Glen Research

REPORT

Volume 4, Number 2 October 1991

Biotinylation in the Sequence and at the 3'-Terminus

Despite their relatively high price, biotin phosphoramidites have achieved rapid and enthusiastic acceptance in the research community. This is undoubtedly because of the simplicity with which these products are adapted to automated synthesis and associated rapid purification techniques. Applications to which our biotin phosphoramidites have been applied include:

1) Diagnostic probe development including occasions where multiple biotins may be introduced into a probe to enhance sensitivity of ultimate detection.
2) Introduction of biotin at the 5'-terminus of a primer prior to PCR amplification and trapping of the derived biotinylated DNA using streptavidin magnetic beads.
3) Introduction of biotin at the 3'-terminus of a probe capable of blocking chain extension in a PCR experiment.

Although branched biotin phosphoramidites may be inserted in the actual oligonucleotide sequence to produce biotinylated abasic sites, it is conceivable that significant disruption of hybridization to the complementary strand may occur. Our preference was, therefore, to introduce the biotin molecule at sites in the oligonucleotide that are removed from duplex formation. It was natural for us to look to a biotinylated version of **Amino-Modifier-dT (1)** to achieve these goals since that modified nucleotide is known[1] to cause minimal disruption of hybridization.

Simple addition of biotin to Amino-Modifier-dT gave a product with low solubility in acetonitrile, making the product unacceptable for use in automated synthesis. Protection of the N1-position of biotin with a lipophylic group seemed likely to induce higher solubility. This was confirmed in a report[2] on a biotinylated ribonucleoside monomer and led to the synthesis and introduction of **Biotin-dT (2)** for the production of oligonucleotides

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References:

(1) J. Telser, K.A. Cruickshank, L.E. Morrison, and T.L. Netzel, *J. Am. Chem. Soc.*, 1989, **111**, 6966-6976.

(2) U. Pieles, B.S. Sproat, and G.M. Lamm, *Nucleic Acids Res.*, 1990, **18**, 4355-4360.

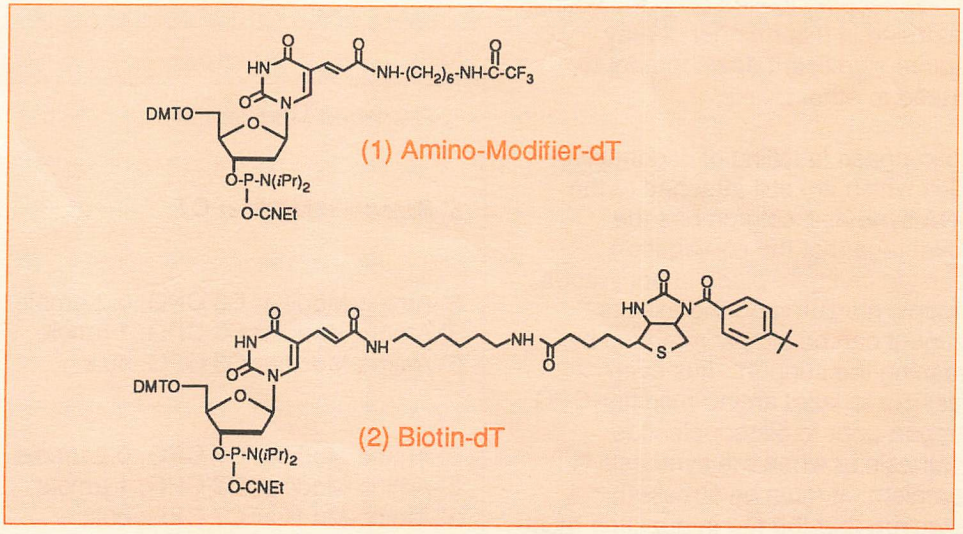

Figure 1: *Structures of Amino-Modifier-dT (1) and Biotin-dT (2)*

Purification of Labelled Oligonucleotides Using Poly-Pak™ Cartridges

The use of oligonucleotides containing a fluorescent label has grown significantly in recent months with applications in sequencing and diagnostic probe development. In common with other manufacturers, Glen Research is almost to the point of releasing phosphoramidites of the most popular fluorescent labelling reagents for use directly on a DNA synthesizer. However, there will always be a need to label oligonucleotides with fluorescent tags which are not available as phosphoramidites.

The process currently used for labelling of oligonucleotides entails the incorporation of an amine functionality at the desired point(s) in the oligonucleotide. The amino-modified oligonucleotide may then be deprotected and purified as necessary. Conjugation of the desired fluorescent tag to the oligonucleotide is then carried out in aqueous buffer, followed by isolation and purification of the labelled oligonucleotide. There are two significant hurdles to overcome in this process. Reaction of a tag containing an active ester or acid chloride with amino groups in aqueous buffer is inefficient and is always accompanied by hydrolysis. These reactions do not, therefore, usually go to completion and the product is heavily contaminated with hydrolyzed fluorescent tag. Although a Poly-Pak procedure is available for purification of fluorescein labelled oligonucleotides produced in this manner, it may require significant development to extend to other labels.

Solid-phase labelling of oligonucleotides which are still attached to the DNA synthesis column has the advantage that the conjugation reaction can take place in anhydrous organic medium and the excess reagent can be simply removed by washing the support. It is even possible to label amino-modifier-CPG support prior to oligonucleotide synthesis or when the synthesis is complete. It must be stressed, however, that the tag in question must

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Procedure

1. On-Column Labelling

a. Over a period of 3 minutes, flush synthesis column with 0.1M DBU in acetonitrile (5mL).
b. Flush synthesis column with acetonitrile (5X1mL) and allow to dry.
c. Insert syringes in both column fittings and gently push fluorescein isothiocyanate (FITC) solution (10mg/mL DMF) (1mL) back and forth through the column. Leave reaction for 2 - 4 hours at RT.
d. Flush synthesis column with DMF (5X1mL).
e. Flush synthesis column with acetonitrile (5X1mL) and allow to dry.

2. Deprotection

f. Carry out ammonium hydroxide deprotection as normal.

3. Poly-Pak Purification

g. Follow the basic procedure for DMT-ON purification as recommended (Page 5 in Poly-Pak Manual).
h. Flush the cartridge with acetonitrile (2mL) followed by 2M TEAA (2mL).
i. Dilute ammonium hydroxide solution containing crude product 3:1 with water.
j. Load the sample solution on to the Poly-Pak cartridge, saving the unretained fraction.
k. Flush the cartridge with ammonium hydroxide/water (1:10) (10mL).
l. Flush the cartridge with water (2mL).
m. Flush the cartridge with 2%TFA (2mL).
n. Flush the cartridge with water (2mL).
o. Flush the cartridge with 10% acetonitrile/0.1M TEAA (5mL).
p. Elute product with 25% acetonitrile/0.1M TEAA (1mL).

What's Happening

1. On-Column Labelling

Remove Fmoc protecting group(s) on the oligonucleotide sequence prepared using an amino-modifier CPG or branched modifiers using 0.1M DBU in acetonitrile. Wash the synthesis column with acetonitrile to remove excess DBU and to prepare for reaction with FITC solution. React the derived amino group(s) with FITC (or other active fluorescent tag) for 2 - 4 hours. Wash excess label from the column and prepare for the normal deprotection step.

2. Deprotection

No change from normal is necessary.

3. Poly-Pak Purification

Steps 3g - 3n are similar to those used in standard DMT-ON cartridge purification. The intent of these steps is to remove non-DMT containing failure sequences. The exception is that Step k is extended to wash off excess label. This step is continued until washes are virtually colorless.

DMT groups are removed from the sequence and excess acid is removed in Steps 3m- 3n.

Step 3o is designed to remove non-fluorescent labelled sequences and the fluorescein labelled product is finally isolated in Step 3p. The product should be checked by RP HPLC using a system such as the one shown in Figure 2 on the back page.

Reference: (1) C. Lehmann, Y.Z. Xu, C. Christodoulou, Z.K. Tan, and M.J. Gait, *Nucleic Acids Res.*, 1989, **17**, 2379.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price ($)
5'-Branched Modifier C3	10-1950-90	100µm	90.00
	10-1950-02	0.25g	300.00
5'-Branched Modifier C7	10-1957-90	100µm	90.00
	10-1957-02	0.25g	300.00
3'-Amino-Modifier C3 CPG, 0.2 µmole	20-2950-42	Pk/4	75.00
3'-Amino-Modifier C3 CPG, 1 µmole	20-2950-41	Pk/4	125.00
3'-Amino-Modifier C3 CPG, bulk	20-2950-01	0.1g	85.00
	20-2950-10	1.0g	600.00
3'-Amino-Modifier C7 CPG, 0.2 µmole	20-2957-42	Pk/4	85.00
3'-Amino-Modifier C7 CPG, 1 µmole	20-2957-41	Pk/4	140.00
3'-Amino-Modifier C7 CPG, bulk	20-2957-01	0.1g	95.00
	20-2957-10	1.0g	675.00

Poly-Pak™ Cartridges - Recent Improvements

In recent months, many of our customers have noted a change in the appearance and performance of our Poly-Pak™ cartridges. By and large, these cartridges have gained acceptance as the basis of a fast, convenient procedure for purification of synthetic oligonucleotides. However, some features of the original cartridges were causing us concern and, for some time, we have been addressing means to improve performance. An improved product has now resulted.

While the original choice of packing material, PRP-1 from The Hamilton Company, is superb for chromatography of oligonucleotides in packed columns, the very fine particles gave problems in both manufacture and performance of Poly-Pak cartridges. The main drawback in performance was the significant back pressure, especially during the wash with 2M triethylamine acetate. We were also limited by back pressure in the load of packing material and so the yields from the original cartridges, although much higher than in some competing products, were still a disappointment to us.

Following an exhaustive study of potential replacement polymeric packing materials, we have adopted a new packing material which operates with a significantly lower back pressure than the PRP-1 which was previously used. The new version of Poly-Pak cartridges also leads to significantly higher yields of purified oligonucleotide, as demonstrated in Table 1.

Following a good quality synthesis of a 20mer on a 0.2 µmole scale, 10 - 12 A_{260} units of purified product are typically isolated. A similar sequence produced on a 1 µmole scale should yield 30 - 40 A_{260} units of product.

Glen Research has recently completed a new booklet, entitled "USER GUIDE TO POLY-PAK™ CARTRIDGE PURIFICATION". As well as covering the principles and procedures of purification of normal oligonucleotides, the guide covers the purification of oligophosphorothioates, and amino-modified, thiol-modified, and biotinylated oligonucleotides. A section also covers large-scale purification using bulk polymeric packing. The guide is well illustrated with representative chromatograms. It is delivered along with Poly-Pak orders but may be requested separately simply by calling Glen Research.

Length	Scale (µmole)	Crude (A_{260} Units)	Flow Through	Yield 1st 0.5mL 20%CH_3CN	Yield 2nd 0.5mL 20%CH_3CN	Yield Total Yield
3	1	21	3	15	0	15
10	1	58	22	15	6	21
15	1	71	21	18	11	29
18	1	66	19	12	15	27
18[1]	1	109	12	37	5	42
20	1	131	53	20	15	35
25	1	151	47	24	8	32
30	1	113	68	13	5	18
19	0.2	27	6	12	4	16
13[2]	1	76	-	35	11	46

[1] Ammonium Hydroxide solution was evaporated and the crude oligonucleotide was applied to the cartridge in 0.1M TEAA solution.
[2] Oligo was amino-modified at the 3'-terminus.

Table 1: *Yields of oligonucleotides purified on Poly-Pak Cartridges*

Notes:

1. Yields of crude and purified oligonucleotides vary with synthesis efficiency. The above results derive from routine syntheses and serve to illustrate yield variations which are unrelated to cartridge performance.
2. Flow through represents the portion of the crude oligonucleotide solution which is unretained by the cartridge. Further product can usually be isolated by applying this fraction to a new cartridge or the same cartridge once the first purification is complete.
3. The remainder of the A_{260} units of crude solution are usually eluted from the cartridge in the Ammonium Hydroxide/Water (1:10) wash.
4. Elution of the purified product from the cartridge predominantly occurs in the first 0.5mL portion of 20% Acetonitrile wash. However, depending on the sequence, significant product may elute in the next 0.5mL wash.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price ($)
Poly-Pak™ Cartridge	60-1100-01	each	8.00
	60-1100-10	10/Pk	80.00
Poly-Pak™ Packing Material	60-1000-05	5g	70.00
	60-1000-25	25g	300.00

Cartridge Purification of Labelled Oligonucleotides

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be stable under the conditions of ammonium hydroxide deprotection of the oligonucleotides following synthesis.

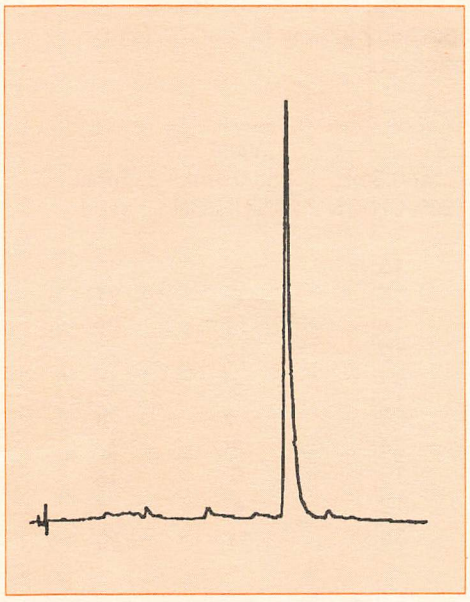

Figure 2: RP HPLC of Poly-Pak purified fluorescein labelled 15-mer following the conditions shown in Table 2.

Time	0.1M TEAA(%)	Acetonitrile(%)
0	93	7
20	75	25
30	75	25
35	93	7
40	93	7

Column: Spherisorb ODS-2 (150X4.6mm)
Flow Rate: 1mL/min.
Detector: UV at 254nm

Table 2: RP HPLC conditions for Amino-Modified Oligonucleotides.

The process shown here has been developed to take advantage of the simple removal of the 9-fluorenyl-methoxycarbonyl (Fmoc) protecting group used in branched amino-modifiers and amino-modifier-CPG supports. In our procedure, we use 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile[1] for specific removal of the Fmoc protecting group(s). It is possible to quantify the amount of Fmoc released by spectroscopic measurement. The DMT-ON cartridge purification procedure is then used to separate failure sequences capped during synthesis from the full-length oligonucleotide. The final steps of the procedure then remove non-labelled oligonucleotides from the desired fluorescent product.

Sequence Biotinylation

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biotinylated within the sequence.

It should be noted that we have found this product to be highly susceptible to deactivation by moisture in the diluent acetonitrile. Biotin-dT will, therefore, be supplied with a vial of anhydrous acetonitrile for use as diluent.

Using a branched biotin phosphor-amidite, biotin may be introduced simply at the 3'-terminus of a desired probe but it is necessary to use a non-coding deoxynucleoside support for the synthesis. As a more direct synthetic route, we have introduced **Biotin-CPG** for the preparation of oligonucleotides containing biotin at the 3'-terminus. This product is available both in columns and in bulk.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
Amino-Modifier-dT	10-1039-90	100µm	180.00
	10-1039-02	0.25g	360.00
Biotin-dT	10-1038-95	50µm	162.50
	10-1038-90	100µm	325.00
	10-1038-02	0.25g	650.00
Biotin-CPG, 0.2 µmole	20-2951-42	Pk/4	120.00
Biotin-CPG, 1 µmole	20-2951-41	Pk/4	200.00
Biotin-CPG, bulk	20-2951-01	0.1g	120.00
	20-2951-10	1.0g	995.00

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