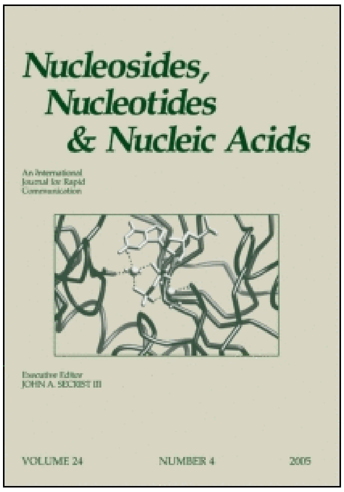

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

3'-Terminal Modification of Oligonucleotides Using a Universal Solid Support

Paul S. Nelson[a]; Sylvester Muthini[a]; Mark A. Kent[a]; Thomas H. Smith[a]
[a] Nucleic Acids Chemistry Division, CLONTECH Laboratories, Inc., Palo Alto, CA, USA

To cite this Article Nelson, Paul S. , Muthini, Sylvester , Kent, Mark A. and Smith, Thomas H.(1997) '3'-Terminal Modification of Oligonucleotides Using a Universal Solid Support', Nucleosides, Nucleotides and Nucleic Acids, 16: 10, 1951 — 1959

To link to this Article: DOI: 10.1080/07328319708002546

URL: http://dx.doi.org/10.1080/07328319708002546

NUCLEOSIDES & NUCLEOTIDES, 16(10&11), 1951-1959 (1997)

3'-TERMINAL MODIFICATION OF OLIGONUCLEOTIDES USING A UNIVERSAL SOLID SUPPORT

Paul S. Nelson*, Sylvester Muthini, Mark A. Kent, and Thomas H. Smith

Nucleic Acids Chemistry Division, CLONTECH Laboratories, Inc., 1020 East Meadow Circle, Palo Alto, CA 94303, USA

Abstract: A practical method to prepare 3'-terminally labeled oligonucleotides using a universal solid support (**1**) was developed. This novel method was demonstrated by incorporating thirteen different label moieties on the 3'-terminus of a common $(dT)_7$ sequence. Label incorporations were verified by ion exchange HPLC analysis and MALDI TOF mass spectrometry.

INTRODUCTION

The importance of methods to covalently attach labels and reporter molecules to the 3'-terminus of synthetic oligonucleotides has been well established by DNA-based diagnostic and antisense therapeutic applications[1-4]. Current techniques to incorporate 3'-terminal labels require specialized solid supports to transfer a pre-attached label moiety or functional group to the 3'-terminus *via* automated solid phase synthesis[5-12]. Such specialized reagents are limited because a different solid support must be used for each kind of 3'-terminal modification. The ability to incorporate any 3'-modification from a single universal solid support would be more advantageous and convenient.

Recently, we reported the use of a universal solid support (**1**) in automated oligonucleotide synthesis[13]. The universal solid support allowed any oligonucleotide sequence to be synthesized from a single type of CPG support regardless of the nature of the 3'-terminal base. In this paper, we investigated the viability of employing the universal support for the purpose of incorporating 3'-terminal oligonucleotide modifications. We have successfully demonstrated this novel labeling method by incorporating various base-modified nucleotides, abasic 2-deoxyribosyl analogs, 2-aminobutyl-1,3-propanediol analogs, 3-amino-1,2-propanediol, hexaethyleneglycol, 2, 2'-sulfonyldiethanol, and mixed base modifications on the 3'-terminus of oligonucleotides.

MATERIALS AND METHODS

All phosphoramidite and CPG reagents were obtained from either Clontech Laboratories, Inc. (Palo Alto, CA) or Glen Research, Inc. (Herndon, VA). OCP polymeric oligonucleotide purification columns were supplied by Clontech Laboratories. Ion exchange HPLC analysis was performed on a Rainin (Emeryville, CA) Rabbit HPX system using a Pharmacia (Piscataway, NJ) MonoQ HR 5/5 column. Oligonucleotide synthesis was performed on a ABI (Foster City, CA) 394 DNA/RNA Synthesizer according to manufacturer's protocol. Mass spectrum analyses were performed on a PHI-EVANS MALDI triple electrostatic analyzer time-of-flight mass spectrometer by Charles Evans & Associates (Redwood City, CA).

Preparation of Universal Solid Support (1)[13]

The succinate precursor was prepared from cis-3-O-(4,4'-dimethoxytrityl) tetrahydrofuran-4-ol using a modified procedure of Hardy *et al*[14] and covalently attached to CPG (500 A^{O}) through a novel approach using benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as coupling reagent[15]. Thus BOP (233 mg, 0.527 mmol), 1-hydroxybenzotriazole (HBT, 71 mg, 0.527 mmol), triethylamine (0.42 ml), and cis-3-O-(4,4'-dimethoxytrityl) tetrahydrofuran-4-yl succinate (304 mg, 0.6 mmol) were dissolved in 32 ml methylene chloride. LCAA-CPG (10 g) was added and the mixture was agitated at room temperature for 16 hrs on an orbital shaker. The CPG resin was collected in a sintered glass funnel, washed 6 X 100 ml methanol, 2 X 100 ml ethyl ether, and dried under high vacuum for 30 min. Capping was accomplished by treating the resin with a pyridine/acetic anhydride/ N-methylimidazole (50:10:6, v/v/v) mixture for 2 hrs. The CPG resin was again collected in a sintered glass funnel and washed 3 X 100 ml pyridine, 3 X 100 ml dimethylformamide, 6 X 100 ml methanol, 2 X 100 ml ethyl ether, and dried under high vacuum. The trityl loading was determined to be 32 umol/gram.

Oligonucleotide Synthesis

3'-terminally modified oligonucleotides **6a-m** were prepared from universal support **1** by the phosphoramidite method using standard conditions on a ABI Model 394 DNA/RNA Synthesizer. 3'-modification was accomplished by coupling a bifunctional phosphoramidite, **2a-m,** in the first cycle followed by synthesis of a common $d(T)_7$ sequence. A bifunctional phosphoramidite is defined as a compound with both a DMT protected hydroxyl for chain elongation and a CE-phosphoramidite group used for coupling. When possible, control oligonucleotides possessing identical 3'-modified structures **6f-g, i-k** were synthesized from specially modified CPG reagents. Syntheses were performed on a 1.0 micromole scale and the TRITYL-ON mode was employed to facilitate subsequent cartridge purification. Coupling efficiencies of phosphoramidites **2a-m** were >98% as determined by measuring the dimethoxytrityl cation concentration.

Deprotection and Purification

Oligonucleotides synthesized from universal support **1** were cleaved from the solid support and deprotected by treatment with 0.5 M LiCl in conc. ammonium hydroxide at 55°C for 16 hrs overnight. Control oligonucleotides synthesized from specially modified CPG reagents were deprotected using standard ammonium hydroxide treatment. Oligonucleotides synthesized in the TRITYL-ON mode were purified on a polymeric cartridge (OCP columns) by directly applying the deprotection solution and following the recommended procedures of the supplier. Yields of the purified oligonucleotides were consistently between 20-30 ODs.

Ion Exchange HPLC Analysis

The 3'-terminally modified $d(T)_7$ oligonucleotides **6a-m**, synthesized from universal solid support **1** were analyzed by ion exchange HPLC on a Pharmacia Mono Q HR 5/5 column (5 mm X 5 cm). The mobile phases employed were A = 50 mM Tris (pH 7.5) and B = 800 mM NaCl, 50 mM Tris (pH 7.5). A gradient of 5 - 45% B over 40 min at a flow rate of 1.0 ml/min was used. 3'-modified $(dT)_7$ peaks, both with and without the 3'-furanyl phosphodiester adapter, were fully resolved. The efficiency of cleaving the 3'-furanyl phosphodiester was determined by area integration.

DISCUSSION AND RESULTS

The design of our 3'-terminal labeling method utilizes a unique universal solid support (**1**) and is outlined in Figure 1. Solid support **1** possesses a non-nucleosidic anhydroerythritol (furanyl) adapter rather than a standard pre-attached nucleoside. A bifunctional labeling reagent (Figure 2), i.e., a reagent possessing both a DMT protected hydroxyl and a CE-phosphoramidite group, is incorporated onto the universal solid support **1** during the first coupling cycle. The oligonucleotide sequence of interest is then synthesized by employing standard automated synthesis conditions using normal base phosphoramidites. Upon deprotection with 0.5 M LiCl in conc. ammonium hydroxide, the furanyl group is transferred from the solid support to the 3'-terminus of the oligonucleotide and cleaved through a cyclic mechanism to give the desired 3'-terminally labeled oligonucleotide.

We found this method to be very convenient in preparing oligonucleotides with a 3'-terminal mixed base site and wanted to investigate the utilization of solid support **1** as a general method for labeling the 3'-terminus. Thus, a series of 3'-modified $(dT)_7$ oligonucleotides (**6a-m**) were synthesized by incorporating their respective bifunctional phosphoramidites **2a-m** in the first coupling cycle. Structural backbones represented in this series were base-modified nucleotides[16,17] (**2a-e**), abasic 2-deoxyribosyl analogs[5,18] (**2f-h**), 3-amino-1,2-propanediol[7,19] (**2i**), 2-aminobutyl-1,3-propanediol analogs[6] (**2j-k**), hexaethyleneglycol[20] (**2l**), and 2,2'-sulfonyldiethanol[21] (**2m**). Oligonucleotides **6a-i** were derived by scission of the 3'-furanyl phosphodiester adapter attached to a secondary hydroxyl, whereas oligonucleotides **6j-m** were derived by adapter scission from a primary hydroxyl. All modified phosphoramidites, **2a-m**, incorporated at >98% coupling efficiency.

The use of LiCl to facilitate scission of the 3'-furanyl phosphodiester adapter has been previously reported[13]. However, it was only studied when attached to the 3' terminal hydroxyl of an oligonucleotide, i.e., a secondary hydroxyl. It was unknown how efficiently the LiCl/ NH_4OH conditions could facilitate the scission mechanism when the adapter is connected to a primary hydroxyl as in **6j-m**. One would expect the mechanism to be less efficient for a primary hydroxyl since it is a poorer leaving group. It was also unknown how well a free rotating secondary hydroxyl **6i** would facilitate furanyl

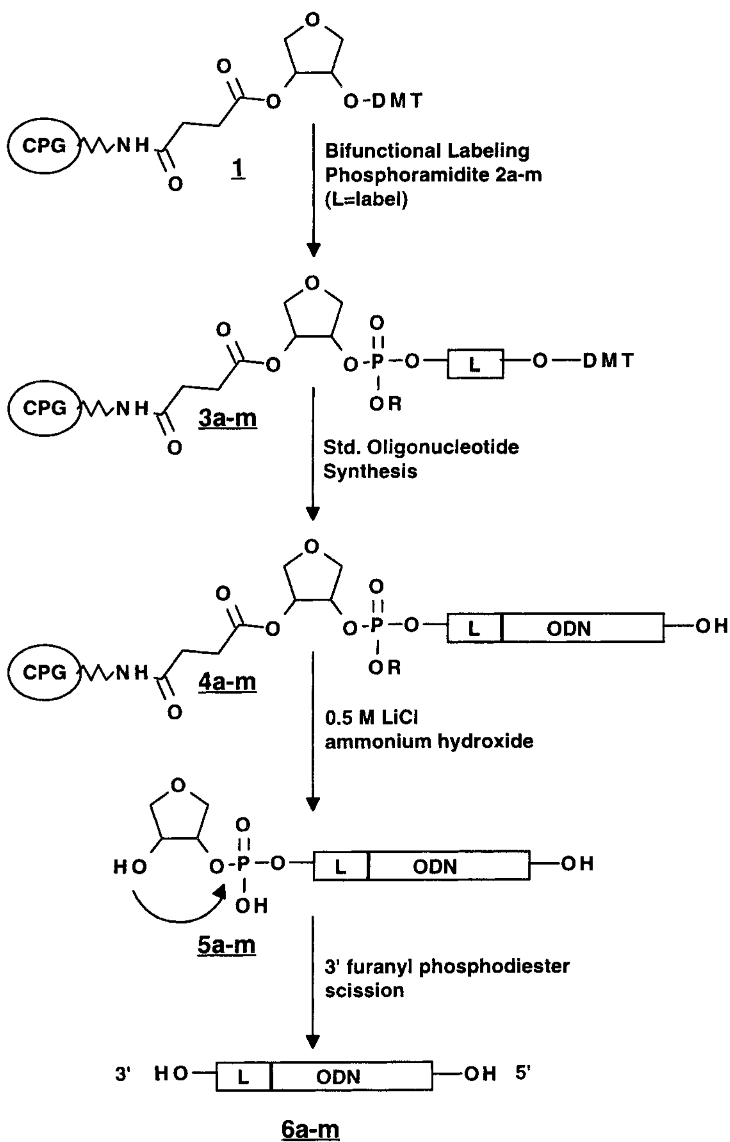

FIGURE 1. Schematic Outline of the 3'-Terminal Labeling Method.

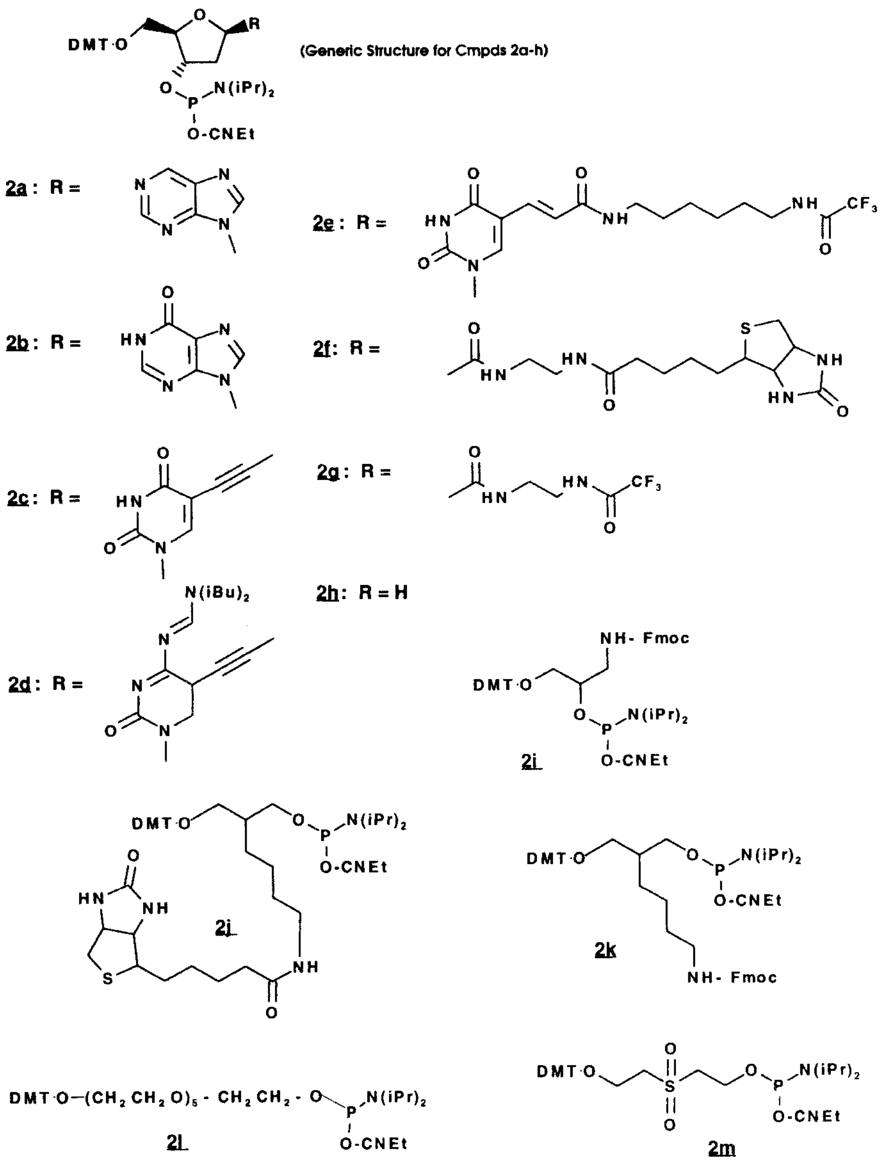

FIGURE 2. Stuctures of Bifunctional Labeling Phosphoramidites **2a-m**.

phosphodiester cleavage relative to a ring constrained secondary hydroxyl (**6a-h**). In order to develop an universal method for incorporating 3'-terminal modifications it was necessary to evaluate the cleaving mechanism efficiency for various non-nucleosidic backbones.

Thirteen different 3'-modified $(dT)_7$ oligonucleotides **6a-m** were synthesized using universal solid support **1** in order to evaluate the scission efficiency of the 3'-furanyl phosphodiester adapter. Established 0.5M LiCl/ conc. NH_4OH deprotection conditions were employed[13] and the degree of adapter scission was quantitated by analytical ion exchange HPLC. The 3'-modified $(dT)_7$ peaks, both with and without the 3' furanyl phosphodiester adapter, were fully resolvable and quantitation was accomplished by area integration. For oligonucleotides **6f-g, i-k**, 3'-modified control oligonucleotides synthesized from corresponding specialized CPG reagents[5-7] were used for identity purposes. In all cases, peak identity was verified by MALDI-TOF mass spectrometry.

The results are summarized in Table 1. Quantitative scission of the adapter was observed for oligonucleotides **6a-e** which were respectively synthesized by employing base modified phosphoramidites **2a-e** in the first coupling cycle. Abasic 3'-modified oligonucleotides **6f-h** synthesized from 2-deoxyribosyl phosphoramidites **2f-h** also resulted in quantitative scission of the adapter. Interestingly, when compound **2i** was incorporated at the 3'-terminus to give oligonucleotide **6i**, slightly lower cleavage of the adapter was observed at 89%. The decreased scission efficiency may be due to fact that the furanyl phosphodiester adapter is bonded to a free-rotating secondary hydroxyl rather than being ring constrained. The most significant decrease in scission efficiency, ranging from 16 - 53%, was observed when the adapter was attached to a primary hydroxyl (**6j-l**). This can be explained by the fact that a primary hydroxyl represents a poorer leaving group. Furanyl phosphodiester scission efficiency for compound **6m** could not be measured since it readily undergoes β-elimination to give the 3'-phosphate derivative.

SUMMARY

We have developed a novel 3'-terminal oligonucleotide labeling method utilizing a universal solid support in automated DNA synthesis. We have successfully demonstrated the utility of the method by incorporating thirteen different modifications

TABLE 1. Summary of ion exchange HPLC and MALDI TOF mass spectroscopy results. 3'-Terminally labeled $(dT)_7$ oligonucleotides **6a-m** were prepared from bifunctional phosphoramidites **2a-m** respectively.

Oligonucleotide #	HPLC Ret. (min.)	% adapter scission	Mol.Wt. calc.	Mol.Wt. actual
Secondary hydroxyl				
6a	24.65	100%	2365.6	2365.2
6b	25.59	100%	2381.6	2382.3
6c	24.00	99%	2395.6	2395.6
6d	23.79	100%	2394.6	2394.7
6e	19.64	100%	2525.8	2525.3
6f	23.60	99%	2559.9	2559.1
6g	18.35	100%	2333.6	2333.9
6h	23.91	96%	2247.5	2247.6
6i	19.01	89%	2220.5	2220.2
Primary hydroxyl				
6j	25.42	16%	2502.9	2502.9
6k	18.93	30%	2276.6	2276.7
6l	21.67	53%	2412.7	2412.8
6m	24.83	100%	2147.4	2147.2

on the 3'-terminus using a common $(dT)_7$ sequence. 3'-Terminal modifications derived from bifunctional phosphoramidites **2a-h** gave the best results, showing quantitative scission of the transferred furanyl phosphodiester adapter. This method is more advantageous than current specialized CPG approaches because it allows incorporation of 3'-terminal labels from a single solid support. Employing this novel method for modifying oligonucleotides on the 3'-terminus may be useful for applications in DNA based diagnostics and antisense therapeutics.

REFERENCES

1. Uhlmann, E. and Peyman, A. (1990) *Chem. Rev.* **90**, 544-584.
2. Tenover, F.C. (1988) *Clin. Microbiol. Rev.* **1**, 82.
3. Cohen, J. (1989) *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression*, Macmillian Press, London.
4. Goodchild, J. (1990) *Bioconj. Chem.* **1**, 165-187.
5. Smith, T.H., Kent, M., Muthini, S., Boone, S., and Nelson, P.S. (1996) *Nucleosides and Nucleotides* **15**, 1581-1594.

6. Nelson, P.S., Kent, M., and Muthini, S. (1992) *Nucleic Acids Res.* **20**, 6253-6259.
7. Nelson, P.S., Frye, R., and Liu, E. (1989) *Nucleic Acids Res.* **17**, 7187-7194.
8. Petrie, C.R., Reed, M.W., Adams, A.D., and Meyer, R.B. (1992) *Bioconj. Chem.* **3**, 85-87.
9. Thaden, J. and Miller, P.S. (1993) *Bioconj. Chem.* **4**, 385-401.
10. Reed, M.W., Adams, A.D., Nelson, J.S., and Meyer, R.B. (1991) *Bioconj. Chem.* **2**, 217-225.
11. Asseline, U. and Thuong, N.T. (1990) *Tetrahedron Lett.* **31**, 81-84.
12. Vu, H., Joyce, N., Rieger, M., Walker, D., Goldknopf, I., Hill, T.S., Jayaraman, K., and Mulvey, D. (1995) *Bioconj. Chem.* **6**, 599-607.
13. Nelson, P.S., Muthini, S., Kent, M., and Smith, T.H. (1997) *BioTechniques* **22**, 752-756.
14. Hardy, P., Holland, D., Scott, S., Garman, A., Newton, C., and McLean, M. (1994) *Nucl. Acids Res.* **22**, 2998-3004.
15. Hudson, D. (1988) *J. Org. Chem.* **53**, 617.
16. Froehler, B., Wadwani, S., Terhorst, T.J., and Gerrard, S.R. (1992) *Tetrahedron Lett.* **33**, 5307-5310.
17. Ruth, J. (1984) *DNA* **3**, 123.
18. Wilk, A., Koziolkiewicz, M., Grajkowski, A., Uznanski, B., and Stec, W.J. (1990) *Nucleic Acids Res.* **18**, 2065-2068.
19. Nelson, P.S., Sherman-Gold, R., and Leon, R. (1989) *Nucleic Acids Res.* **17**, 7179-7186.
20. Durand, M., Chevrie, K., Chassignol, M., Thuong, N.T., and Maurizot, J.C. (1990) *Nucleic Acids Res.* **18**, 6353-6359.
21. Horn, T. and Urdea, M. (1986) *Tetrahedron Lett.* **27**, 4705-4708.

Received January 20, 1997
Accepted June 27, 1997