Glen Research

REPORT

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Chemical Modification of the 5'-Terminus of Oligonucleotides

In the previous issue of the *Glen Research Report*, we described the use of nucleoside H-phosphonates to effect modification at the phosphate backbone of synthetic oligonucleotides. Specifically, the preparation of phosphorothioate, phosphotriester, and phosphoramidate linkages was described using H-phosphonates, while the remainder of the synthetic oligonucleotide was prepared by phosphoramidite chemistry. In this issue, we focus on reagents and techniques which may be used for modification of the 5'- or 3'-terminus of a synthetic oligonucleotide.

Introduction

As the use of synthetic oligonucleotides in biomedical research becomes more and more sophisticated, the need for rapid and simple modification of oligonucleotides becomes increasingly apparent. Because conventional automated synthesis proceeds from 3' to 5', the 5'-terminus is clearly readily available for manipulation. Indeed, the ability to attach a suitable molecule to the 5'-terminus for use as a label is significant in the continuing development of non-radioactive probes and in DNA sequencing.

The reactivity of the 5'-hydroxyl group of oligonucleotides is not sufficiently different from the other active groups to allow attachment of labels directly to the 5'-position. Consequently, several modifications, including 5'-amines[1] and 5'-thiols[2], have been examined. There are, however, several disadvantages to direct substitution of the 5'-hydroxyl group: 1) the resulting link between the oligonucleotide and an attached molecule is short, an undesirable feature in many applications; and 2) the phosphoramidites of all four modified nucleosides must be prepared to use present day synthesis protocols and still permit flexibility of sequence.

References: 1a. L.M. Smith, S. Fung, M.W. Hunkapiller, T.J. Hunkapiller, and L.E. Hood, *Nucleic Acids Res.*, 1985, **13**, 2399.
1b. B.S. Sproat, B. Beijer, and B. Rider, *Nucleic Acids Res.*, 1987, **15**, 6181.
2. B.S. Sproat, B. Beijer, B. Rider, and P. Neuner, *Nucleic Acids Res.*, 1987, **15**, 4837.

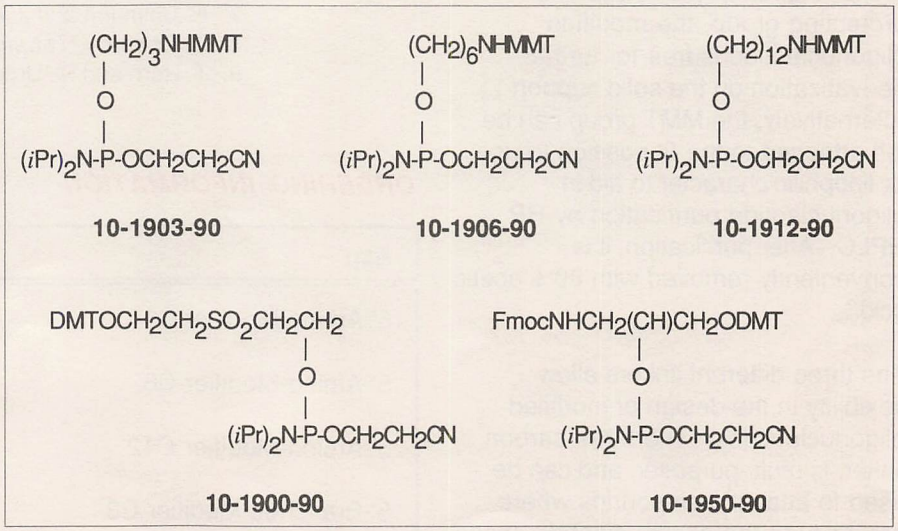

Figure 1: *Structures and Catalog Numbers of 5'-Modifiers*

5'-Modifiers

A more general approach to the modification of the 5'-terminus is to use reagents which would couple to the 5'-hydroxyl of an oligonucleotide. To be most effective, these reagents should be compatible with automated DNA synthesizers. A method which satisfies the above criteria is the use of phosphoramidite reagents, as shown in Figure 1 overleaf, which are readily adapted for use in automated synthesizers with little or no modification to existing protocols.

5'-Amino-Modifiers

5'-Amino-Modifiers are ß-cyanoethyl (CE) phosphoramidites which, when activated with 1H-tetrazole, can couple to the 5'-terminus of the oligonucleotide in the same time frame and with similar efficiency as nucleoside phosphoramidites.

The linker portion of the 5'-Amino Modifiers which Glen Research has made available, consists of an alkyl chain of three[3], six, or twelve carbons attached to phosphorus as a CE phosphoramidite. The primary amine is blocked with the monomethoxytrityl (MMT) group which is a more stable amine protecting group than dimethoxytrityl (DMT) and, therefore, more likely to survive synthesis procedures. It can be removed in the final step of the synthesis while the oligonucleotide is still support-bound. (Following removal of the amine protecting group, the modified oligonucleotide is free for further derivatization on the solid support.) Alternatively, the MMT group can be left attached to the 5'-position to use its lipophilic character to aid in oligonucleotide purification by RP HPLC. After purification, it is conveniently removed with 80% acetic acid.

The three different linkers allow flexibility in the design of modified oligonucleotides. The three carbon linker is multi-purposed and can be used to attach[4] compounds where proximity to the oligonucleotide poses no problem. The longer carbon chain linkers have specific uses in affinity chromatography where the oligonucleotide must be adequately spaced from the support surface, and for labeling with biotin[5] or fluorescent labels where interaction with the oligonucleotide, or the duplex it forms, may quench some of the fluorescence.

5'-Branched Modifier

The 5'-Branched Modifier is a three carbon linker molecule with a primary amine protected with base-labile Fmoc and a primary hydroxyl protected with DMT. This compound can be added to the 5'-terminus during several cycles to generate multiple amine functionalities. Also, it can be used in combination with a 5'-Amino-Modifier or even protected nucleoside phosphoramidites. After removal of the Fmoc protecting group(s), a modified oligonucleotide with multiple primary amines is obtained. In this way, a modified oligonucleotide can be designed such that the addition of several molecules of biotin or a fluorescent label may enhance its sensitivity in a particular assay procedure.

5'-Chemical Phosphorylation Reagent

Chemical phosphorylation has been described[6] in the past but has not supplanted the enzymatic technique. Two new methods recently described also have significant shortcomings. In one method[7], no simple means of following coupling efficiency exists and, in the other[8], the deprotection step requires the use of additional reagents, i.e., iodine or silver nitrate. These limitations were recently solved[9] by the 5'-Chemical Phosphorylation Reagent. This reagent can be used with a standard phosphoramidite protocol and has two significant attributes. It contains a DMT group which can be removed to determine coupling efficiency, and a sulfonylethyl group which is conveniently removed by ß-elimination with ammonia at the same time as the cyanoethyl protecting groups. These features allow complete compatibility with automated DNA synthesis.

Glen Research offers these 5'-modifiers, packaged in industry-standard vials. They are available in 100µM amounts, allowing approximately five couplings on a 1µM scale, and in bulk for custom orders.

References:
3. B.A. Connolly, *Nucleic Acids Res.*, 1987, **15**, 3131.
4. E. Jablonski, E.W. Moomaw, R.H. Tullis, and J.L. Ruth, *Nucleic Acids Res.*, 1986, **14**, 6115.
5. T. Kempe, W.I. Sundquist, F. Chow, and S. Hu, *Nucleic Acids Res.*, 1985, **13**, 45.
6. F. Eckstein and M. Goumet, *Nucleic Acids Chem.*, 1978, **2**, 861.
7. E. Uhlmann and J. Engels, *Chemica Scripta*, 1986, **26**, 217.
8. B. Connolly, *Tetrahedron Lett.*, 1987, **28**, 463.
9. T. Horn and M. Urdea, *Tetrahedron Lett.*, 1986, **27**, 4705.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price ($)
5'-Amino-Modifier C3	10-1903-90	100µM	50.00
5'-Amino-Modifier C6	10-1906-90	100µM	60.00
5'-Amino-Modifier C12	10-1912-90	100µM	90.00
5'-Branched Modifier C3	10-1950-90	100µM	90.00
5'-Chemical Phosphorylation Reagent	10-1900-90	100µM	60.00

Use of 5'-Modifiers

Solution Preparation

Glen Research 5'-Modifiers are designed for use in automated synthesizers to functionalize the 5'-terminus of a target oligonucleotide. With the exception of the 5'-Branched Modifier, they are viscous oils. The vials should therefore be stored upright to allow the oil to gather in the bottom. To prepare the solution, add 1mL of anhydrous acetonitrile to each vial and swirl the liquid occasionally for about 5 minutes to ensure complete dissolution of the oil. The concentration of the resulting solution is 0.1M and this is suitable for use in any DNA synthesis system.

Coupling Reaction

Since the 5'-Modifiers are CE phosphoramidites they are conveniently coupled to the 5'-terminus of a synthetic oligonucleotide. No change from standard addition protocols is required.

Deprotection and Purification

The terminal DMT or MMT group can be removed on the synthesizer by the standard deblocking method. Note that the solution of MMT cation produced by acid deprotection is yellow and can not be quantified by internal trityl monitors.

The Fmoc protecting group of the 5'-Branched Modifier is removed during base deprotection with ammonia.

Alternatively, after iodine oxidation and ammonia deprotection, the synthetic oligonucleotide can be purified by RP HPLC. The MMT group is then removed using acetic acid : water (80:20) at room temperature for 1 hour.

Chemical Modification of the 3'-Terminus

Techniques and reagents for the modification of the 5'-terminus and the phosphate backbone of synthetic oligonucleotides have been described in this and the previous issue of the *Glen Research Report* and are in fairly widespread use. However, interest in the development of anti-sense oligonucleotides has focused attention on the ability to attach marker and carrier molecules to the 3'-terminus. The 3'-hydroxyl group, although accessible for chemical modification, does not support specific reaction at that position because of the presence of competitive reactive centers. It may be possible to design a support which would allow the protected oligonucleotide to be released while all other protecting groups remain intact. Chemical modification reactions could then proceed at the free 3'-hydroxyl group. To our knowledge, no such support has been described.

Reaction at the 3'-Terminus

One approach to 3'-modification is to prepare an oligonucleotide with a ribonucleoside terminus. Periodate oxidation of the 2',3'-diol cleaves the 2'-3' bond and generates reactive aldehyde groups which are available for specific chemical manipulation, as shown in Figure 2.

Two recent papers[1,2] describe the attachment of a ribonucleoside to the 3'-terminus of an oligodeoxynucleotide using T4 RNA ligase. Following periodate oxidation, the cleaved ribose ring can be converted to a morpholino ring by the addition of a primary amine. In this way, the authors describe the attachment of poly (L-

References:
1. M. Lemaitre, B. Bayard, and B. Lebleu, *Proc. Nat. Acad. Sci. USA*, 1987, **84**, 648.
2. M. Lemaitre, C. Bisbal, B. Bayard, and B. Lebleu, *Nucleosides & Nucleotides*, 1987, **6**, 311.

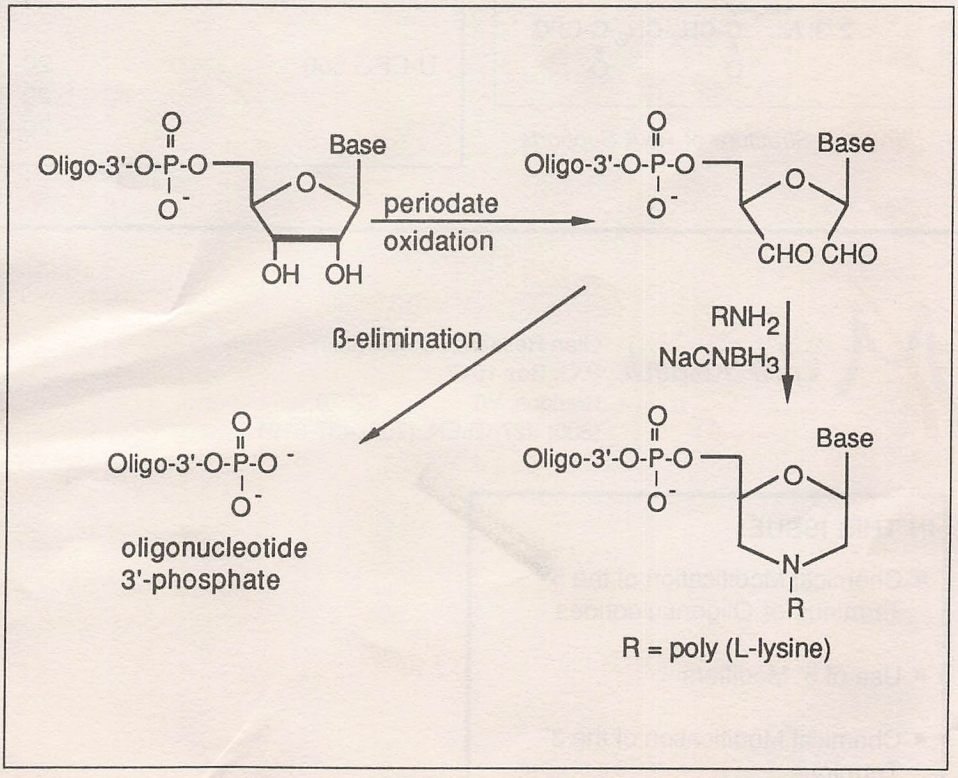

Figure 2: *Reactions of 3'-Terminal Ribonucleoside*

lysine) at the 3'-terminus of an anti-sense oligonucleotide. The addition of the macromolecular carrier significantly improved intracellular delivery of the anti-sense oligonucleotide.

Similarly, other molecules could be attached through a primary amine, creating the opportunity to label oligonucleotides at the 3'-terminus. Also, the possibility for direct attachment of an oligonucleotide to an amino support to form an affinity support exists.

3'-Phosphate

One of the earliest uses[3] for the ribonucleoside terminus was in the preparation of oligonucleotide 3'-phosphates. Following standard deprotection, the ribonucleoside 2',3'-diol is oxidatively cleaved with periodate. Then, under basic conditions, ß-elimination of the remnants of the ribonucleoside results in the oligodeoxynucleotide 3'-phosphate (Figure 2). The 3'-phosphate has been used to reconnect the purified oligonucleotide to a support for use in affinity chromatography.

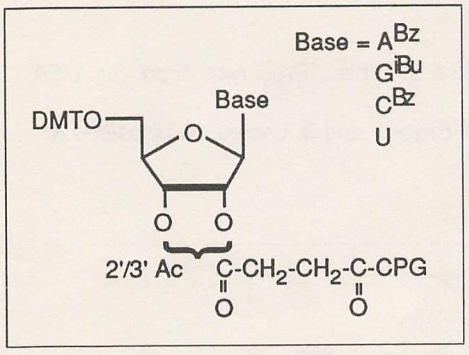

Figure 3: *Structure of RNA Supports*

RNA Supports

A simpler approach to the preparation of an oligonucleotide containing a ribonucleoside terminus is to carry out DNA synthesis on an RNA support.

Glen Research recently introduced RNA supports in which protected ribonucleosides are attached to the most popular CPG support using an aminopropyl spacer. With 5'-DMT protection and all other protecting groups base-labile, the use of these supports is identical to the corresponding DNA supports. The structure of the ribonucleoside supports is shown in Figure 3. These supports are suitable for use in producing oligodeoxynucleotides modified at the 3'-terminus but are, of course, also used in automated RNA synthesis.

References: 3. G.R. Gough, M.J. Brunden, and P.T. Gilham, *Tetrahedron Lett.*, 1981, **22**, 4177.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
A-CPG 500	20-3000-01	0.1g	40.00
	20-3000-02	0.25g	95.00
	20-3000-10	1.0g	355.00
C-CPG 500	20-3010-01	0.1g	40.00
	20-3010-02	0.25g	95.00
	20-3010-10	1.0g	355.00
G-CPG 500	20-3020-01	0.1g	40.00
	20-3020-02	0.25g	95.00
	20-3020-10	1.0g	355.00
U-CPG 500	20-3030-01	0.1g	40.00
	20-3030-02	0.25g	95.00
	20-3030-10	1.0g	355.00

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