is properly chosen at the beginning of the synthesis. Unfortunately, the longer the oligonucleotide to be prepared, the larger is the PEG to be used; consequently, the amount of the derivative produced is proportionally reduced, since no more than two anchoring functional groups are present in a linear, commercial PEG. Moreover, the use of PEG, as well as of any other support, adds a further step for its final removal, and, more important, proper recovery and recycling required for economical reasons.

In conclusion, both the described solution syntheses appear to be superior to the solid-phase method in the large-scale preparation of oligonucleotide and oligonucleotide phosphorothioates of moderate size, since the upscaling of the organic solution syntheses is properly designable without special apparatus. On the other hand, it is well known that the Merrifield-like procedures can be readily automated, allowing very fast and efficient coupling reactions along with a more friendly operativity, which does not demand any special skill. Large scale synthesizers operating on an up to 0.2 mol scale are currently available, but it is quite hard to imagine a synthesizer that could furnish all the quantity of the oligonucleotide derivative needed for the market in one, or a few, single batches.

As recently reported, we agree that »further oligonucleotide manufacturing processes are likely to integrate a variety of options, ultimately delivering safe and cost-effective methods for large-scale production of oligonucleotides«.[15]

Acknowledgement. – We are greatly indebted to Prof. Reese and his coworkers who, with their synthetic efforts at the same target, gave us the opportunity for a better evaluation of the advantages inherent to the solution-based protocols for oligonucleotide synthesis.

REFERENCES

1. H. Seliger, *Methods in Molecular Biology*, Vol. 20, S. Agrawal (Ed.), Humana Press Inc, Totowa, New Jersey (USA), 1993, pp. 391–435.
2. S. T. Crooke, *Therapeutic Applications of Oligonucleotides*, S. T. Crooke (Ed.), R. G. Landes Co., Austin (USA), 1995.
3. P. H. Seeberger and M. H. Caruthers, *Applied Antisense Oligonucleotide Technology*, C. A. Strein and M. D. Krieg (Eds.), Wiley-Liss, New York (USA), 1998, pp. 51–72.
4. Y. S. Sanghvi, M. Andrade, R. P. Deshmukh, L. Holmberg, A. N. Scozzari, and D. L. Cole, *Manual of Antisense Methodology*, G. Hartman and S. Endras (Eds.), Kluwer Academic Publisher, Dordrecht (The Netherlands), 1999, pp. 3–23.
5. D. L. Cole, presented at *IBC's 3rd International Conference on Oligonucleotide Technology*, San Diego (USA), 1999.
6. S. L. Beaucage and P. P. Iyer, *Tetrahedron* **49** (1993) 6123–6154.
7. M. Andrade, A. N. Scozzari, D. L. Cole, and V. Ravikumar, *Bioorg. Med. Chem. Lett.* **4** (1994) 2017–2022.
8. E. Bayer and M. Mutter, *Nature* **237** (1972) 512–513.
9. J. M. Harris, *Poly(ethylene glycol) Chemistry. Biotechnical and Biomedical Applications*, J. M. Harris (Ed.), Plenum Press, New York (USA), 1992, pp. 1–14.
10. G. M. Bonora, *Appl. Biochem. Biotechnol.* **54** (1995) 3–17.
11. G. M. Bonora, R. Rossin, S. Zaramella, D. L. Cole, A. Eleuteri, and V. Ravikumar, *Org. Proc. Res. Dev.* **4** (2000) 225–231.
12. A. H. Krotz, P. Klopchin, D. L. Cole, and V. Ravikumar, *Bioorg. Med. Chem. Lett.* **7** (1997) 73–78.
13. C. B. Reese, Q. Song, V. M. Rao, and I. Beckett, *Nucleosides & Nucleotides* **17** (1998) 451–470.
14. J. K. Mihaichuk, T. B. Hurley, K. E. Vagle, R. S. Smith , J. A. Yegge, G. M. Pratt, C. J. Tompkins, D. P. Sebesta, and W. A. Pieken, *Org. Proc. Res. Dev.* **4** (2000) 214– 224.
15. Y. S. Sanghvi, *Org. Proc. Res. Dev.* **4** (2000) 168–169.

SAŽETAK

Sinteza fosfortioat-oligonukleotida u otopini na veliko: usporedba fosforamidilnog i fosfortriesterskog dimernog pristupa

Gian Maria Bonora, Simone Zaramella i Vasulinga Ravikumar

Opisana su i uspoređena dva različita pristupa sintezi oligonukleotida u otopini. Oba pristupa zasnivaju se na primjeni sinteze dimera kao građevnih blokova, čime se postiže ušteda u vremenu, te poboljšano iskorištenje i kvaliteta konačnog produkta. Opisanim se strategijama lako može povećati mjerilo, što je ujedno i njihova prednost pred dobro poznatim (uobičajenim) metodama sinteze u krutoj fazi.