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SOLID-PHASE SYNTHESIS OF PROTECTED OLIGONUCLEOTIDE BLOCKS

APPLICATIONS TO BLOCK CONDENSATION ON A POLYMER SUPPORT AND SYNTHESIS OF A 3'-MODIFIED OLIGONUCLEOTIDES

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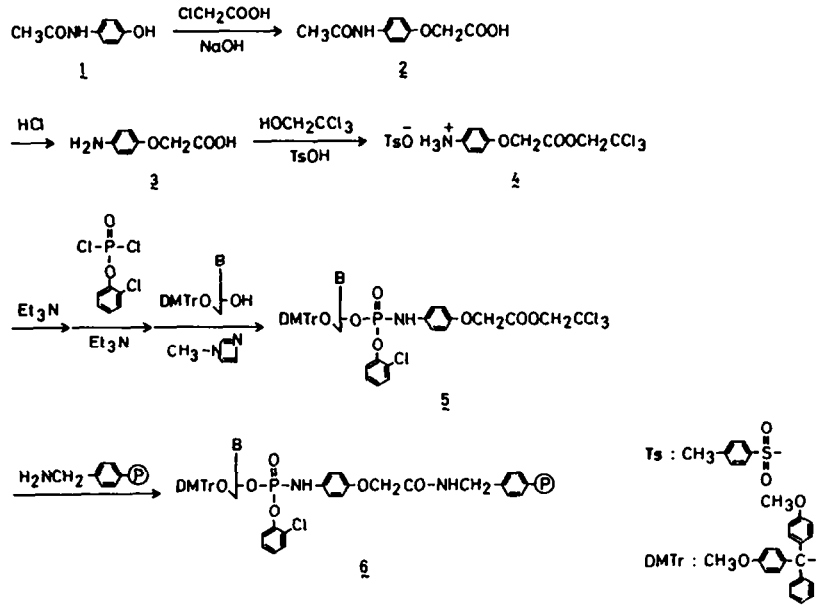
Abstract--The solid-phase synthesis of protected oligodeoxyribonucleotide blocks using a new phosphoramidate linkage between the 3'-terminal nucleoside and the polymer support is described. The oligonucleotide block cleaved from the support contains a phosphodiester group at its 3'-terminus and can be used as a coupling unit in the solid-phase synthesis of long-chain oligonucleotides. A 35mer has been synthesized using hepta- and nonanucleotide blocks. By deprotection of the appropriate oligonucleotide block, a 16mer containing an o-chlorophenyl phosphate group at the 3'-terminus has been obtained.

Recent developments in the phosphite triester method have made it possible to synthesize long-chain oligodeoxyribonucleotides rapidly and easily.[1-3] The phosphotriester approach, however, is limited to the synthesis of shorter oligonucleotides because of the occurrence of side-reactions and the lower coupling yield. Although some attempts have been made to solve these problems,[4] block condensation on a polymer support seems to be a useful method to synthesize long-chain oligonucleotides by the phosphotriester approach and the solid-phase synthesis of oligonucleotide blocks is required because the synthesis of these blocks in solution is very time-consuming.

We formerly synthesized protected oligoribonucleotides containing a 3'-phosphomonoester group by the phosphodiester approach using a 4'-aminophenoxymethylpolystyrene support.[5] Efimov et al. used a sulfonylethyl group as the phosphate-support linkage and synthesized tetramer blocks by the phosphotriester approach.[6] A phenylmercaptoethyl linkage has also been used.[7] In this paper, we report the solid-phase synthesis of oligonucleotide blocks using a phosphoramidate linkage and their application to the synthesis of a long-chain oligodeoxyribonucleotide (35mer) and an oligonucleotide containing an o-chlorophenyl phosphate group at the 3'-terminus. The 35mer has the sequence of the HindIII-EcoRI fragment of the synthetic human nerve growth factor (hNGF) gene[8] and the use of the 3'-modified oligonucleotide is reported elsewhere.[9]

Nucleoside phosphoramidate resin

To synthesize protected oligonucleotide blocks on a polymer support, a phosphate triester linkage is required between the 3'-terminal nucleoside and the support, which is cleaved specifically to yield a 3'-phosphodiester group. We used nucleoside 3'-phosphoro-p-anisidate[10] for this linkage. It is stable



Scheme 1 Synthesis of the nucleoside phosphoramidate resin

against acids and bases and can be cleaved by isoamyl nitrite.[10] As shown in Scheme 1, 1% cross linked polystyrene was used as a support and the carboxyl group of the nucleoside phosphoramidate was used for the attachment to the support. The carboxyl group was protected by the 2,2,2-trichloroethyl ester[11] which could be removed selectively by zinc under mild conditions.[12] We found that the 2,2,2-trichloroethyl ester reacted with primary amines to yield amides, so we used a fully protected nucleoside phosphoramidate (5) purified by column chromatography to make an amide linkage with the aminomethylated polystyrene.

Cleavage from the support was accomplished by treating the thymidine phosphoramidate resin (6, B=T) with isoamyl nitrite in pyridine-acetic acid when 5'-O-dimethoxytrityl thymidine 3'-(o-chlorophenyl)phosphate was obtained. The treatment with isoamyl nitrite for 3 hours was enough for recovering the nucleotide (Fig. 1).

To investigate the stability of the phosphoramidate linkage against the capping reaction, that is acetylation of the unreacted 5'-hydroxyl group, 5'-O-dimethoxytrityl thymidine 3'-(o-chlorophenyl)phosphoro-p-anisidate (7) was

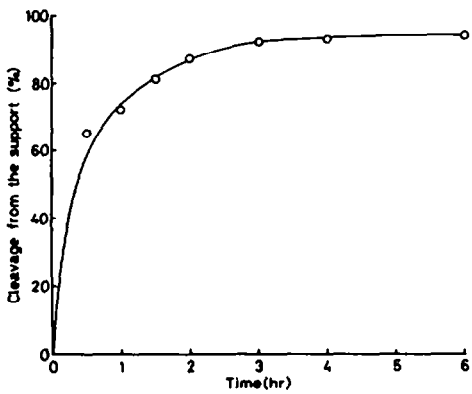
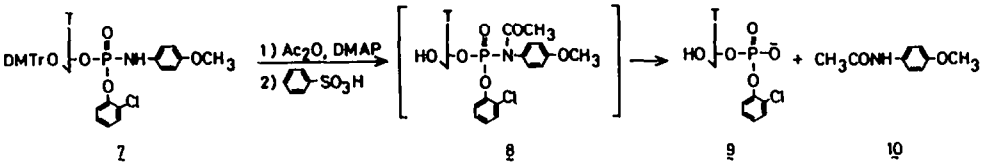


Fig. 1 Cleavage of the phosphoramidate linkage. The thymidine phosphoramidate resin (10 mg) was treated with isoamyl nitrite (0.13 ml, 1 mmol) in pyridine-acetic acid (1:1, v/v)(1 ml). The amount of nucleotide left on the support was determined by spectrophotometric measurement of the trityl cation.



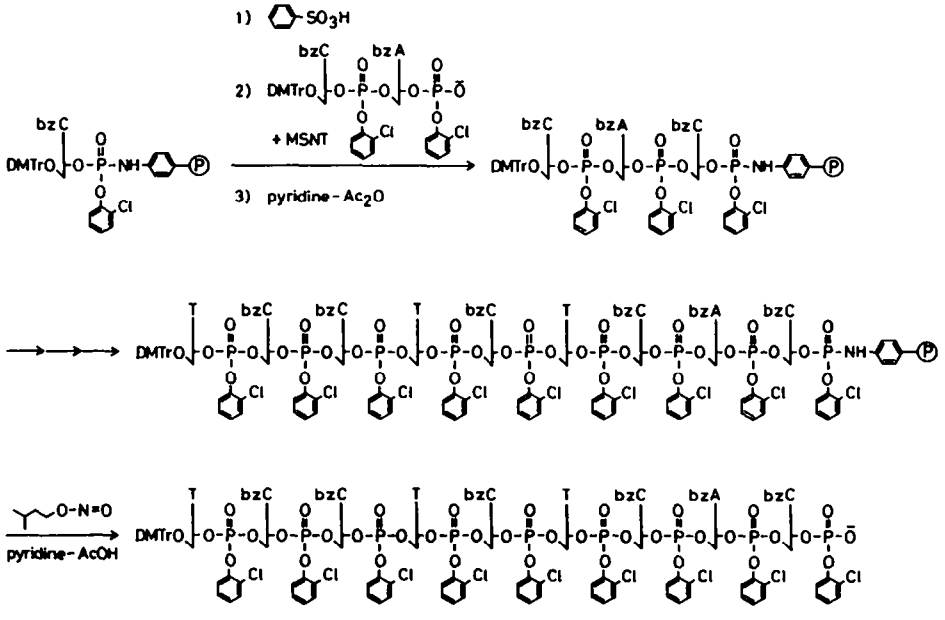
Scheme 2 Acetylation of the protected nucleoside phosphoramidate

treated with acetic anhydride using 4-dimethylaminopyridine (DMAP) as catalyst. It was found that the phosphoramidate was acetylated and hydrolyzed to thymidine 3'-(o-chlorophenyl)phosphate (9) and N-acetyl-p-anisidine (10) after the acid treatment (Scheme 2). This means that the nucleotide-support linkage is being cleaved during the chain elongation. Acetylation without DMAP, however, did not cause this side-reaction, so we used pyridine-acetic anhydride (3:2, v/v) for the capping reaction in the solid-phase synthesis of oligonucleotide blocks (Table 1).

Solid-phase synthesis of oligonucleotide blocks

The solid-phase synthesis of oligonucleotide blocks is illustrated in Scheme 3 and Table 1. Deoxycytidine phosphoramidate resin [0.3 g(50 μmol)] was used and detritylation with benzenesulfonic acid was repeated three times. Dinucleotide blocks were used as coupling units and the unreacted 5'-hydroxyl group was capped by acetylation. All reactions were performed in a reaction vessel which had a capacity of 40 ml and was equipped with a sintered glass filter and a two-way stop-cock.

After four cycles of the reactions, the resin containing a nonamer block was treated with isoamyl nitrite in pyridine-acetic acid (1:1, v/v) for 3 hours. The nonamer block was purified by reversed-phase column chromatography with a linear gradient of acetonitrile in 0.1 M triethylammonium bicarbonate (TEAB) buffer (Fig. 2). It was eluted at an acetonitrile concentration of 70% and



Scheme 3 Synthesis of an oligonucleotide block

precipitated with n-hexane-ether (1:1, v/v). The sequences and yields of the oligonucleotide blocks synthesized by this method are listed in Table 2. The coupling yield was calculated from the spectrophotometric determination of trityl cation and 15-20 μmol of each oligonucleotide block was obtained. This was sufficient for application in oligonucleotide synthesis.

Table 1 Reaction cycle for the synthesis of oligonucleotide blocks.

STEP	SOLVENT or REAGENT	AMOUNT	REACTION TIME	NUMBER OF OPERATIONS
1	CH_2Cl_2-MeOH (7:3,v/v)	6 ml	0.1min.	3
2	2% Benzenesulfonic acid in CH_2Cl_2-MeOH (7:3,v/v)	6 ml	1 min.	1
3	CH_2Cl_2-MeOH (7:3,v/v)	6 ml	0.1min.	1
4	2% Benzenesulfonic acid in CH_2Cl_2-MeOH (7:3,v/v)	6 ml	1 min.	1
5	CH_2Cl_2-MeOH (7:3,v/v)	6 ml	0.1min.	1
6	2% Benzenesulfonic acid in CH_2Cl_2-MeOH (7:3,v/v)	6 ml	1 min.	1
7	CH_2Cl_2-MeOH (7:3,v/v)	6 ml	0.1min.	2
8	Pyridine	6 ml	0.1min.	3
9	Pyridine	1 ml	coevaporation	1
10	Dimer block in Pyridine	0.2g/1.5ml	coevaporation	1
11	MSNT in Pyridine	0.2g/1.5ml	20 min.(40°C)	1
12	Pyridine	6 ml	0.1min.	2
13	Pyridine Ac_2O	6 ml 4 ml	60 min.	1
14	Pyridine	6 ml	0.1min.	3

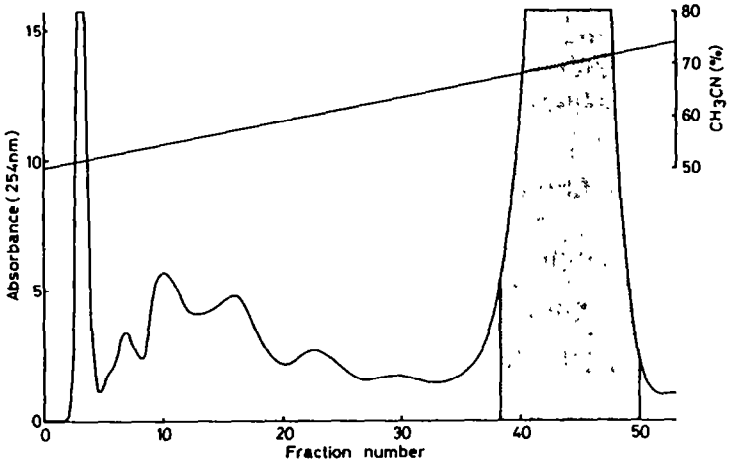


Fig. 2 Purification of the protected nonamer block, dTCCTCTCACp, using a column (1.7 cm I.D. x 15 cmL.) of Preparative C18 with a linear gradient of acetonitrile in 0.1 M TEAB buffer.

Table 2 Sequences and yields of the oligonucleotide blocks synthesized on the polymer support.

Sequence	Yield Average of condensation	Isolation	
dAGCTTCC	90%	68 mg	(40%)
dTCCTCTCAC	85	73	(31)
dCCGATCTTC	90	79	(33)

We have synthesized another oligonucleotide block with a guanine-rich sequence, but the isolated yield was low (about one half that of other blocks). This may be attributed to a side-reaction between the guanine base and the condensing agent, 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT),[13] because our method does not contain the deprotection step in which the modification to the base is reversed. Base protection[14-16] is expected to increase the isolated yield of the oligonucleotide block.

Block condensation on a polymer support

Using oligonucleotide blocks synthesized on the polymer support, the pentatriacontamer (35mer), d(AGCTTCCTCCTCTCACCCGATCTTCCACCGTGGCG), which had the sequence of the HindIII-EcoRI fragment of the synthetic human nerve growth factor (hNGF) gene[8] has been synthesized. Controlled-pore glass (CPG) was used as a support and the oligonucleotide blocks were condensed with the decamer on the support elongated with monomer and dimer units. The operations for the chain elongation were repeated as reported previously[17] with 55 mg (1.5 μmol) of deoxyguanosine-CPG. The reaction time for condensation was 120 minutes at 28°C using 12 μmol of hepta- or nonamer and 60 μmol of MSNT in pyridine (190 μl), but the coupling yield was not high.

To investigate the relationship between the chain length of the coupling unit and the yield, protected oligothymidylates of various lengths were condensed on CPG or polystyrene. The coupling yield decreased according to the chain length under the conditions described in the legend of Fig. 4. Despite the low coupling yield, block condensation is still effective because the final product can be purified more easily than that produced by stepwise elongation.

The 35mer was deprotected (except for the dimethoxytrityl group) and purified by reversed-phase column chromatography (Fig. 5a). One fraction containing the tritylated 35mer was treated with acetic acid to remove the trityl group and analyzed by high pressure liquid chromatography (HPLC). This was

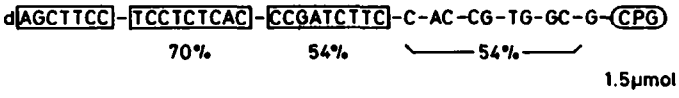


Fig. 3 Synthesis of the 35mer.

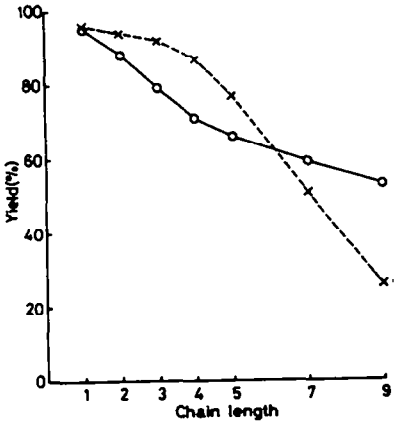


Fig. 4 Relationship between the chain length of the coupling unit and the yield. Oligothymidylate of various lengths (10 μmol) was condensed with deoxyguanosine on CPG (33 mg, 0.9 μmol)[O———O] or polystyrene (10 mg, 2 μmol)[X-----X] in pyridine (150 μl) using MSNT (15 mg, 50 μmol) at 40°C for 20 min.

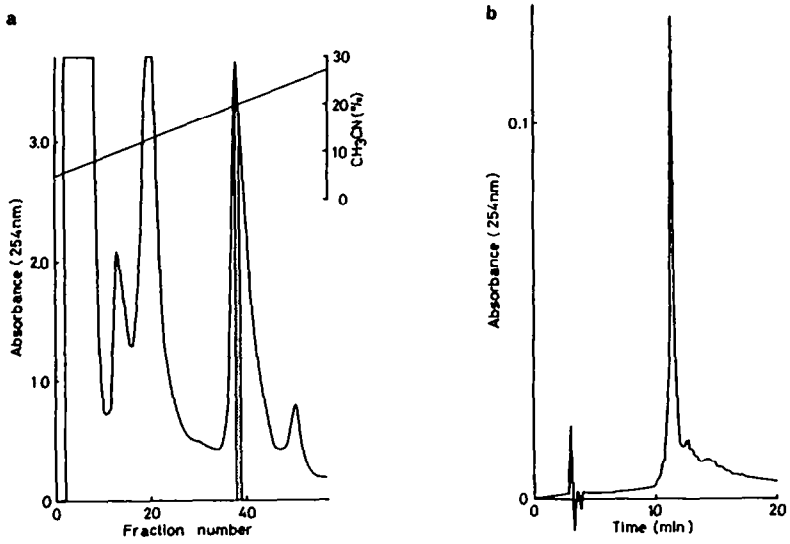


Fig. 5 a) Purification of the tritylated 35mer by reversed-phase column chromatography (Preparative C18, 0.7 cmI.D. x 10 cmL.).
b) HPLC analysis of the deprotected 35mer (fraction 38) using a TSK gel ODS-120A column with a linear gradient of acetonitrile (from 11 to 19 % during 20 min) in 0.1 M TEAA buffer.

further purified by HPLC and the sequence was identified by the Maxam-Gilbert method.[18]

An oligonucleotide containing an o-chlorophenyl phosphate at the 3'-terminus

An oligonucleotide containing an o-chlorophenyl phosphate group at the 3'-terminus was found to inhibit chain elongation by DNA polymerase at the expected site, so it was used to prepare partially-double-stranded DNA in combination with a primer.[9] We have synthesized such oligonucleotides in the 3'-direction,[10] but they can also be synthesized by deprotection of the oligonucleotide blocks because the o-chlorophenyl phosphate derivative cleaved from the support is already a phosphodiester which can be left during the deprotection steps. We have synthesized a hexadecamer, d(ATGGAGAACACAACATp), containing an o-chlorophenyl phosphate group at the 3'-terminus.

The solid-phase synthesis of the protected hexadecamer block was accomplished in the same way as described above, using 52 mg (5 μmol) of the thymidine phosphoramidate resin. The average of the coupling yield was 89%. After cleavage from the support, the hexadecamer was purified by reversed-phase column chromatography and was deprotected by the successive use of N^1,N^1,N^3,N^3-tetramethylguanidinium syn-pyridine-2-aldoximate in dioxane-water, conc. ammonia, and 80% acetic acid. The deprotected hexadecamer was purified by HPLC (Fig. 6) and its sequence was identified by two-dimensional homochromatography[19,20] (Fig. 7). As the mobility of the last spot in the second dimension is small, the hexadecamer is considered to contain the o-chlorophenyl phosphate group at the 3'-terminus which resists the nuclease P_1 digestion.

In conclusion, protected oligonucleotide blocks have been prepared rapidly using a phosphoramidate resin, and a long-chain oligodeoxyribonucleotide has been synthesized by the phosphotriester approach, although investigations are still needed to improve the coupling yield. An oligonucleotide containing an o-chlorophenyl phosphate group at the 3'-terminus has also been synthesized using the phosphoramidate resin. This method is more convenient than elongation in the

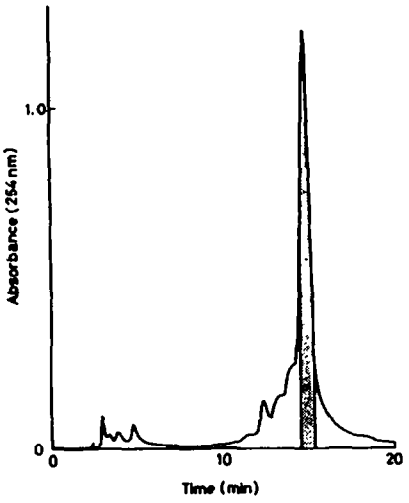


Fig. 6 Purification of the deprotected 16mer containing a 3'-(o-chlorophenyl) phosphate by HPLC using a Nucleosil $5C_{18}$ column with a linear gradient of acetonitrile (from 15 to 19 % during 20 min) in 0.1 M TEAA buffer.

Fig. 7 Sequence analysis of the 16mer. The 5'-labeled oligonucleotide was digested with nuclease P1[19] and analyzed by two-dimensional homochromatography using Homo-mix III.[20]

3'-direction,[10] because the coupling yields can be calculated by determining the color produced by the trityl cation and the product is more easily purified.

EXPERIMENTAL

Protected mono- and dinucleotides were prepared as reported previously.[17] The general method for the solid-phase synthesis of oligonucleotides has also been reported.[17] MSNT and pyridine for condensation were purchased from Dojindo Laboratories and deoxyguanosine-CPG (27.03 umol/g) was obtained from Applied Biosytems. Coupling yields were determined from the absorbance of the filtrate at the detritylation steps at 500 nm in $HClO_4$-EtOH (3:2, v/v) using a Shimadzu UV-240 spectrophotometer.

TLC was performed on Kieselgel 60 F_{254} plates (Merck) with $CHCl_3$-MeOH (10:1, v/v). For reversed-phase TLC, Kieselgel 60 F_{254} silanisiert (Merck) was used in the solvent system of acetone-20mM triethylammonium acetate (TEAA) buffer (7:3 or 6:4 , v/v). Protected nucleosides and nucleotides were purified using a column of Wakogel C-300 (Wako Pure Chemical) with $CHCl_3$-MeOH or Preparative C18 (Waters Associates) with acetone-0.1% aqueous pyridine.

^{1}H-NMR spectra of protected nucleotides were measured at 100 MHz with a Jeol JNM-FX100 spectrometer. For other compounds a Hitachi R-600 spectrometer was used at 60 MHz.

Reversed-phase column chromatography for oligonucleotides was performed using Preparative C18 (Waters Associates) packed in an Econo-Column (Bio-Rad) with a pump (Eldex Model E), a UV monitor (Gilson, Model 111B), and a fractionator (Gilson, Model FC-80K).

HPLC was performed using a TSK gel ODS-120A column (Toyo Soda Manufacturing) or Nucleosil $5C_{18}$ (Macherey-Nagel) packed in a stainless column (4.6 mmI.D. x 300 mmL.) eluted with a linear gradient of acetonitrile in 0.1 M TEAA buffer (pH 7.0). Purified oligonucleotides were analyzed by anion-exchange HPLC using a TSK gel DEAE-2SW column (Toyo Soda Manufacturing) with a linear gradient of ammonium formate in 20 % aqueous acetonitrile.

2,2,2-Trichloroethyl ester of 4-aminophenoxyacetic acid (4)

4-Aminophenoxyacetic acid 3 (8.36 g, 50 mmol) prepared from p-acetaminophenol and monochloroacetic acid as described[21] was mixed with 2,2,2-trichloroethanol (38.4 ml, 0.4 mol) and p-toluenesulfonic acid monohydrate (19.0 g, 0.1 mol) in toluene (250 ml) and the mixture was refluxed in a device with a Dean-Stark trap for 22 hr. The reaction mixture was concentrated in vacuo and the product was precipitated by addition of ether (200 ml). The tosylate of the trichloroethyl ester was recrystallized from ethanol (200 ml).
yield: 19.11 g (81%); mp: 165-167°C; R_f($CHCl_3$-MeOH, 10:1):0.55; ^{1}H-NMR (DMSO-d_6):δ =2.30(s, 3H), 4.99(s, 2H), 5.05(s, 2H), 7.16(d, 2H), 7.19(d,2H), 7.20(d, 2H), 7.50(d, 2H).

Fully-protected thymidine phosphoramidate (5, B=T)

To the tosylate 4 (4.71g, 10 mmol) suspended in ether (40 ml), triethylamine (2.5 ml, 18 mmol) was added at 0°C. The mixture was stirred for 5 min and poured into an ether solution (5 ml) of o-chlorophenyl phosphorodichloridate (1.3 ml, 8 mmol). After stirring for 1 hr, triethylamine hydrochloride was removed by filtration and the solvent was evaporated. The residue was coevaporated with pyridine and dissolved in dioxane (12 ml), followed by addition of 5'-O-dimethoxytrityl thymidine (1.63 g, 3 mmol) and 1-methylimidazole (1.0 ml, 12 mmol). After 2 hr, the reaction solution was concentrated, dissolved in $CHCl_3$, and washed with saturated aq. $NaHCO_3$ and water successively. The product was purified by silica gel column chromatography eluted with 3% MeOH in $CHCl_3$ and precipitated in n-hexane.
yield: 1.86 g (61%); R_f($CHCl_3$-MeOH, 10:1):0.51 and 0.56 (diastereomers); ^{1}H-NMR ($CDCl_3$): δ =1.41(s, 3H, $-CH_3$), 2.3-2.7(m, 2H, 2'-H), 3.3-3.5(m, 2H, 5'-H), 3.26(s, 6H, CH_3O-), 4.2-4.3(m, 1H, 4'-H), 4.70(s, 2H, $-CH_2$-), 4.80(s, 2H, $-CH_2$-), 5.2-5.5(m, 1H, 3'-H), 6.13(t, 1H, 1'-H), 6.6-7.5(m).

Thymidine phosphoramidate resin (6, B=T)

A mixture of aminomethylated polystyrene (1% divinylbenzene, 100-200 mesh, NH_2:0.21 meq/g)(0.95 g, 0.2 mmol), fully protected thymidine phosphoramidate 5(1.02 g, 1 mmol), and triethylamine (0.03 ml, 0.22 mmol) in N,N-dimethylformamide (DMF)(5 ml) was shaken for 42 hr at 30°C. The reaction mixture was filtered and the resin was washed with DMF and pyridine. Then pyridine (6 ml) and acetic anhydride (4 ml) were added, and after shaking for 1 hr, the resin was washed with pyridine, dichloromethane, and ether successively. The amount of nucleotide was 97 μmol/g.

Synthesis of oligonucleotide blocks

The reaction cycle in Table 1 was repeated with the deoxycytidine phosphoramidate resin (6, B=bzC, 165 μmol/g)(0.3 g, 50 μmol). After the final condensation the resin was washed with pyridine-acetic acid (1:1, v/v) three times and mixed with pyridine-acetic acid (1:1, v/v)(6 ml) and isoamyl nitrite (0.67 ml, 5 mmol). The mixture was shaken for 3 hr at 30°C and then filtered. After the resin was washed with pyridine, the filtrate was concentrated to 5 ml and added dropwise into n-hexane-ether (1:1, v/v)(200 ml) with stirring. The precipitated gum was dissolved in $CHCl_3$ and washed with 0.1 M TEAB buffer. The product was purified by reversed-phase column chromatography shown in Fig. 2. The fractions were checked by reversed-phase TLC and the expected oligonucleotide block was precipitated in n-hexane-ether (1:1, v/v)(10ml) containing triethylamine (0.1 ml). Yields are listed in Table 2.

Deprotection of the oligonucleotide block

After reversed-phase column chromatography, the fractions containing the oligonucleotide block (about 70 A_{254} units) were collected and concentrated. TEAB was removed by coevaporation with water. To this residue, a 0.5 M solution of N^1,N^1,N^3,N^3-tetramethylguanidine and syn-pyridine-2-aldoxime in dioxane (1 ml) and water (1 ml) was added and the mixture was stirred for 12 hr at 30°C. The solvent was then evaporated and pyridine (1 ml) and conc. ammonia water (10 ml) were added. After 5 hr at 60°C, the mixture was concentrated and applied to a column (1.5 cm I.D. x 38 cmL.) of Sephadex G-25. The fractions of the first peak were collected and concentrated, followed by coevaporation with water. Acetic acid (80%, 2 ml) was added to the residue and after 20 min, the acetic acid was removed by coevaporation with water. The deprotected oligonucleotide containing an o-chlorophenyl phosphate group at the 3'-terminus was purified by HPLC as shown in Fig. 6. The sequence of the oligonucleotide so obtained was identified by two-dimensional homochromatography.[19,20]

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