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**PREPARATION OF THE FLUORESCHEIN REAGENT
FOR SOLID-PHASE OLIGONUCLEOTIDE 5'-LABELLING
AND ITS USE FOR THE SYNTHESIS OF FLUORESCENTLY
LABELLED PCR PRIMERS FOR HIV-1 DETECTION**

The preparative synthesis of fluorescein H-phosphonate reagent for solid phase labelling of oligonucleotides is described. Fluorescein-labelled thymidine and two PCR primers for HIV-1 detection were synthesized using this reagent.

Introduction. The progress in our understanding of gene structure and function has depended upon methods for detecting small amounts of nucleic acids. The screening, isolation, structural and functional analysis of genes all involve the use and detection of labelled oligonucleotides. Radioactive labels are still very popular because they are easy to handle and provide a great sensitivity of detection. But an increasing number of highly sensitive non-radioactive bioanalytical systems are being developed to reduce or eliminate the problems associated with radioactivity [1, 2]. Non-isotopic reporter groups, in contrast to isotopic labels, are usually stable and have safety advantages. Fluorescent labelling is now widely used since fluorescent dyes are detected directly and at high sensitivity [3]. The latter is adequate for most applications in the nucleic acids studies except those which require ultimate sensitivity [1]. Oligonucleotides carrying fluorescent reporter groups are used as hybridization probes for the detection of specific nucleic acids, including diagnostic procedures in medicine [1—5], as primers for automated sequencing [6], in fluorescence microscopy [7], etc. We have recently started the studies of the fluorescent DNA labelling, particularly for AIDS detection. Polymerase chain reaction (PCR) is now used as diagnostic procedure in screening for HIV sequences [4]. It would be convenient for clinical laboratories to detect amplified fragments using a fluorescently labelled primers or probes. Here we describe the preparation of the reagent for solid-phase oligonucleotide labelling with fluorescein and its use for the synthesis of labelled primers for HIV-1 detection.

Materials and Methods. 4,4'-Dimethoxytrityl chloride (DMTrCl) and 1,2,4-triazole (Tri) were purchased from Fluka (Switzerland), acrylamide and N,N'-methylene bisacrylamide for electrophoresis from Reanal (Hungary). Another reagents and solvents were of home production. Solvents were dried according to [8]. Silicagel 60 F₂₅₄ plates (Merck, Germany) were used for TLC analysis in the following solvent systems: CHCl₃-CH₃OH 9:1 (A), CHCl₃ (B), iso-PrOH-conc. NH₃-H₂O 7:2:1 (C). Trimethylsilyl-silica (TMS-silica) was prepared as described [9]. Reverse-phase HPLC was performed on Pharmacia FPLC System (Sweden) using HR 5/2 column in the gradient of CH₃CN in 0,1 M triethylammonium acetate (pH 7.5) with elution rate 0,5 ml/min. Absorbance spectra were recorded on Specord UV-VIS spectrometer (Karl Zeiss Jena, Germany), fluorescence spectra were obtained on Hitachi MPF-4 spectrofluorimeter (Japan). Victoria-6M gene synthesizer (Novosibirsk, Russia) was used

for oligonucleotide synthesis by standard H-phosphonate method [10] except that couplings were performed in acetonitrile-pyridine (4:1) mixture. Nucleoside H-phosphonates were prepared as described [10]. Our own polymer support based on Silochrom-2 silica [11] was used for the solid-phase synthesis.

1-(4,4'-dimethoxytrityloxy)-4-chlorobutane. 4-Chlorobutanol-1 (1,0 ml, 10 mmol) was treated with DMTrCl (4,1 g, 12 mmol) in dry pyridine (Py) (50 ml) for 2 hrs. The reaction mixture was diluted with chloroform (200 ml), and washed with 5% NaHCO₃ (2×50 ml) and water (50 ml). The organic phase was dried with Na₂SO₄ and evaporated with toluene. After the chromatography on silica gel in the gradient of chloroform (0–40%) in hexane the colorless oil was obtained (4,5 g, 85%). *R_f* 0,73 (system B).

Fluorescein methyl ester (1). Fluorescein (4,0 g, 12 mmol) was refluxed in 100 ml of methanol in the presence of conc. H₂SO₄ (5 ml) during 16 hrs. The mixture was poured into 0,5 l of water and NaHCO₃ was added by small portions to neutral pH. Red crystals were filtered, washed with water and dried. After recrystallization from methanol 2,85 g (71%) of fluorescein methyl ester was obtained. Mp 273–274 °C (lit. 272 °C, Beilstein); *R_f* 0,36 (system A); λ_{\max} (EtOH) 459,487 nm.

6-(4-dimethoxytrityloxybutyl)fluorescein methyl ester (2a). Fluorescein methyl ester (1,30 g, 3,75 mmol), 1-dimethoxytrityloxy-4-chlorobutane (3,10 g, 7,5 mmol) and fine powders of K₂CO₃ (1,03 g, 7,5 mmol) and KJ (1,25 g, 7,5 mmol) were stirred in dimethylformamide (DMF) (15 ml) at room temperature for 2 weeks until all starting dye was converted into fluorescent trityl-containing product with higher *R_f*. The mixture was diluted with CHCl₃ (100 ml) and washed with 0,25 M TEAB (2×50 ml) and water (50 ml), dried over Na₂SO₄ and evaporated to dryness. The product was isolated by chromatography on silica gel in the gradient of methanol (0–2%) in chloroform. The resulting resin was precipitated into hexane yielding 2,40 g (87%) of orange powder. Mp 92–95 °C. *R_f* 0,46 (system A). λ_{\max} (EtOH) 460,489 nm. Anal. Calc. for C₄₆H₄₀O₈: C 76,67; H 5,56. Found: C 76,01; H 5,27.

6-(4-hydroxybutyl)fluorescein methyl ester (3a). The solution of (2a) (2,16 g, 3 mmol) in chloroform (200 ml) was cooled to 0 °C and trifluoroacetic acid (2 ml) was added. After 5 min the solution was washed with 5% NaHCO₃ (2×50 ml), and water (50 ml), dried with Na₂SO₄ and evaporated.

The product was recrystallized from benzene. Yield 1,22 g (94%) of orange crystals. Mp 205–206 °C. λ_{\max} (EtOH) 460,489 nm. Anal. Calc. for C₂₅H₂₂O₆: C 71,77; H 5,26. Found: C 71,98; H 5,01.

6-(4-hydroxybutyl)fluorescein methyl ester H-phosphonate, triethylammonium salt (4a). To a stirred solution of Tri (2,90 g, 42 mmol) and N-methylmorpholine (8,30 ml, 75 mmol) in anhydrous acetonitrile (40 ml) PCl₃ (1,10 ml, 12,5 mmol) was added. After 30 min the reaction mixture was cooled to 0 °C and (3a) (1,04 g, 2,5 mmol) in 20 ml of anhydrous pyridine was added dropwise over 20 min. The mixture was stirred for additional 20 min without cooling and poured into 0,25 M triethylammonium bicarbonate (TEAB) (200 ml). The mixture was extracted with chloroform (3×100 ml), the organic phase was washed with 0,25 M TEAB and water (100 ml each), dried with Na₂SO₄ and evaporated to dryness with toluene. The residue was applied to a silica gel column (50×150 mm) which was first washed with 3–5% methanol in chloroform, and then the product was eluted with 10% methanol in chloroform containing 1% of triethylamine. The eluate was washed with 0,25 M TEAB and water, dried with Na₂SO₄ and evaporated to dryness. Our attempts to precipitate the resulting resins were unsuccessful. It was dried in a vacuum of oil pump to give a dark-orange solid foam. The yield of hygroscopic powder was 0,91 g (63%). *R_f* about 0,05 (system A), 0,72 (system C). λ_{\max} (EtOH) 460,489 nm. Anal. Calc. for C₃₁H₃₈NO₈P: C 63,81; H 6,52; P 5,32. Found: C 63,25; H 6,92; P 5,05.

Fluorescein-thymidine conjugate (5a, R=T). 58 mg (0,1 mmol) of the reagent (4a) and 3'-O-acetylthymidine (37 mg, 0,13 mmol) were evaporated twice with anhydrous pyridine, dissolved in 1 ml of this solvent and pivaloyl chloride (PivCl) (60 μ l, 0,5 mmol) was added. After 5 min the solution of iodine (127 mg, 0,5 mmol) in 3 ml of pyridine-water (95:5) mixture was added. After 20 min the reaction mixture was diluted with 0,5 M TEAB (5 ml) and Na₂S₂O₃ solution was added dropwise to neutralize excess J₂. The reaction mixture was extracted with CHCl₃ (3 \times 3 ml), the organic layer was washed with 0,25 M TEAB and water (3 ml each), dried over Na₂SO₄ and evaporated to dryness with toluene. The residue was chromatographed on a small silica gel column (10 \times 50 mm) under conditions described above for fluorescein H-phosphonate isolation.

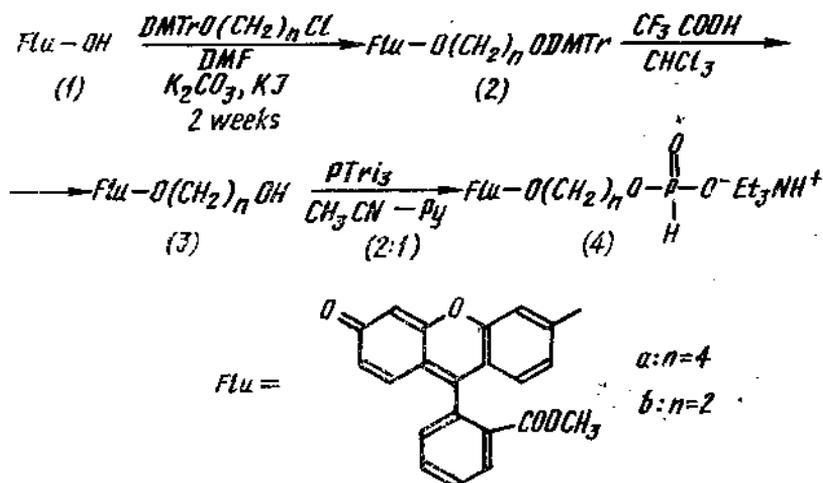
The residue was dissolved in dioxan-conc. NH₃ (1:1) mixture (5 ml) and left overnight at room temperature for deblocking. The mixture was evaporated to dryness and the deblocked fluorescein-labelled thymidine was isolated by reverse-phase chromatography on TMS-silica in the gradient of dioxan (20–80 %) in 0,05 M triethylammonium acetate (pH 7,5) according to [9]. The yield of product after drying was 25 mg (30 %). R_f 0,70 (system C). λ_{\max} (H₂O) 454,480 (shoulder) nm.

Synthesis of fluorescein-labelled oligonucleotides. After the last chain elongation steps the oligonucleotide bound to the polymer support was detritylated. The solution of (4a) (0,04 M in acetonitrile-pyridine 4:1, 200 μ l) and PivCl (0,2 M in the same solvent, 200 μ l) were added to the polymer simultaneously. After 4–5 min the support was washed with pyridine and after standard oxidation (2 % iodine in pyridine-water 98:2, 15–20 min) it was treated with conc. NH₃ at 50 °C overnight (or 3 days room temperature). Fluorescein-labelled oligonucleotides were isolated by standard polyacrylamide gel electrophoresis and desalted by gel-filtration on PD-10 cartridge (Pharmacia).

Discussion. Fluorescent dyes as well as other reporter molecules can be covalently linked to oligonucleotides by a wide variety of methods (see comprehensive reviews [12–14] for detailed discussion). Generally, oligonucleotide labelling can be performed at both termini or internally during chemical synthesis or by the post-synthetic procedures. The post-synthetic functionalization of deprotected oligonucleotide derivatives containing aminoalkyl or mercaptoalkyl linkers is more popular, and many reagents for this purpose are commercially available. But complex reaction mixtures are usually formed in this case containing starting oligonucleotide together with labelled product which should be purified by HPLC; moreover, removal of the excess dye can also be a serious problem [15]. As for us, another general approach, namely direct labelling, is more attractive since it avoids laborious post-synthetic functionalization. In this approach, a suitably protected chemical moieties are used which can be coupled at the 5'-terminus of protected oligonucleotide directly during the solid-phase synthesis in a similar manner to the internucleotide condensation reaction. It is possible when the reporter molecule to be linked can be converted into phosphoramidite (H-phosphonate, phosphodiester) derivative and withstands the coupling and deprotection steps. There are several examples of the use of phosphorylating derivatives of fluorescent dyes and other reporter groups of the direct labelling [12–14].

As is generally known, the fluorescence intensity is proportional to extinction coefficient (ϵ) and quantum yield of fluorescence (q) of the substance being irradiated. Thus, the detection limit of fluorescent label depends mainly on $\epsilon \cdot q$ value. We have chosen a well-known fluorescein as fluorescent marker since it has both intense absorbance ($\epsilon_{496} = 75\ 000$) and high quantum yield (q 0,6–0,9) and can be therefore detected in lower concentration than most of common fluorophores [16]. Fluorescein-labelled primers are used in automated DNA sequencing where a sensitivity level was achieved ($3 \cdot 10^{-18}$ M per band) comparable to that for isotopic labels [6].

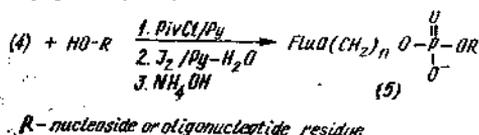
Oligonucleotides are usually labelled with fluorescein by post-synthetic approach using fluorescein isothiocyanate (FITC) or iodoacetamidofluorescein via amino- or mercaptoalkyl groups, respectively [6, 12-17]. We elaborated a preparative synthesis of the fluorescein H-phosphonate derivatives (4) for oligonucleotide 5'-labelling during solid phase synthesis. A similar fluorescein phosphoramidite reagent was proposed previously by F. Schubert et al. in short communication [18], but experimental details are still unavailable, as far as we know. In present article we describe a full synthetic protocol for the preparation of the reagent (4a). It was synthesized as illustrated in Scheme 1.



Fluorescein methyl ester (1) was first prepared by the esterification of fluorescein with methanol in the presence of H_2SO_4 . Its phenolic hydroxyl was then alkylated by modified Claisen method to introduce a linker group. The alkylation in DMF in the presence of potassium carbonate and catalyzed by iodide. O-Protected 4-chlorobutanol-1 was easily prepared by tritylation of alcohol in pyridine. Intermediate (2a) was detritylated and the resulting 6-(4-hydroxybutyl)fluorescein methyl ester (3a) was phosphorylated with tris(1,2,4-triazolyl)phosphine according to [10]. The latter reaction was performed in acetonitrile-pyridine mixture due to low solubility of (3a) in acetonitrile. H-phosphonate (4a) was isolated by silica gel chromatography. Compounds (1)-(4) were characterized by absorbance and fluorescence spectroscopy and elemental analysis.

Reagent (4b) containing an ethylene linker was also synthesized by the way described starting from ethylene chlorohydrin (data not presented; see below).

Reagent (4) can be used as P-component for the coupling to oligonucleotide or nucleoside on solid phase or in solution in H-phosphonate coupling reaction, according to general Scheme 2. As in H-phosphonate method of oligonucleotide synthesis, the coupling is performed via activation of the phosphonate building block by condensing reagent (PivCl) and then H-phosphonate diesters are converted to the native phosphodiesters by aqueous iodine oxidation:



To check the reagent (4a) 5'-fluorescein-labelled nucleoside (5a, R = thymidine) was first synthesized successfully in solution according to this scheme. Fluorescein H-phosphonate (4a) was coupled with a slight excess of 3'-O-acetylthymidine in pyridine in the presence of PivCl as con-

densing reagent. TLC analysis showed that starting (4a) was disappeared immediately and H-phosphonate diester was formed quantitatively. The latter was oxidized with aqueous iodine without isolation, and after the deblocking the fluorescein-thymidine conjugate was purified by reverse-phase chromatography on TMS-silica and its properties were studied.

The absorbance spectra of fluorescein intermediates (1)–(4) are almost identical showing two bands in the visible region at 459–460 and 487–489 nm. Fluorescein has a similar spectrum too. 5'-Fluorescein-labelled thymidine (5a) shows slightly different pattern of spectrum which is

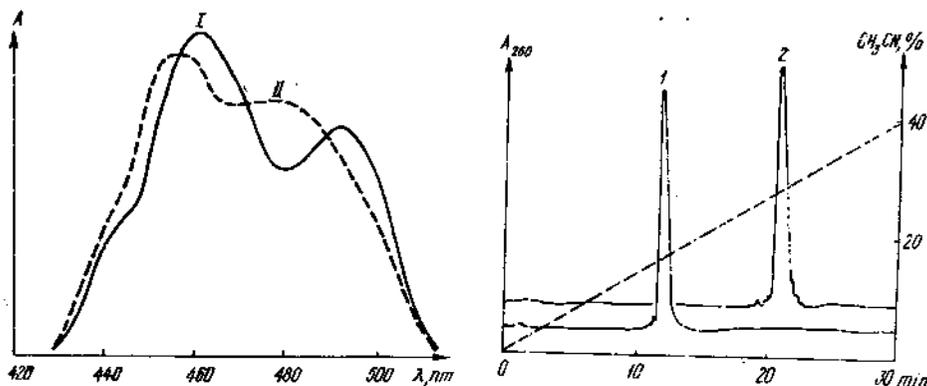


Fig. 1. The absorbance spectra of intermediate (3a) (I) and conjugate (5a, R=T) (II) in the visible region

Fig. 2. Reverse-phase HPLC of unlabelled (1) and 5'-fluorescein-labelled (2) primer SK 68 after purification

shifted to the short-wave region for 5–6 nm (Fig. 1). This difference is probably due to the conversion of methyl ester function present in (1)–(4) into amide group during the ammonolysis of fluorescein conjugate.

The limit of detection of 5'-fluorescein-labelled thymidine by the naked eye was determined as approximately 1–5 pmol by spotting various quantities of labelled nucleoside on filter paper and employing a light of the Chromatoscop lamp. Required sensitivity level usually depends on the amount of nucleic acid sample to be studied. The PCR with its ability to amplify DNA exponentially would permit the use of even relatively insensitive non-radioactive labels [1, 4]. So, this detection limit should be adequate for use of fluorescein-labelled primers or probes in PCR. Of course, one could increase the sensitivity considerably using a suitable equipment.

Fluorescein H-phosphonate reagent (4a) was used as a building block for solid phase oligonucleotide synthesis at the last step. We have synthesized two fluorescein-labelled primers for the amplification of *env* region of HIV-1 genome. These primers are known as SK 68 and SK 69 [19]:

SK 68 5'AGCAGCAGGAAGCACTATGG 3'

SK 69 5'CCAGACTGTGAGTTGCAACAG 3'

Oligonucleotides were synthesized by H-phosphonate method using Victoria 6M gene synthesizer with average coupling yields 98–98.5% per step. After the chain elongation was complete the fluorescein reagent (4a) was coupled to the 5'-hydroxy group of oligonucleotide. The coupling was carried out manually under standard conditions, as for nucleoside H-phosphonates, except that coupling time was prolonged (4–5 min). The coupling yields at this step were almost quantitative. After oxidation and deprotection the desired 5'-fluorescein-labelled oligonucleotides were purified by polyacrylamide gel electrophoresis where the labelled primers were visible as bands with greenish fluorescence. The products were eluted and desalted by gel-filtration. The purity of the labelled oligonucleotides was checked by reverse-phase HPLC (Fig. 2).

The UV VIS spectra of labelled primers showed two maxima at 260 and 454 nm. The contribution to the absorbance at 260 nm is due mostly to the oligonucleotide part of conjugate whereas the absorbance at 454 nm is due exclusively to dye moiety.

The biological part of work (the use of labelled PCR primers for HIV detection) is now in progress, as well as more detailed spectroscopic studies.

We tried to use the fluorescein derivative (4b) for oligonucleotide labelling too, but it showed poor results. We could give the following explanation, since fluorescein is electron withdrawing group, the conjugates (5b) containing an ethyl linker are probably susceptible to β -elimination and would therefore be cleaved at the ammonolysis step. This type of decomposition is however impossible in the conjugates (5a).

So, the use of the fluorescein reagent (4a) permits the efficient introduction of the fluorescent label into oligonucleotides and nucleosides on solid-phase as well as in solution. This reagent can be used in solid-phase oligonucleotide synthesis without changing current synthesis, de-blocking and purification procedures, thus significantly simplifying the preparation of fluorescein-labelled oligonucleotides. It would be useful also for the fluorescent labelling of another classes of biologically active compounds.

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ОТРИМАННЯ ФЛЮОРЕСЦЕНТНОГО РЕАГЕНТА ДЛЯ ТВЕРДОФАЗНОГО 5'-МІЧЕННЯ ОЛІГОНУКЛЕОТИДІВ ТА ЙОГО ВИКОРИСТАННЯ В СИНТЕЗІ ФЛЮОРЕСЦЕНТНО МІЧЕНИХ ПРАЙМЕРІВ ДЛЯ ПЛР ПРИ ДЕТЕКЦІЇ ВІЛ-1

Резюме

Описано препаративний метод синтезу Н-фосфонатного реагента для флюоресцентного мічення олігонуклеотидів безпосередньо в процесі їх твердофазного синтезу. За допомогою цього реагента синтезовано флюоресцеїн-мічені тимідин та два праймери для ампліфікації ділянки гена HIV-1.

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