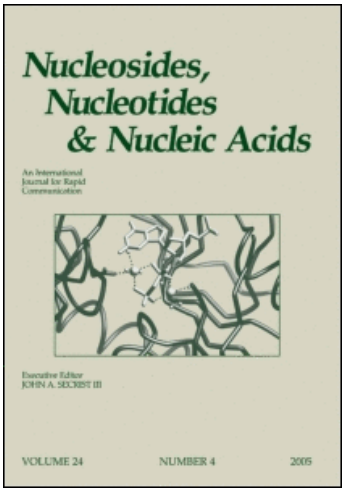

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A NEW METHOD OF SYNTHESIS OF FLUORESCENTLY LABELLED OLIGONUCLEOTIDES AND THEIR APPLICATION IN DNA SEQUENCING

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ABSTRACT

A new approach to the chemical synthesis of oligonucleotides bearing reporter functional groups based on the modification of cytosine residues during the final deprotection step is reported. The application of the fluorescently labelled primer for the automated DNA sequencing is shown.

INTRODUCTION

Non-radioactively labelled oligonucleotides are widely used in molecular biology and diagnostics[1]. Many chemical studies are directed toward a development of new and still simpler procedures to obtain such derivatives. Fluorescently labelled oligonucleotides are successfully used in automated DNA sequencing[2]. Methods used to prepare these primers include automated chemical synthesis of oligonucleotides containing additional reactive functions which are subsequently used to attach fluorescent markers usually at a 5'-end[2,3]. As many of the popular labelling agents are designed for reactions with an amino group the linkers with this function are most often used. Usually, the linker molecules are attached to the oligonucleotide in an extra step of a solid-phase synthesis.

We decided to use the previously discovered[4] reactivity of 4-N-p-arylsulfonylcytosine residues toward primary amines to introduce aminoalkyl linker groups into oligonucleotides[5]. This approach should allow, in principle, the synthesis of a series of functionalized oligonucleotides from one fully protected oligonucleotidic precursor[6]. 4-

N-Substituted cytosine moieties are interesting also for DNA (RNA) modification as sites with low steric hindrance[7]. We reported recently another application of 4-N-aminoalkylcytosine derivatives obtained from 4-N-arylsulfonylcytosine nucleosides[8]. Their 5'-triphosphates labelled with non-radioactive tag groups are substrates for DNA polymerases[8].

RESULTS AND DISCUSSION

4-N-p-Toluenesulfonyldeoxycytidine[4] (dC^{Ts}) was chosen for the introduction into oligodeoxynucleotides through phosphoramidite chemistry[9].

Thus, deoxycytidine was transiently O-protected with trimethylsilyl chloride in pyridine[10] and the excess of the silyl reagent was removed under diminished pressure. Then p-toluenesulfonyl chloride (2 molar equivalents) was added and the reaction was continued overnight at 50 °C. After the work-up including 4 hr treatment with ammonia in aqueous pyridine, crude 4-N-p-toluenesulfonyldeoxycytidine (**1**) was obtained. The latter was subsequently reacted with 4,4'-dimethoxytrityl chloride in pyridine to give, after silica gel column chromatography, the desired 5'-O-dimethoxytrityl-4-N-p-toluenesulfonyl-2'-deoxycytidine (**2**) in 74% overall yield.

Then the reactivity of 4-N-p-toluenesulfonylcytosine function toward 1,2-bis(2-aminoethoxy)ethane (**3**) was examined. Treatment overnight of **2** with 3 M solution of the amine **3** in pyridine at 50 °C gave quantitatively the 4-N-aminoalkyl derivative, 5'-O-dimethoxytrityl-4-N-(8-amino-3,6-dioxaoctyl)-2'-deoxycytidine, **4** (DMT-$dC^{aminoalkyl}$).

The amine **3**, 1,2-bis(2-aminoethoxy)ethane, was chosen due to its properties including solubility both in water and organic solvents. In contrast to other "long chain" amines such as 1,12-diaminododecane, solubility in water allows easy work-up (water wash of dichloromethane solution) and later chromatographic purification. In our opinion, this indicates that the aminoalkyl chain derived from ethyleneoxy elements should be hydrated when introduced into an oligonucleotide and have lower tendency to form coiled structures in aqueous media than polymethylene linkers. This is of crucial importance as the reporter groups that are attached to a synthetic oligonucleotide will be ultimately bound (or to interact) with other biomolecules (nucleic acids and/or enzymes). Moreover, the results of studies on the influence of the structure of linkers on the efficiency of solid-supported chemical synthesis of oligonucleotides corroborate our choice of the polyethyleneoxy linker[11]. The same linker was used also in some other recent reports[12].

In the next step 5'-O-dimethoxytrityl-4-N-p-toluenesulfonyl-2'-deoxycytidine 3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (**5**) was prepared in 88% yield in the reaction of **2** with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite[13].

In order to examine the transformation of dC^{Ts} residues in oligonucleotide chains, an octamer d-C^{Ts}TTTTTTT (**6**) was prepared. The octamer was released from the CPG

Table 1. Oligonucleotides and their characteristics.

No	Oligonucleotide	HPLC[a]	A_{260}/A_{480}[b]	Enzymatic digestion[20] nucleoside ratios[c]				
		R_t [min]		dA	dC	dG	dT	dC*
6	d-C^{Ts}TTTTTTT	33.7 (A)	-	-	-	-	-	-
7	d-$C^{aminoalkyl}$TTTTTTT	26.0 (A)	-	-	-	-	-	-
8	d-CTTTTTTT	25.6 (A)	-	-	-	-	-	-
10	d-$C^{aminoalkyl}$AGGAAACAGCTATAGC	23.9 (A)	-	-		-	-	-
11	d-$C^{aminofluoresc}$AGGAAACAGCTATAGC	34.9 (B)	10.0	6.8	3.0	4.0	1.95	1.0

[a]HPLC separations were run with following gradients: linear gradient of acetonitrile in TEAA buffer (0.1 M, pH 7.0) from 2% to 17% (in 30 min.) and to 30% (in 10 min.) - (A); from 2% to 17% (in 50 min.) and to 57% (in 10 min.) - (B); flow rate 0.7 mL/min.

[b]UV/VIS spectra in water.

[c]Digestion mixture was analyzed by the HPLC (Experimental); dC* = $dC^{aminofluoresc}$.

support by 0.5 hr concentrated aqueous ammonia treatment at room temperature and was purified by HPLC (Table 1). The octamer was dissolved in dry **3** with the help of an ultrasonic bath and kept at 80 °C. The clean transformation of **6** into octamer d-$C^{aminoalkyl}$TTTTTTT (**7**) was observed by HPLC (Fig. 1). The transformation reaction was completed in 10 hr and was not accompanied by the formation of d-CTTTTTTT (**8**).

Similarly, the 17-mer d-C^{Ts}AGGAAACAGCTATAGC (**9**) was synthesized using dC^{Ts} phosphoramidite **5**. Primer **9** is a precursor of reverse sequencing primer d-$C^{aminoalkyl}$AGGAAACAGCTATAGC (**10**), appropriate for sequencing of DNA inserted into cloning vectors derived from bacterial phage M13[14].

We observed the deposition of the aminoalkylated and fully deprotected product oligonucleotide during the transformation step with the amine **3.** Under these reaction conditions the removal of other amino protecting groups of oligonucleotides takes place. This observation is analogous to an earlier one concerning the deprotection of exo-amino groups of oligonucleotides with ethanolamine, ethylenediamine and butylenediamine[15]. Similar observations were reported recently for the mixture of ethanolamine with hydrazine[16]. The precipitation of a crude product simplified very much the work-up procedure (wash of the sedimentation with ethanol). Actually, we have observed the deposition of the transformed and fully deprotected oligonucleotide from amine **3** solution in the case of many other primers prepared by this approach. However, it should be pointed out that the precipitation of crude product was not observed for dT-rich

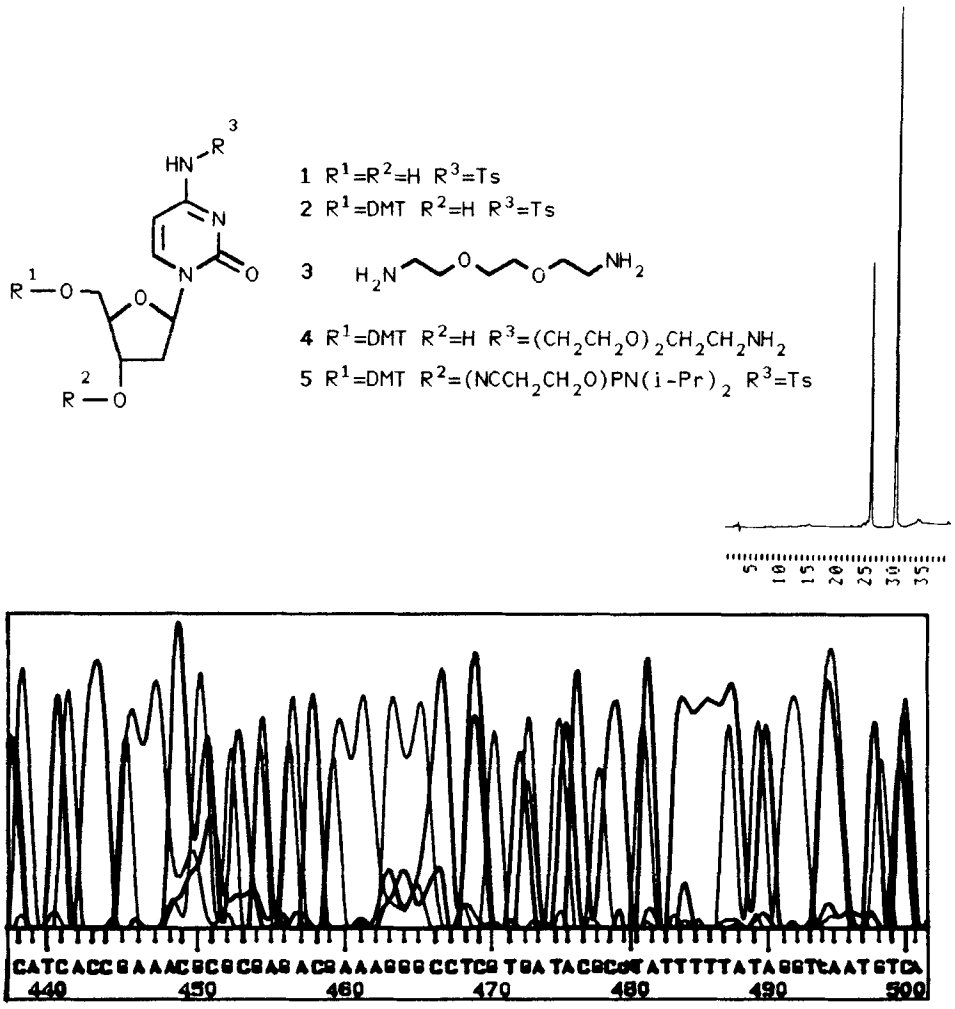

Fig. 1. The HPLC separation (conditions (A), Table 1.) of oligonucleotides 6 and 7 after 1.5 hr reaction with 3. Result of sequencing of pUC plasmid by the dideoxy method using primer 11 with A.L.F. DNA Sequencer™ of Pharmacia LKB.

oligonucleotide 7 thus indicating the dependence of this property on the composition of the oligomer.

The d-C^{aminoalkyl}AGGAAACAGCTATAGC 17-mer (**10**) was purified by HPLC as described in Table 1 and was subsequently reacted with fluoresceine isothiocyanate in sodium borate buffer pH 9.2 according to recommendation of the supplier[17]. Purification by HPLC and polyacrylamide gel electrophoresis gave the desired fluoresceinated primer

11. The UV/VIS spectra of **11** were consistent with the expected ratio of A_{260}:A_{480} (Table 1). Enzymatic digestion of **11** according to Eadie[18] gave all nucleosidic components in the expected ratio (Table 1).

Fluorescently labelled 17-mer primer **11** was then employed in the automated DNA sequencing using A.L.F. DNA Sequencer™ from Pharmacia LKB. This primer allowed consistently for a clear DNA analysis of sequences of length at least 500 base pairs (dideoxy chain termination method[19]). The result of sequencing of the pUC plasmid[20] with **11** is shown in Fig. 1.

Further studies on the application of 4-N-p-toluenesulfonyl-2'-deoxycytidine (dC^{Ts}) precursor are in progress.

EXPERIMENTAL

General methods

All the solvents used in the reaction were purified and dried according to the earlier published procedures. ^1H NMR spectra were recorded with 300 MHz Varian Unity 300 NMR spectrometer using tetramethylsilane (TMS) as an internal standard. ^{31}P NMR spectra were recorded with the 34.6 MHz (90 MHz for ^1H) Jeol 90 FX NMR instrument using 85% aq H_3PO_4 as an external standard. UV-VIS spectra were measured on Beckman DU-65 spectrophotometer.

Thin layer chromatography was performed on E. Merck pre-coated plates: (i) silica gel 60 HF_{254} in the following solvent systems: A - dichloromethane-methanol (9:1); B - dichloromethane-methanol (95:5); C - isopropanol-concentrated aqueous ammonia-water (7:1:2); D - dichloromethane-ethyl acetate-triethylamine (45:45:10); (ii) Merck silica gel 60 F_{254} silanized (RP-2) in solvent: E - acetone-water (7:3). All solvent ratios are by volume. Short column chromatography was performed on silica gel Merck H 60 in dichloromethane containing methanol or in a mixture of dichloromethane, methanol and triethylamine. 1,2-Bis(2-aminoethoxy)ethane (Fluka) was dried over molecular sieves 4Å. Fluoresceine isothiocyanate isomer I (FITC) was obtained from Fluka.

Sequencing with A.L.F. DNA Sequencer™ from Pharmacia LKB was carried out essentially according to instructions of the manufacturer and using Pharmacia LKB AutoRead™ Sequencing Kit (27-1690-01).

The HPLC analyses were done using Applied Biosystems Model 1783A Absorbance Controller, 1480A Injector/Mixer, 1400A Solvent Delivery System (2x) equipped with Merck D-2000 Chromato-Integrator. The analyses were performed on Merck C18 (5 μm) column with linear gradients of acetonitrile in 0.1 M triethylammonium acetate (TEAA) pH 7.0 with flow rate 0.7 mL/min; gradients and retention times (R_t) are reported in Table 1.

Synthesis of 5'-O-dimethoxytrityl-4-N-p-toluenesulfonyl-2'-deoxycytidine (2)

2'-Deoxycytidine hydrochloride (1.32 g, 5 mmole) was reacted with trimethylsilyl chloride (2.5 mL, 20 mmole) in anhydrous pyridine (20 mL) under stirring for 1 hr. Then the reaction mixture was concentrated under exclusion of moisture to the volume of ca 15 mL and p-toluenesulfonyl chloride (1.9 g, 10 mmole) was added. The tightly stoppered reaction vessel was placed in the oven at 60 °C and left overnight. The TLC analysis showed that the reaction went to completion. The reaction mixture was partitioned between dichloromethane (50 mL) and aqueous saturated sodium bicarbonate (60 mL). The aqueous layer was extracted twice with dichloromethane (20 mL). The combined extracts were concentrated under reduced pressure (water pump) and pyridine was added to the volume of 15 mL. Concentrated aqueous ammonia (15 mL) was added and the progress of the desilylation was followed by TLC analysis: R_f(A) 0.35, R_f(E) 0.89. The reaction was completed in 4 hr. Then the reaction mixture was concentrated under diminished pressure and the resultant crude 4-N-p-toluenesulfonyl-2'-deoxycytidine was dried by co-evaporation with anhydrous pyridine (3 x 20 mL) and redissolved in anhydrous pyridine (20 mL). 4,4'-Dimethoxytrityl chloride (1.70 g, 5 mmole) was added. The tritylation reaction went to completion in ca 2 hr: R_f(B) 0.50, R_f(C) 0.70. The mixture was partitioned between dichloromethane (50 mL) and 0.5 M aqueous sodium bicarbonate (50 mL). The aqueous layer was extracted with dichloromethane (2 x 50 mL) and the combined organic extracts were dried over anhydrous sodium sulfate and concentrated. Pure 5'-O-dimethoxytrityl-4-N-p-toluenesulfonyl-2'-deoxycytidine (**2**) was obtained after silica gel column chromatography with dichloromethane-methanol mixture as an eluting solvent. The foam of pure product was dissolved in benzene (20 mL), frozen and lyophilized under oil pump pressure to give the title compound as a white solid: 2.52 g, 73.7 % yield. ^{1}H NMR ($CDCl_3$): δ 8.60 (m, 1H, NH), ca 7.8 (m, 3H, H-6 and 2H of Ts), 6.8-7.4 (m, 15H, 2H of Ts and DMT), 6.24 (t, 1H, J 8.8 Hz, H-1'), 5.68 (bs, 1H, H-5), 4.55 (m, 1H, H-3'), 4.03 (m, 1H, H-4'), 4.04 (s, 6H, 2xOCH_3), 3.45 (dq, 2H, J 3 and 10 Hz, H-5' and 5"), 2.48 and 2.27 (2m, 2H, H-2' and 2"), 2.41 (s, 3H, CH_3) ppm; MS-FAB: M^+ 683, calc. for $C_{37}H_{37}N_3O_8S$ 683.

Synthesis of 5'-O-dimethoxytrityl-4-N-(8-amino-3,6-dioxaoctyl)-2'-deoxycytidine (4)

Anhydrous 1,2-bis(2-aminoethoxy)ethane (750 µL, 5.12 mmole) was added to the solution of 5'-O-dimethoxytrityl-4-N-p-toluenesulfonyl-2'-deoxycytidine (205 mg, 0.30 mmole) in pyridine (750 µL). The flask was tightly closed, placed in the oven and kept overnight at 50 °C. The reaction mixture was partitioned between water (15 mL) and dichloromethane (7.5 mL). The organic extracts were dried over anhydrous sodium sulfate, concentrated and analyzed by the TLC, R_f(A) 0.0, R_f(C) 0.62, indicating that the reaction went to completion. Pure 5'-O-dimethoxytrityl-4-N-(8-amino-3,6-dioxaoctyl)-2'-deoxycytidine (**4**) was obtained after a silica gel chromatography in dichloromethane-methanol mixture as a white foam: 170 mg, 0.257 mmole, 85.7% yield; ^{1}H NMR

($CDCl_3$): δ 7.73 (d, 1H, J 7.2 Hz, H-6), 6.77-7.35 (m, 13H, DMT), 6.27 (t, 1H, J 6.0 Hz, H-1'), 6.10 (m, 1H, NH), 5.32 (d, J 6.6 Hz, H-5), 4.42 (q, 1H, J 5.5 Hz, H-3'), 3.96 (q, 1H, J 3 Hz, H-4'), 3.72 (s, 6H, 2xOCH_3), 3.54 (s, 4H, OCH_2CH_2O), 3.45 (t, 2H, J 5.2 Hz, CH_2), 3.4 (m, 2H, H-5' and 5"), 2.78 (t, 2H, CH_2), 2.14 and 2.34 (m, 2H, H-2' and 2") ppm.

Reaction of 5'-O-dimethoxytrityl-4-N-(8-amino-3,6-dioxaoctyl)-2'-deoxycytidine (4) with fluoresceine isothiocyanate (FITC)

To the solution of **4** (1.75 mg, 2.6 μmole) in pyridine (100 μL) and water (5 μL) FITC (0.75 mg, 1.9 μmole) was added. The reaction mixture was left overnight at room temperature. The reaction mixture was concentrated under oil pump pressure and the crude product was detritylated with 20% acetic acid in dichloromethane (1 hr). After concentration of the mixture on Speed-vac and co-evaporation with ethanol, the residue gave $dC^{aminofluoresc}$ after purification by HPLC, R_t 27.4 min., with a linear gradient of acetonitrile in 0.1 M TEAA from 30% (for 10 min.), then to 50% (in 40 min.); UV-VIS (H_2O): λmax 237 and 490 nm, λsh 270 nm, A_{250}/A_{260} 1.0, A_{260}/A_{490} 0.5.

Synthesis of 5'-O-dimethoxytrityl-4-N-p-toluenesulfonyl-2'-deoxycytidine 3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (5)

5'-O-dimethoxytrityl-4-N-p-toluenesulfonyl-2'-deoxycytidine (**2**) (1.368 g, 2 mmole) was dissolved in anhydrous dichloromethane (10 mL) with diisopropylethylamine (2 mL, 11.5 mmole). Then to the above solution 2-cyanoethyl-N,N-diisopropyl-chlorophosphoramidite[13] (0.53 mL, 2.4 mmole) was added under exclusion of moisture. The reaction went to completion in ca 15 min; R_f(D) 0.20. Methanol (0.1 mL) was added and after 10 min. the reaction mixture was partitioned between saturated aqueous sodium bicarbonate (20 mL) and dichloromethane (30 mL). The aqueous layer was extracted twice with dichloromethane (10 mL). The extracts were dried, concentrated and purified by a short column silica gel chromatography in petroleum ether-acetone mixture with 5% of triethylamine (by vol.). The foam of a pure product was dissolved in benzene (20 mL), frozen and lyophilized under oil pump pressure to give **5** as a white solid: 1.555 g, 87.6% yield. ^1H NMR (CH_3CN): δ 7.76 and 7.72 (2xd, 1H, J 8 Hz, 2 diastereoisomeric H-6), 7.65-7.88 (m, 2H of Ts), 6.8-7.44 (m, 15H, DMT and 2H of Ts), 6.12 and 6.04 (q and d, J 5.7 and 7.7 Hz, 1H, 2 diaster. H-1'), 4.60 (o, 0.5H, J 6.1 Hz, H-3'), 4.04 (m, 0.5H, H-3'), 3.77 (3s, 6H, 2xOCH_3), 2.88 (q, 2H, J 3 Hz, $CH_2\underline{CH_2}CN$), 2.36 (s, 3H, CH_3), 1.31 (m, 12H, CH_3 of N-i-Pr) ppm; ^{31}P NMR (CH_3CN): δ 147.95 ppm; MS-FAB: M^+ 883, calc. for $C_{46}H_{54}N_5O_9PS$ 883.

General procedure for the synthesis of oligonucleotides with 4-N-p-toluenesulfonyl-2'-deoxycytidine units and their transformation into long chain aminoalkyl derivatives

Synthesis of oligonucleotides with p-toluenesulfonyl groups and their reaction with 1,2-bis(2-aminoethoxy)ethane

Oligonucleotides were synthesized with Gene Assembler Plus from Pharmacia LKB (Sweden) according to the manufacturer protocol using appropriate supports (0.2 μmole) and phosphoramidites. Yields of 98% were achieved in couplings with 0.1 M solution of **5** in acetonitrile. The oligonucleotide was released from the support in an Eppendorf tube by treatment with concentrated aqueous ammonia at room temperature for ca 1 hr. Then the collected solution was concentrated with Speed-vac evaporator to dryness. Anhydrous 1,2-bis(2-aminoethoxy)ethane (100 μL) was added to the partially deprotected oligonucleotide, the tube was closed and the oligonucleotide was dissolved at room temperature with the help of the ultrasonic bath (5 to 10 min). The reaction solution was placed in the oven at 80 °C overnight. The fully deprotected final product with the aminoalkyl groups precipitated from the amine solution. The tube was centrifuged and the solution was gently removed. Anhydrous ethanol (100 μL) was added to wash the precipitate with the help of the ultrasonic bath (5 to 10 min) and centrifuged. The supernatant was discarded and the wash procedure was repeated once again. The sedimentation of the crude oligonucleotide was dried with Speed-vac evaporator and used in further experiments.

Several A_{260} O. D. units of a crude oligonucleotide could still be recovered from the amine supernatant after its gel filtration through NAP-10 (Pharmacia LKB) or similar columns.

Reaction of the aminoalkyloligonucleotides with fluoresceine isothiocyanate (FITC)

The procedure used to introduce fluoresceine residues into oligonucleotides was essentially the same as advised by Pharmacia LKB for AutoPrimer™ Synthesis Kit (27-9290-01). The precipitate of crude oligonucleotide (up to 30 A260 O. D. units) was dissolved in 0.05 M sodium borate buffer pH 9.2 (100 μL) and a freshly prepared solution of fluoresceine isothiocyanate isomer I (FITC, 1 mg) in anhydrous dimethylsulfoxide (30 μL) was added. The reaction was carried out in darkness at room temperature for 4 to 20 hr with essentially the same results. The reaction mixture was then applied to NAP-10 column (Pharmacia LKB) and washed with double distilled water (pH 8 to 9.5, adjusted with aqueous ammonia). The fraction containing oligonucleotides (ca 1.3 mL) was collected and the desired labelled oligonucleotide was isolated after polyacrylamide gel electrophoresis (20 %, 7 M urea) or HPLC (Table 1.) and characterized by the UV-VIS spectra (Table 1.) as well as digestion with snake venom phosphodiesterase and alkaline bovine phosphatase according to Eadie[18]. Analysis of the digestion mixture was performed on Merck C18 HPLC column using a linear gradient of 90% aqueous methanol in 50 mM potassium phosphate buffer (pH 7.0) containing 1% of methanol. The gradient consisted of the following segments: 0% (for 30 min.), to 15% (in 30 min.), to 100% (in 20 min.) and 100% (for 10 min). The following retention times R_t were observed: 7.1 min. (dC), 25.3 min. (dG), 27.4 min. (dT), 52.7 min. (dA) and 71.4 min. ($dC^{aminofluoresc}$).

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REFERENCES

1. Landegren, U., Kaiser, R., Caskey, C.T., Hood, L., *Science*, **242**, 229 - 237 (1988)
2. Ansorge, W., Sproat, B.S., Stegemann, B., Schwager, C., *J. Biochem. Biophys. Methods*, **13**, 315 - 323 (1986); Rosenthal, A., Sproat, B.S., Voss, H., Stegemann, J., Schwager, C., Erfle, H., Zimmermann, J., Coutelle, C., Ansorge, W., *DNA Sequence - J. DNA Sequencing and Mapping*, **1**, 63 - 71 (1990)
3. Agrawal, S., Christodoulou, C., Gait, M.J., *Nucl. Acids Res.*, **14**, 6227 - 6245 (1986)
4. Markiewicz, W.T., Kierzek, R., Hernes, B., *Nucleosides, Nucleotides*, **6**, 269 - 272 (1987)
5. Preliminary results were presented during International Symposium "*Synthetic Oligonucleotides: Problems and Frontiers of Practical Application*", Moscow, June 23 - 30, 1991
6. When the present studies were completed we learned about a similar approach based on 4-O-modified 2'-deoxyuridine derivatives: MacMillan, A.M., Verdine, G.L., *J. Org. Chem.*, **55**, 5931 - 5933 (1990)
7. Telser, J., Cruickshank, K.A., Morrison, L.E., Netzel, T.L., *J. Am. Chem. Soc.*, **111**, 6966 (1989)
8. Kierzek, R., Markiewicz, W.T., *Nucleosides, Nucleotides*, **10**, 1257 - 1275 (1991)
9. Matteucci, M.D., Caruthers, M.H., *J. Am. Chem. Soc.*, **103**, 3185 (1981)
10. Ti, G.S., Gaffney, B.L., Jones, R.A., *J. Am. Chem. Soc.*, **104**, 1316 - 1319 (1982)
11. Katzhandler, J., Cohen, S., Rahamim, E., Weisz, M., Ringel, I., Deutsch, J., *Tetrahedron*, **45**, 2777 (1989)
12. Pieles, U., Sproat, B.S., Lamm, G.M., *Nucl. Acids Res.*, **18**, 4355 - 4360 (1990)
13. Sinha, N.D., Biernat, J., Köster, *Nucl. Acids Res.*, **12**, 4539 - 4557 (1984)
14. Messing, J. et al., *Proc. Natl. Acad. Sci. USA*, **74**, 3642 (1977); Gronenborn, B., Messing, J., *Nature*, **272**, 375 (1978); Messing, J. et al., *Nucl. Acids Res.*, **9**, 309 - 321 (1981); Messing, J. and Vieira, J., *Gene*, **19**, 269 (1982);
15. Berner, S., Gröger, G., Mühlegger, K., Seliger, H., *Nucleosides, Nucleotides*, **8**, 1165 - 1167 (1989)
16. Polushin, N.N., Pashkova, I.N., Efimov, V.A., *Nucl. Acids Res., symp. ser. No* **24**, 49 - 50 (1991)
17. Pharmacia LKB AutoPrimer™ Synthesis Kit (27-9290-01)
18. Eadie, J.S., *Anal. Biochem.*, **165**, 442 - 447 (1987)
19. Sanger, F., Nicklen, S., Coulson, A.R., *Proc. Natl. Acad. Sci. USA*, **74**, 5463 - 5468 (1977)
20. Vieira, J., Messing, J., *Gene*, **19**, 259 (1982); Yanisch-Perron, C., et al., *Gene*, **33**, 103 (1985)

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