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OLIGONUCLEOTIDE LABELLING: A CONCISE SYNTHESIS OF A MODIFIED THYMIDINE PHOSPHORAMIDITE

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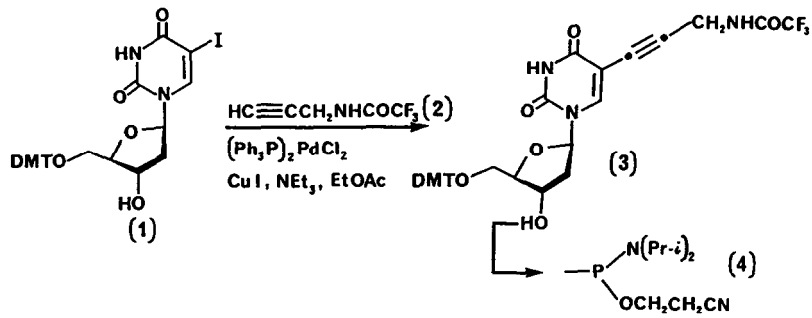
Abstract: The condensation of 5'-dimethoxytrityl-5-iodo-2'-deoxyuridine (1) with N-trifluoroacetylpropargylamine (2) in the presence bis(triphenylphosphine) Pd(II) chloride and copper(I)iodide and subsequent conversion to the 3'-phosphoramidite (4) is an efficient and rapid method of preparing modified nucleosides valuable in oligonucleotide labelling studies. We believe this to be the most expedient route yet devised to these types of molecules.

Oligonucleotides modified to contain chemical functionality, especially primary aliphatic amines, not normally found in nucleic acids are increasingly being used in many technological processes.[1] One approach to the synthesis of such molecules is to incorporate aliphatic amine containing nucleotide phosphoramidites to the oligonucleotide synthesis cycle. The aliphatic amine groups are subsequently used to co-valently attach a variety of ligands such as DNA affinity cleaving reagents,[2,3] enzymes,[4] paramagnetic spin probes[5] or affinity binding molecules such as biotin,[6] to the oligonucleotides. For example fluorescently labelled sequencing primers and fluorescently labelled nucleotide triphosphates form the basis of two commercial non-radioisotopic DNA sequencing methods likely to be used in large-scale sequencing projects.[7] Several published methods describing the syntheses of modified nucleotides are known, but these involve time-consuming multi-step procedures and/or the use of toxic organomercurial reagents.[2,8] Another set of syntheses are based on the work of Robins and Barr[9,10] who first described the Pd(II) catalysed coupling of 3',5'-di-O-p-toluyl-2'-deoxyuridine with terminal alkynes to yield 5-alkynyl-2'-deoxyuridines. Several groups[5,11] have utilized this methodology to prepare labelled oligonucleotides, but without exception all have followed the initiative of Robins and Barr and used p-toluyl protecting groups for hydroxyl protection. This necessitates the addition of unwieldy protection/deprotection steps to the syntheses. In addition, the work by Haralambidis et al[11] uses the tBOC group for protection of the propargylic amine function which requires a post-synthesis treatment of the oligonucleotide with

ethane thiol-trifluoroacetic acid to effect complete deprotection. We wish to describe here (Scheme) a three-step conversion of 5-iodo-2'-deoxyuridine to a phosphoramidite protected with groups fully compatible with state-of-the-art automated DNA synthesis methods and also to demonstrate the utility of this intermediate in the preparation of labelled oligonucleotides.

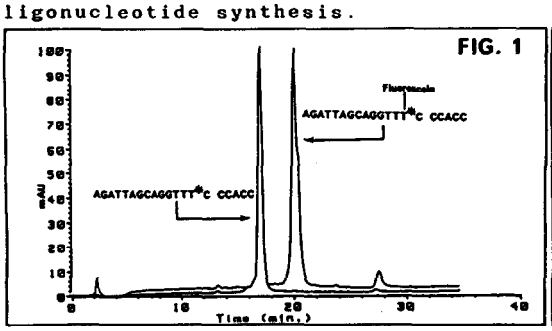
It occurred to us that since the Pd(II) coupling of terminal alkynes with 5-iodonucleosides takes place in basic media[10] the dimethoxytrityl ether should be stable under these conditions. Moreover, it appeared non-essential to protect the 3'-hydroxyl function since we had carried out successful coupling experiments with 3',5'-di-O-p-toluyl derivatives in which methanol was present in the reaction mixture in equimolar concentration to the nucleoside component. In addition, the N-trifluoroacetyl function was also likely to be stable under these conditions.

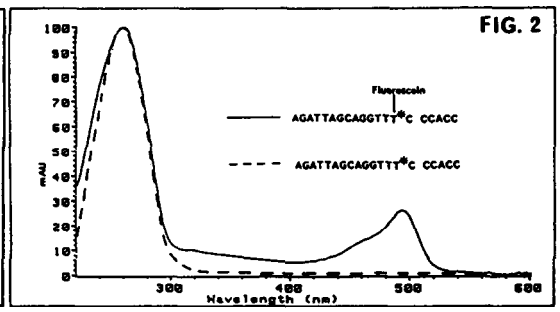


Scheme

A typical preparation proceeded as follows. Ethyl acetate (240ml) was freshly distilled into a dry 250ml round-bottom flask. To this solution was added in sequence, 5'-dimethoxytrityl-5-iodo-2'-deoxyuridine[12] (4.0g, 6.1mmol), N-trifluoroacetylpropargylamine[13] (1.84g, 12.2mmol), bis(triphenylphosphine)-palladium (II) chloride (0.091g, 0.13mmol), copper(I)iodide (0.091g, 0.48mmol) and triethylamine (3.08g, 30.5mmol). The headspace was purged with nitrogen and the solution magnetically stirred at room temperature. After 40h, analysis of the dark reaction mixture by TLC (ethyl acetate) revealed that no starting material was present. (Relative to the tBOC group the N-trifluoroacetyl-group facilitates subsequent cyclization to the fluorescent side-product, therefore reactions should be monitored carefully so as to maximize product formation). The ethyl acetate solution was extracted with EDTA solution (5% w/v, 2 X 300ml) followed by sodium bisulfite solution (5% w/v, 300ml) and evaporated in vacuo to a dark-red foam which was further purified by flash chromatography[14] on a column of silica gel 60 (25 X 5.5cm) packed using ethyl acetate - hexane (1:1, v/v).

Elution with ethyl acetate-hexane (1:1, v/v, 1000mls) followed by ethyl acetate-hexane (3:2, v/v) eluted the product as a pale-yellow foam (3.23g, 53%). Elution with ethyl acetate yielded a fluorescent by-product (0.21g, 4%), which was presumably the furano[2,3-d]pyrimidin-2-one nucleoside formed by a cyclization process originally described by Robins and Barr.[10] Compound (3) was then converted in good yield to the phosphoramidite (4) using published methods.[16] A 0.1M solution in dry acetonitrile was then prepared for use in oligonucleotide synthesis.





To examine the utility of phosphoramidite (4) in oligonucleotide synthesis we undertook the preparation[17] of several oligodeoxyribonucleotides, the twenty-mer fragment of the E. Coli enterotoxin gene 5'AGA TTA GCA GGT TT*C CCA CC3' is shown here as an example, the asterisk signifying the presence of the modified thymidine. Colorimetric analysis of released trityl cation in syntheses utilizing (4) typically indicated coupling efficiencies of 98.8% per step. After cleavage from the solid support and removal of all protecting groups the oligonucleotides were analysed and purified to a single homogeneous peak by HPLC[18] (Fig 1). To confirm the presence of the primary amino group within the oligonucleotide we reacted the substance with fluorescein isothiocyanate (FITC 150 equiv., 100mM aq Na_2CO_3, 2.5 hr, RT) and observed (HPLC, Fig 1) a quantitative conversion of the starting material to a new substance which had a UV profile containing oligonucleotide and fluorescein chromophores in the correct relative ratio (Fig 2). A control experiment utilizing an oligonucleotide containing thymidine instead of (4) did not react with FITC in this way. The isolated, labelled oligonucleotide appeared as a single fluorescent band on a polyacrylamide gel electrophoretogram that migrated closer to the origin relative to the free-amino or unmodified oligonucleotides.

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13) To a magnetically-stirred, ice-cold solution of propargylamine (5.51g, 100mmol) in methanol (100ml) was added ethyl trifluoroacetate (18.5g, 130mmol). The resulting solution was stirred at room temperature until TLC indicated that no starting material was present (24hr). The solution was evaporated in vacuo, the residue dissolved in chloroform (100ml), extracted with saturated sodium bicarbonate (2 X 100ml), then water (100ml) and finally evaporated in vacuo to a dark-red oil. The residue was distilled under reduced pressure to give a colorless liquid (11.17g, 74%), which solidified at -20 ^{o} C. B.p. 51 ^{o} C at 2.5 torr. ^{1}H NMR ($CDCl_3$): 2.34 (t, J=2.7Hz, 1H), 4.16 (dd, J=5.4,2.7Hz, 2H), 7.0 (br s, 1H). Electron ionization mass spectrum: M^+ observed 151.0246, $C_5H_4NOF_3$ requires 151.0245. Also m/z 131(M-HF), 123(M-CO), 102(M-HF-CHO), 82($M-CF_3$), 69(CF_3), 39(C_3H_3).

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15) ^{1}H NMR (DMSO-d_6 + D_2O): 7.94 (s, 1H, H-6), 7.42-7.22 (m, 9H, phenyl), 6.90 (m, 4H, phenyl), 6.10 (t, J=6.6Hz, 1H, H-1'), 4.33 (m, 1H, H-3'), 4.06 (br s, 2H, $CCCH_2$), 3.91 (m, 1H, H-4'), 3.28 (m, 1H, H-5'), 3.08 (m, 1H, H-5''), 2.28 (m, 2H, H-2', H-2''). GC/MS (Thermabeam interface): 679(M^+, 1%), 572, 418, 322, 320, 303(100%).

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17) Oligonucleotide synthesis was carried out using beta-cyanoethyl phosphoramidites on an Applied Biosystems 380B DNA synthesizer using, unless otherwise stated, reagents and protocols as supplied by the manufacturer. 1-Methylimidazole was used instead of dimethylaminopyridine in the acetic anhydride/lutidine/THF capping reagent.

18) Figures show product after HPLC purification. HPLC was carried out on a Vydac C-18 column #218TP10415 using as eluant buffer B (100mM triethylammonium acetate, pH 7.2) supplemented by 1% per min with buffer A (acetonitrile). Flow rate 1.0 ml per min. At 0 min the eluant consisted of 2% A: 98% B.

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