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SYNTHESIS OF FLUORESCEINYLATED 2'-DEOXYADENOSINE. MONO, DI AND TRIPHOSPHATE DERIVATIVES.

S.R. SARFATI* and A. NAMANE

Unité de Chimie Organique, associée au CNRS 487, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris Cedex 15, France.

Abstract: The synthesis of 2'-deoxyadenosine nucleotides which contain an aliphatic amino chain at the 8 position, covalently attached to fluorescein, provide fluorescent non-radioactive DNA probes. Through a fluoresceinylated 5'-phosphoromorpholidate, the 5'-triphosphate can be obtained.

DNA sequence determination is one of the most important tools of molecular biology. For DNA sequence analysis, two methods [1,2] exist, both of which are in wide use. Although these methods are extremely powerful, they are also very laborious, time consuming, expensive and require the use of hazardous and short lived radioisotopes.

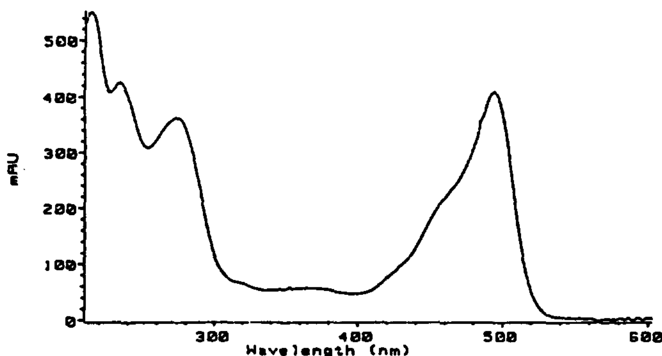
Recently, several labelling procedures for the detection of specific nucleic acid sequences in situ, using non-radioactive tags such as biotin [3,4] or fluorophores [5], have been developed, to reduce or eliminate the problems associated with radioactivity. In biochemical research there is an increasing use of natural products rendered fluorescent by the addition of a fluorophore because they enable molecular events to be visualized rapidly at high sensitivity.

We report in this paper our own investigations on the preparation of a non-radioactive probe. This probe is an analogue of a dATP bearing a twelve-atom linker arm at the C-8 position. This spacer arm possesses a fluoresceinylated primary amino group which can be used to visualize, by fluorescence spectroscopy, a chosen sequence of DNA into which the modified nucleotide is incorporated. A wide range of applications can be imagined for this probe, such as genome screening in molecular genetics.

The basic procedure used for the attachment of the dye molecule to the aminonucleotides is to combine the aminonucleotide and the dye in a buffered aqueous solution (pH 9) at room temperature for several hours and then to purify the product in two steps. The first purification step removes the bulk of the unreacted or hydrolysed dye by gel filtration. The second purification step separates the dye conjugate from any unreacted nucleotide by reverse phase HPLC.

The benzyloxycarbonyl protecting group of compound 1 [4] was removed by hydrogenolysis with a 10% Pd-C catalyst. The resulting 8-(10-aminodecyl)-amino-5'-dAMP 2 on reaction with fluorescein isothiocyanate in a mixture of 1M carbonate-bicarbonate buffer at pH 9 in the dark at room temperature for 18 hrs afforded the fluoresceinylated monophosphate 5 [8] in good yield. The dye-nucleotide conjugate was purified by gel filtration on Sephadex G25 (medium) using water as eluent followed by reverse phase HPLC on Nucleosil 5μm C18 column. It has been characterized by mass and U.V spectroscopies.

When 8-(10-aminodecyl)-amino-5'-dATP 4 [4] was treated in this way with fluorescein isothiocyanate, TLC showed several decomposition products. After purification by gel filtration and analysis of the void volume on reverse phase HPLC coupled with a diode-array detector (HP 1040 M) the chromatogram presented a multitude of peaks, none of which presented an UV spectrum identical to the fluoresceinylated monophosphate.

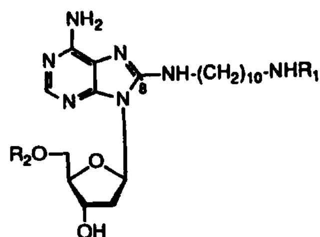


UV spectra of fluoresceinylated 2'-deoxyadenosine mono- , di- and triphosphate derivatives.

The synthesis of the fluoresceinylated triphosphate analog 8 can, however be carried out using the monophosphate [6] (one pot) or the phosphoromorpholidate, the latter using the Moffatt procedure [7].

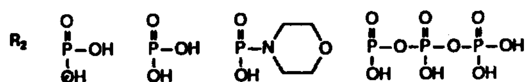
Coupling the 8-(10-aminodecyl)-amino-5'-phosphoromorpholidate 3 with FITC using the experimental conditions described above, led after purification on Sephadex G25 (medium) and reverse phase HPLC, to the desired compound 6 in good yield. Characterization of 6 was accomplished using mass and UV spectroscopies. Its UV spectrum is superimposable on that of compound 5.

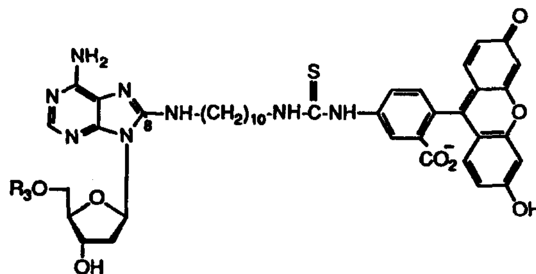
Treatment with one eq. of tetra-n-butylammonium pyrophosphate in dry DMSO for three days afforded the fluoresceinylated diphosphate 7 and triphosphate 8. After purification by ion exchange chromatography on a DEAE cellulose column (bicarbonate form) using a linear gradient of triethylammonium bicarbonate (0 to 1M), followed by reverse phase chromatography on a Nucleosil 5 μm C18 column, we have isolated 7 and 8 in relatively poor yields (9% and 24%, respectively). The fluorescein labelled dADP arise by degradation of the desired fluorescein labelled dATP.



	1	2	3	4
R_1	Cbz	H	H	H

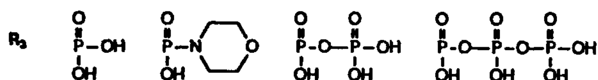
R_2





5 6 7 8

R_3



The di- and triphosphate derivatives (7 and 8) have been characterized by UV, mass and NMR spectroscopies [10,11]. Studies of their applications in molecular biology are currently in progress.

__Abbreviations:__ FITC, fluorescein isothiocyanate ; DMSO, dimethylsulfoxide ; Cbz, benzyloxycarbonyl; TLC, thin layer chromatography ; HPLC, high performance liquid chromatography ; NMR, nuclear magnetic spectroscopy ; dAMP, dADP, dATP ; 2'-deoxyadenosine mono, di, triphosphate ; TEAB, triethylammonium bicarbonate.

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8. Compound **5**. M.S. (FAB$^+$) M$^+$: 890, m/e : 891 ; λmax. (TEAB +CH_3CN) 234, 274 and 494 nm.

9. Compound **6**. M.S. (FAB$^+$) M$^+$: 959, m/e : 960 (m+H$^+$) ; λ max. (TEAB + CH_3CN) 234, 274 and 494 nm.

10. Compound **7**. M.S.(FAB$^+$) M$^+$: 970, m/e : 971 (m+H$^+$) ; λ max (TEAB + CH_3CN), 234, 274 and 494 nm. ^{31}P-NMR 162 MHz : D_2O : δ (PO_4H_3 as reference) : -8.58 (d, 1P, P_β, $J_{P_\alpha-P_\beta}$ = 13Hz); -9.69 (d,1P,P_α, $J_{P_\beta-P_\alpha}$ = 13 Hz). λ max. (TEAB + CH_3CN) 234, 274 and 494 nm.

11. Compound **8**. M.S. (FAB$^+$) M$^+$: 1050, m/e : 1051 (m+H$^+$); ^{1}H-NMR 400 MHz : (D_2O) : δ = 1.28 (m, 14H, decyl protons) ; 1.43 (m, 2H, decyl protons) ; 1.63 (m, 2H, decyl protons); 2.13 (dd, 1H, H'-2) ; 2.53 (m, 1H, H"-2) ; 3.52 (m, 2H, H-5'and H-5") ; 4.22 (m, 1H, H'-4) ; 4.07 (m, 1H, H'-3) ; 4.82 (HDO) ; 6.03 (q, 1H, H'-1) ; 6.62-7.72 (9H, fluorescein protons) ; 7.91 (s, 1H, H-2). ^{31}P-NMR 162 MHz : D_2O : δ (PO_4H_3 as reference) : -8.59 (d, 1P, P_γ) ; -9.61 (d, 1P, P_α) ; -20.24 (t, 1P, P_β).

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