

## **Fluorescent Labelling at 5'-Terminus of Oligonucleotides Preceded by One Step Alkylamino Generation**

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**Abstract.** Synthetic oligonucleotides have been 5' - aminoalkylated at the end of the stepwise synthesis on the polymer support. This was achieved by the coupling of 5'-OH tetramer d(TGCT) with N-(4-bromobutyl) phthalimide, which on ammonia treatment yielded the title compound. Covalent labelling of 4-acetylamino-1, 8-naphthalimido-N-caproic acid (NCA) with butylaminotetramer is also described. The later on coupling with the fluorescent 4-acetylamino-1,8-naphthalimido-N-caproic acid (NCA) yielded the tagged oligonucleotide.

### **1. Introduction**

Derivatization of synthetic oligonucleotides at the 5'- terminus has been difficult due to the unreactive nature of primary hydroxyl group. There are only a few reports for preparation of oligodeoxynucleotides having a 5' alkylamino derivative. Hood and coworkers<sup>1</sup> recently prepared synthetic oligomers containing a 5'-amino group through the introduction of suitably protected 5'-amino-5'-deoxy thymidine monomer. The second method as developed by two different groups<sup>2,3</sup>, reported the preparation of 5'- alkylamino phosphoramidite derivatives of unprotected oligonucleotides by reaction of diamines with a 5'- phosphorimidazolide. However, this method suffers from the disadvantages of introducing a phosphoramidate linkages between the 5'- end of the oligomer and the diamine which is prone to ready-acid catalyzed hydrolysis. The third method of Kemp et al<sup>4</sup> involves introduction of 2-(biotinylamide) ethanol by reaction with a 5'-phosphorylated polymer supported nucleotide. Connolly and Rider<sup>5</sup> have introduced a thiol group at the 5'-terminus of a synthetic oligomer using S-trityl-6- mercaptohexanol as morpholinophosphite derivative and subsequent deprotection of S-trityl group. The same authors have reported introduction of primary amino group at 5' terminus of oligonucleotides<sup>6</sup>. In a latest report 5'-alkylamino derivatives of synthetic oligomers have also been synthesized on controlled pore glass solid support using carbonyldiimidazole (CDI) and hexamethylenediamine (HMDA)<sup>7-8</sup>. Although this method is quite rapid and useful but requires modification

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for one step procedure for alkylamino generation at 5'-OH end of oligonucleotides.

In the present report we wish to communicate a very simple and efficient procedure using commercially available reagents for the synthesis of 5'-alkylamino derivatives of synthetic oligomer i.e., a tetramer (TGCT) on controlled pore glass support. For this purpose a tetramer, d(TGCT) an oft repeat sequence in tat initiator of HIV-1 genome was synthesized in our laboratory on "Omnifit Dual Column DNA Bench Synthesizer" using phosphotriester chemistry<sup>8</sup>. The 5'-OH group of d(TGCT), still attached to CPG via 3' end, was reacted with N-(4-bromobutyl) phthalimide under anhydrous conditions. This on hydrolysis with ammonia yielded the desired molecule. The ethereal linkage thus generated withstands the deprotection conditions used in oligonucleotide synthesis. Thus, a butyl amino group has been generated on the 5'-terminus of a synthetic oligonucleotide, while still attached to the resin. The advantages of this method can be enumerated as below;

1. the length of the alkyl chain can be varied as desired.
2. the end amino group can be kept protected with phthaloyl group for storage purpose and deprotected when needed and,
3. the coupling can be carried out with any reporter group having any functionality reactive towards amino group.

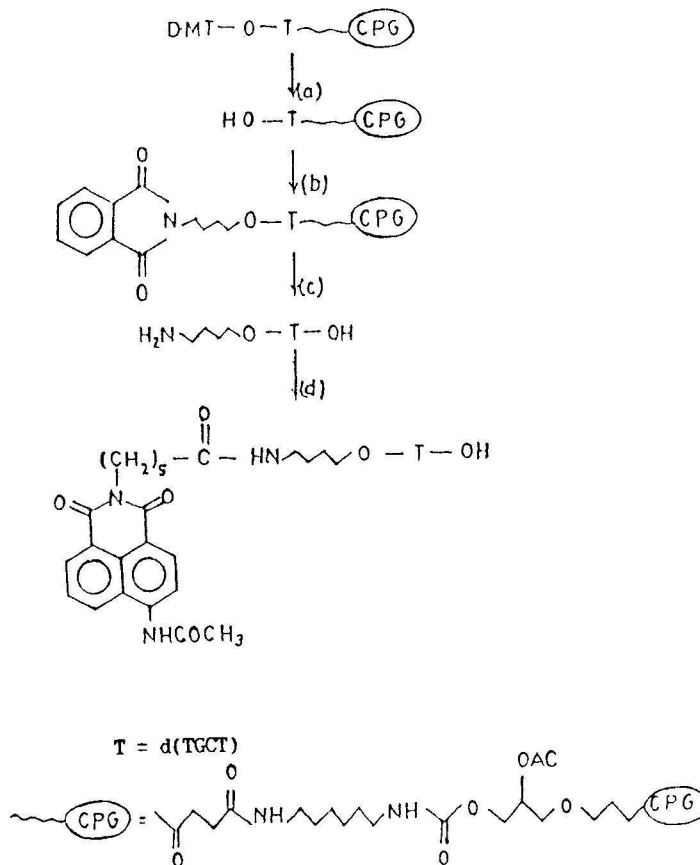
## 2. Experimental Procedures

The phospho-triesters of thymidine, guanosine and cytidine were purchased from Yoshitomi Chemical Company, Japan. 1-Methylimidazole, mesitylenesulphonyl nitrotriazole (MSNT) were obtained from Fluka, Buchs, Switzerland. N-(4-Bromobutyl) phthalimide and DCC were purchased from Sigma Chemical Company, USA. p-Nitrophenol, HPLC grade water and methanol were obtained from E. Merck company, Bombay, India. Fluorescent studies were carried out on SFM 25 spectrofluorometer and HPLC on LKB DBF Pharmacia, using RPC C-18 column.

**Synthesis of Tetramer DMT d(TGCT):** It was synthesized using phosphotriester approach on 0.2  $\mu$ M scale. The 5' - DMT - group using control pore glass solid support was kept intact.

**5'-Aminobutyl Tetramer:** The solid support still attached to the tetramer, was taken in a small reaction vessel having a medium porosity frit and stop-cock with a controlled inlet for nitrogen. The resin was washed well with anhydrous HPLC grade dioxane (3x 3 ml) and dimethoxytrityl group was deprotected with 3% trichloroacetic acid in dichloromethane. Resin was again washed well with dichloromethane followed by dimethyl- formamide (3 x 3ml) and pyridine ( 3 x 3 ml). The resin containing free

5'-hydroxyl on tetramer was then suspended in anhydrous pyridine (1ml). N-(4-bromobutyl) phthalimide (0.014 mM; 4.0 mg) dissolved in pyridine (0.4 ml) was introduced into the reaction vessel with the use of a syringe. The activation was allowed to proceed for 4h at r.t. with occasional shaking. After this time the resin was washed well with pyridine ( 5 × 3ml).



**Scheme 1. General reaction scheme for synthesis of 5'-aminobutyl teramer and its further labelling with NCA tag**

(a) 3% trichloroacetic acid. (b) N-(4-bromobutyl) phthalimide in pyridine. (c) ammonia, and (d) p-nitrophenyl ester of 4-acetylamino-1,8-naphthalimido-N-caproic acid (NCA).

**Cleavage, deprotection and purification of 5'-butylamino-tetramer:** The 5'-butylamino tetramer still attached with resin was taken in eppendorf tube and treated with concentrated ammonia (30%, 4ml) for 2h at r.t. to cleave the support and phthaloyl group. Aqueous phase was filtered using sintered funnel to collect the derivatized tetramer in round bottom flask. Flask was sealed carefully and immersed in a shaking water bath for 8 h at 55°C to remove amino protecting groups.

After ammonia treatment the flask was taken out, cooled, under vacuo to a gum and taken in ammonium acetate buffer for purification on reverse phase HPLC. A gradient HPLC system was set up using C-18 column (4 × 250 mm) using 0.1M ammonium acetate as buffer A and acetonitrile as buffer B. Fractions were monitored at 260 nm and pooled accordingly. These fractions were lyophilised to dryness.

**Preparation of *p*-nitrophenyl ester of 4-acetylamino-1, 8-naphthalimido-N-caproic acid (NCA):** Ester of NCA was prepared by dissolving 4-acetylamino-1, 8-naphthalimido-N-caproic acid (0.054 mM; 20mg) in dry dioxane (1.0ml) containing dry pyridine (0.2 ml), triethylamine (0.2 ml) and *p*-nitrophenol (0.54 mM; 7.5 mg). Dicyclohexyl-carbodiimide (0.135 mM, 27.8 mg) was added to it. Reaction was complete in 2.5 h. Dicyclohexyl urea was removed by filtration to obtain the nitrophenyl ester of NCA.

**Coupling of 5'-aminobutyl tetramer with NCA:** 5'-Aminobutyl tetramer was dissolved in 250 µL of a mixture of 1.0M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> (pH 9.0). To this solution 500 µL solution of *p*-nitrophenyl ester of NCA dissolved in a mixture of 1.0 M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> buffer (pH 9.0):DMF:H<sub>2</sub>O (5:2:3; v/v) was added and the reaction mixture was vortexed and wrapped with aluminium foil to prevent light exposure. The reaction mixture was stirred for 8h at r.t. in dark. The labelled tetramer was then passed through Sephadex G-25 using ethanol; water (8:2 v/v) as eluent. The filtrate containing NCA labelled tetramer was collected, concentrated and resuspended in aq. ammonium acetate buffer (1.0 ml) and purified by HPLC in the same way as described for aminobutyl tetramer. The NCA labelled tetramer was monitored at 260 nm. Pure labelled samples showing absorption at 260 nm were pooled and lyophilized.

**Measurement of fluorescence of NCA labelled tetramer:** The NCA labelled tetramer was dissolved in 0.1 M aq. ammonium acetate buffer (pH 7.0) and its emission spectrum was recorded at excitation wavelength of 360 nm in 0.04 OD concentration.

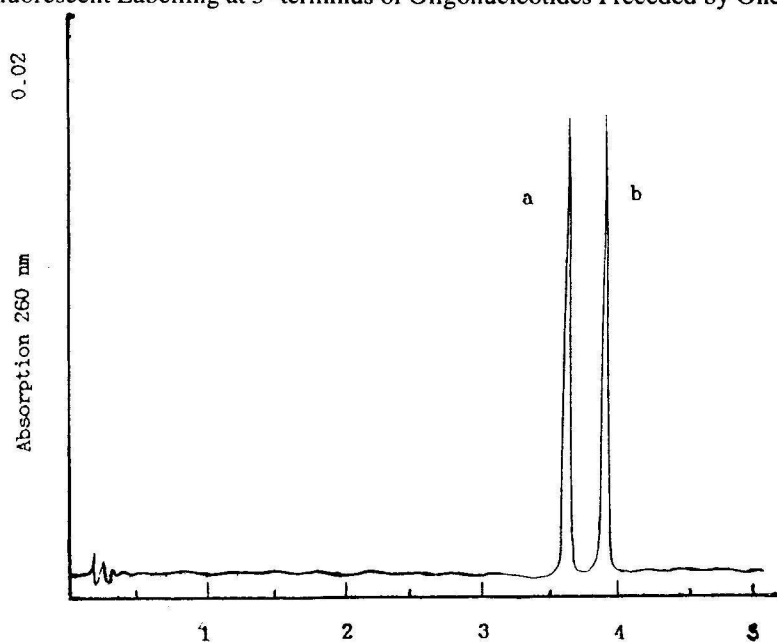


fig. 1. HPLC  $C_{18}$  reverse phase elution profile of approximately 0.02  $A_{260}$  units of 5'-aminobutyl tetramer (peak a, retention time 3.6 min) and 5'-NCA labelled tetramer (peak b, retention time 3.9 min). The initial conditions of the chromatogram are solvent A for 2 minutes and then a linear gradient of solvent A to 60% solvent B over 20 minutes at a flow rate of 0.5 ml/min. Solvent A, aqueous ammonium acetate (0.1 M), solvent B acetonitrile, pH 6.93.

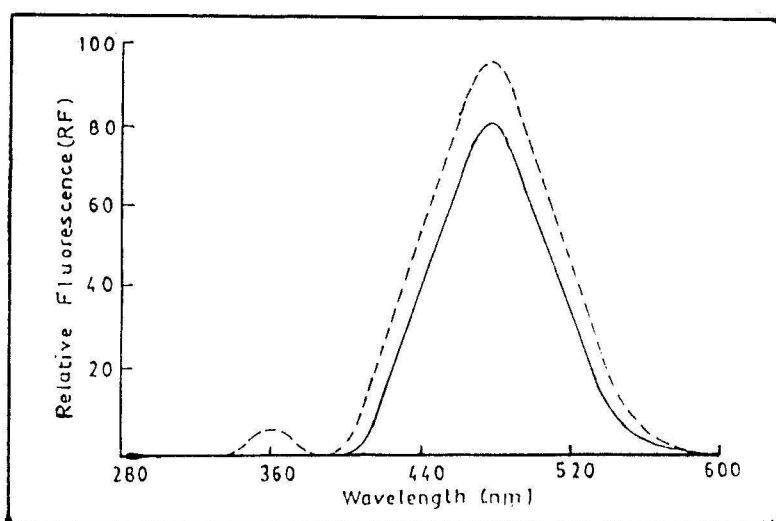


Fig. 2. Relative fluorescence of NCA tag (---) and 5'-NCA labelled tetramer (---), solvent 0.1 M aqueous ammonium acetate, conc. 0.04 OD,  $\lambda_{ex}$  360 nm.

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