SYNTHESIS OF OLIGODEOXYNUCLEOTIDES ELIMINATING THE USE OF ACETONITRILE

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H-Phosphonate synthesis of oligodeoxynucleotides is described in which acetonitrile is replaced by dichloromethane. This solvent is thereafter easily regenerated and reused. Practically "one solvent" procedure of oligonucleotide synthesis has been established. The preparation of 5'-O-(4,4'-dimethoxytriphenylmethyl)-N^2-(N,N-dimethylaminomethylene)-2'-deoxyguanosine (*Ic*) and 5'-O-(4,4'-dimethoxytriphenylmethyl)-N^6-(N,N-dimethylaminomethylene)-2'-deoxyadenosine (*Id*) is described.

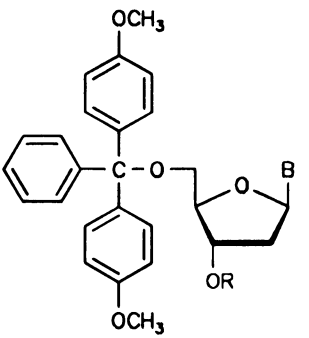
Currently, polymer-supported syntheses of oligodeoxynucleotides are carried out in commercial synthesizers. The phosphoramidite chemistry[1] mostly used by these machines requires the application of acetonitrile. Although the phosphoramidite chemistry enables synthesis of oligonucleotides exceeding 100 bases[2], the purity of these fragments for genetical purpose is questionable[3]. Recently, the increasing interest in applications of PCR* methodology[4] is leading to demand for chains approximately 20 bases long. These chains can also be prepared by use of the slightly less efficient but chemically more simple H-phosphonate method[5-8]. Originally, mixture of pyridine and acetonitrile was used as the solvent for H-phosphonates and activators (pivaloyl chloride[5] or 1-adamantanecarbonyl chloride[9]).

The aim of this paper is to avoid the use of acetonitrile in routine H-phosphonate oligonucleotide synthesis. Acetonitrile is poisonous and expensive solvent and its purification or regeneration is difficult. We replaced it by cheaper and relatively less harmful dichloromethane. This solvent is frequently used instead of 1,2-dichloroethane (declared carcinogen) in deblocking step. Dichloromethane is easy to regenerate and its purification is much simplier than in the case of acetonitrile. Pyridine, although its

* Abbreviations used: PCR, polymerase chain reaction; Ada, adamantyl.

toxicity is comparable with acetonitrile, was preserved from chemical reasons. In our standard experiments pyridine as the solvent for H-phosphonates and dichloromethane for an activator were used. The last solvent was utilized both for all washing steps and detritylation (Table I). Practically "one solvent" procedure has been established. The attempt to decrease the amount of pyridine using its mixture with dichloromethane (1 : 1) as a solvent for H-phosphonate failed. After 2 days of standing at room temperature, white crystals of quaternary pyridinium salt were formed. This salt did not have any influence on the chemistry (coupling yield) but its formation can be dangerous for hydraulic system of the synthesizer. On this place we would like to point out, that using of both solvents separately, as mentioned above, is absolutely safe.

The modified H-phosphonate synthesis was checked by preparation of $(dT)_6$, $(dC)_6$, $(dA)_6$ and $(dG)_3$, starting from immobilized 5′-O-(4,4′-dimethoxytriphenylmethyl)-N-protected 2′-deoxynucleosides bound to controlled pore glass over an aliphatic chain[10]. As monomers, 3′-H-phosphonates (*IIa – IId*) prepared from 5′-O-(4,4′-dimethoxytriphenylmethyl) derivatives of dT (ref.[11]), N^4-benzoyl–dC (ref.[12]), N^6-(N,N-dimethylaminomethylene)–dA (ref.[13]) and N^2-(N,N-dimethylaminomethylene)–dG (ref.[14]) were used. 1-Adamantanecarbonyl chloride[8] was used as the activator. Capping procedure was omitted. The syntheses were performed on Pharmacia LKB Gene Assembler Plus



Ia – Id, R = H

IIa – IId, R = P(O)(H)(O^- $^+NEt_3$)

In formulae *I* and *II*: *a*, B = thymine

b, B = N^4-benzoylcytosine

c, B = N^2-(N, N-dimethylaminomethylene)guanine

d, B = N^6-(N, N-dimethylaminomethylene)adenine

system. Analytical reverse phase HPLC of crude products after ammonia deblocking showed satisfactory results in all cases (Fig. 1).

A series of oligonucleotides (20 – 24 bases long) was prepared by the above-mentioned procedure and products were partially purified by ethanol precipitation of their potassium salts[10]. Such primers were successfully used either for amplification of target DNA using polymerase chain reaction (PCR) or for sequencing by dideoxynucleotide chain termination method (for results see Figs 2 and 3).

EXPERIMENTAL

Ultraviolet spectra were performed by Hewlett–Packard 8451A UV spectrometer. Analytical HPLC was performed on the Vydac C18 column (The Separations Group, U.S.A.) (4.6 × 250 mm; 10 μm) in 0.1 M ammonium acetate buffer pH 7.0 at 20 °C, using linear concentration gradient of methanol in water. HPLC apparatus consisted of Constametric I pump (LDC, Milton–Roy Co., U.S.A.) with low pressure gradient former GP-3 (Laboratorní přístroje, Praha, The Czech Republic), UV-detector operating at 254 nm and 7125 Rheodyne injector. Thin-layer chromatography (TLC) was performed on Silufol UV 254 (Kavalier, Votice, The Czech Republic) in chloroform–methanol (9 : 1) (S1) or chloroform–methanol (8 : 2) (S2).

Preparative column chromatography was performed on Kieselgel 60 (60741 Fluka, Switzerland). Evaporations were carried out at 40 °C or lower on rotary evaporator in vacuo. Solids were dried at 25 °C, 13 Pa.

2′-Deoxynucleosides were purchased from Pharma–Waldhof (Germany). Pyridine (dry), dichloromethane (dry), N,N-dimethylformamide dimethylacetal, 4,4′-dimethoxytriphenylmethyl chloride, imidazole (puriss.), dichloroacetic acid (puriss.), 1-adamantanecarbonyl chloride (all from Fluka) were used without further purification. N,N-Dimethylformamide (analytical grade, Fluka) was dried over 3 Å molecular

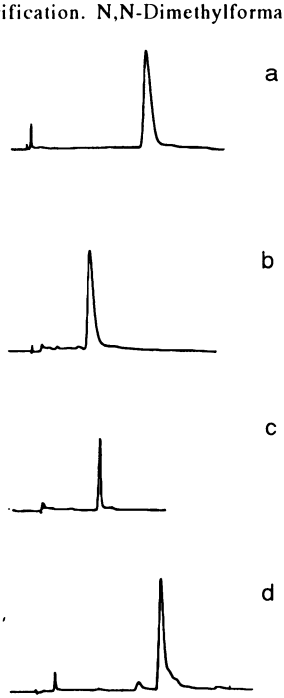


FIG. 1

Reverse phase HPLC pattern of crude homo-oligomers; Vydac C18 10 μm (4.6 × 250 mm), 1.5 ml/min, linear gradient of B solution (50% methanol in 0.1 M TEAA pH 7.0) in A solution (10% methanol in 0.1 M TEAA pH 7.1) during 30 min, *a* dT_6 (40 – 55% B), *b* dA_6 (40 – 55% B), *c* dC_6 (0 – 40% B), *d* dG_3 (0% B)

sieves. Methanol and ethanol were dried with magnesium ribbon. Diethyl ether and benzene were purified by distillation. Phosphorus trichloride (2 M solution in dichloromethane) was purchased from Aldrich. 5'-O-(4,4'-Dimethoxytriphenylmethyl)thymidine[10], N^4-benzoyl-2'-deoxy-5'-O-(4,4'-dimethoxytriphenylmethyl)-cytidine[11] and 3'-H-phosphonates[7] (*IIa – IId*) were prepared according to the published procedures.

2'-Deoxy-5'-O-(4,4'-dimethoxytriphenylmethyl)-N^2-(N,N-dimethylaminomethylene)guanosine (*Ic*)

2'-Deoxyguanosine (2.7 g; 10 mmol) was coevaporated with N,N-dimethylformamide (50 ml) using an oil pump. N,N-Dimethylformamide (45 ml) and N,N-dimethylformamide dimethylacetal (4 ml; 30 mmol) were added and the mixture was stirred overnight. A sample (20 μl) evaporated with pyridine (100 μl) in vacuo and then with toluene (100 μl) showed on TLC single UV-absorbing spot R_F 0.35 (in S2). Water (5 ml) was added under stirring and, after 20 min, the mixture was evaporated (oil pump). The residue was coevaporated with pyridine (3 × 50 ml). To the residue, pyridine (60 ml) and 4,4'-dimethoxytriphenylmethyl chloride (3.6 g; 10.5 mmol) were added and the mixture was stirred overnight. (A sample of the reaction

TABLE I
Solid phase oligonucleotide synthesis protocol

Step No.	Procedure	Duration s	Flow rate ml/min	Reagent
			Coupling	
1	Washing	60	2.5	dichloromethane
2	Deprotection	50	2.5	3% dichloroacetic acid in CH_2Cl_2
3	Washing	45	2.5	dichloromethane
4	Coupling	1.2	2.5	0.1 M H-phosphonate in pyridine
		1.2	2.5	0.4 M AdaCOCl[a] in dichloromethane
5	Repeat step 4 four times			
6	Recycling	60	1.0	
7	Washing	30	2.5	dichloromethane
			Deprotection	
1	Washing	60	2.5	dichloromethane
2	Deprotection	50	2.5	3% dichloroacetic acid in CH_2Cl_2
3	Washing	45	2.5	dichloromethane
			Oxidation	
1	Oxidation	20 min	[b]	0.1 M I_2 in THF–water–pyridine–N-methylimidazole (90 : 5 : 5 : 1 v/v)
2	Oxidation	10 min	[b]	0.1 M I_2 in THF–water–triethylamine (90 : 5 : 5 v/v)

[a] 1-Adamantanecarbonyl chloride. [b] Batch oxidation.

mixture prepared as mentioned above showed on TLC main UV-absorbing and DMTr-positive spot R_F 0.15 (in S1)). Methanol (5 ml) was added and, after 15 min, the mixture was partitioned between chloroform (150 ml) and water (100 ml). The aqueous layer was extracted with chloroform (50 ml), the combined extracts were diluted with toluene (100 ml) and evaporated. The residue was coevaporated with toluene (2 × 50 ml) to a foam. The foam was dissolved in a mixture of chloroform (32 ml) and ethanol (8 ml) and injected into vigorously stirred diethyl ether (450 ml). The precipitate was collected, washed with diethyl ether (4 × 50 ml) and dried under vacuum. Yield 5.8 g (92%) of *Ic*. UV-spectrum: λ_{max} 304 nm, λ_{min} 258 nm.

2'-Deoxy-5'-O-(4,4'-dimethoxytriphenylmethyl)-N^6-(N,N-dimethylaminomethylene)adenosine (*Id*)

The compound *Id* was prepared by the same procedure as *Ic* except the final precipitation which was carried out in the same volume of petroleum ether. Yield 91%. R_F 0.25 (S1). UV-spectrum: λ_{max} 312 nm, λ_{min} 261 nm.

Oligonucleotide Synthesis

Syntheses were carried out on 0.3 μmol scale of immobilized nucleoside on the solid support. Pyridine 0.1 M solutions of H-phosphonates and 0.4 M solution of 1-adamantanecarbonyl chloride in dichloromethane were used. Both reagents (total volume 0.5 ml per coupling) were delivered onto the column (3 × 20 mm) in alternating pulses (50 μl of each) and the resulting mixture was recycled for 60 s (for the synthetic procedure see Table I.).

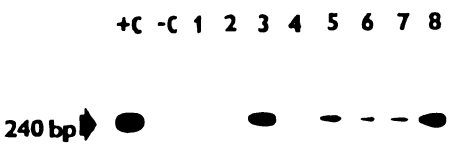


FIG. 2

Detection of proviral sequences in ducks persistently infected with avian leucosis virus (ALV-C). DNA from various organs was subjected to PCR with oligonucleotide primers (5'-CGCGAAGGTGAAGAC-GGTATCA-3' and 5'-TGATTGAACCTAGCGAATTTCC-3') corresponding to *gag* gene region giving rise to 240 bp product. For the PCR amplification standard PERKIN–ELMER CETUS reagents and protocol were used, except reaction volume (25 μl). Final primer concentration was 10 μg/ml. The reaction mixture contained 1.25 mM $MgCl_2$ and 2.5 U Amplitaq polymerase (PE CETUS). PCR products were blotted on Zeta-Probe nylon membrane (BIO-RAD) after agarose gel electrophoresis and then hybridized with radioactively labelled *gag*-specific probe. The autoradiography was performed for 24 h at −70 °C using intensifying screen. Numbers 1 to 8 denote kidney, heart muscle, Harder gland, lung, brain, gonads, spleen and stomach muscle, respectively. DNA from H20 cells[15] with one proviral copy per cell genome was used as a positive control (+C) and DNA from uninfected duck as a negative control (−C)

Following the last step of the synthesis, the solid support was washed with ethanol, diethyl ether and after short drying in vacuo, treated with 35% aqueous ammonia (1.5 ml) in a sealed tube at 55 °C for 16 h. After cooling (ice bath), the CPG was filtered off and washed with concentrated aqueous ammonia (1 ml). The filtrate was heated to 55 °C in an open conical flask for 1 h, chilled on ice and concentrated to approximately 1 ml in vacuo. Samples of this crude oligomer solution were directly injected into the analytical HPLC column (for results see Fig. 1).

Regeneration of Dichloromethane

The effluent from one reaction cycle contains 8 ml of dichloromethane, 0.25 ml of pyridine, 0.06 ml of dichloroacetic acid and rests of H-phosphonates and 1-adamantanecarbonyl chloride. To the effluent, after approx. 100 synthetic steps, sulfuric acid (10 ml) was added under stirring. Dichloromethane was distilled off (40 cm long Vigreux column). The distillate was passed through a bed (50 ml) of basic aluminum oxide and then used for oligonucleotide synthesis.

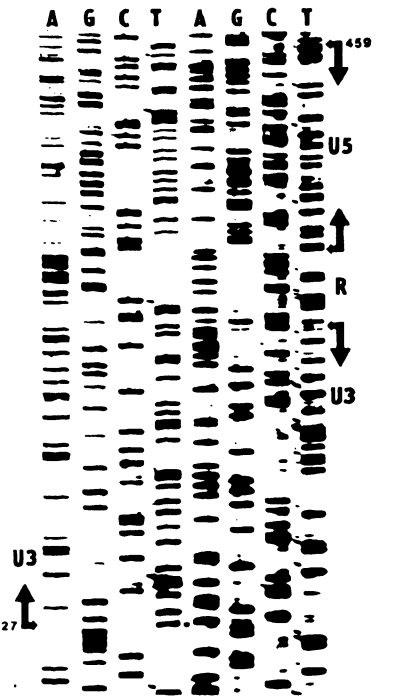


FIG. 3
Sequence of the 5'-LTR structure from LTR,v-*src*, LTR proviral genom integrated in hamster tumor cells[16] reverted to normal phenotype. Cell DNA was subjected to PCR amplification with oligonucleotide primers derived from flanking chromosomal DNA (Kpn I site) and provirus DNA (Bgl II site). PCR product was cloned into pCR 1000 vector (INVITROGEN) and U3,R,U5 region of insert was sequenced using Sequenase (USB) reagent kit. Oligonucleotide 5'-CTCCTGTAGTCTTGCAACATG-3' was used as a sequencing primer at the amount of 50 ng per annealing reaction. 5 μCi of ^{35}S-dATP was used for labelling and autoradiography was performed for 3 days at room temperature. The whole LTR region from the reverted cells perfectly matches the sequence from H19 cells[17]

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