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Studies on deoxyribonucleic acids and related compounds. V. Synthesis of pentadecanucleotide duplex containing the ideal Pribnow sequence of promoter by the phosphotriester method using a new phosphorylating reagent

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ABSTRACT

A phosphorylating reagent o-chlorophenyl phosphoro-p-anisidochloridate was synthesized to phosphorylate the 3'-hydroxyl group of N, 5'-protected deoxynucleosides. These nucleotides served as 3'-terminal units for the synthesis of oligonucleotide blocks. By condensation of these oligonucleotide blocks the partially complementary deoxypentadecanucleotides dAGCTTATAATGCTCG and dAGCTCGAGCATTATA, which contained the ideal Pribnow sequence TATAATG, were synthesized.

INTRODUCTION

We have previously reported syntheses of deoxyoligonucleotides by the phosphotriester method involving phosphorylating reagent p-chlorophenyl phosphoranilido chloridate[2] and p-chlorophenyl phosphoro-p-anisido chloridate.[3] The phosphoranilidate protection method has also been used for synthesis of ribopolynucleotides with chain length up to 20 and proved to be suitable for preparation of protected oligonucleotide intermediates.[4] In this paper we describe the synthesis of two partially complementary pentadecadeoxyribonucleotides dAGCTTATAATGCTCG (upper chain), dAGCTCGAGCATTATA (lower chain) (Chart 1) by using a new phosphorylating reagent, o-chlorophenyl phosphoro-p-anisido chloridate (1). This reagent was used to phosphorylate the 3'-hydroxyl groups of N, 5'-O-protected deoxynucleosides. Nucleotides thus obtained served as the 3'-terminal unit in preparation of oligonucleotide blocks as shown in Chart 2. The 3'-phosphoro-

5' dAGCTTATAATGCTCG 3'
3' dATATTACGAGCTCGA 5'

Chart 1

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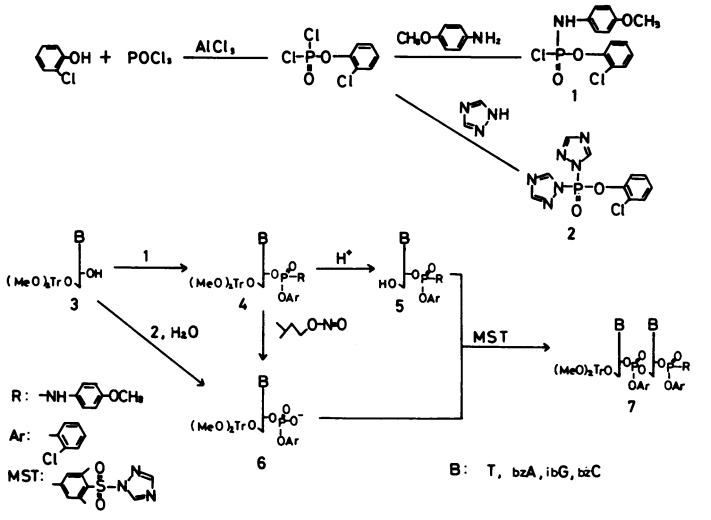

Chart 2

p-anisidate can be removed by treatment with isoamyl nitrite faster than the corresponding anilidate[3] when the chain is to elongate in the 3'-direction and the time for removal of o-chlorophenyl groups on internucleotide phosphates at the final stage should be shorter than that for p-chlorophenyl groups.[5] Experiments to prove this deblocking reaction are described in this report. The pentadecanucleotide duplex synthesized contains the ideal Pribnow sequence and clonable to Hind III sites.

RESULTS

Preparation of o-chlorophenyl phosphoro-p-anisidochloridate (1) and mononucleotides (5)

o-Chlorophenyl phosphoro-p-anisido chloridate (1) was prepared by reacting p-anisidine with o-chlorophenyl phosphorodichloridate[6] as described for the synthesis of the corresponding p-chlorophenyl derivative[3] and used to phosphorylate N, 5'-O-protected nucleosides[7] to give 4. (Chart 2) Some of diastereoisomers of 4 were separated by chromatography on silica gel. The dimethoxytrityl group was removed by treatment with benzenesulfonic acid[7c] to yield 5, which are purified by silica gel

chromatography. Phosphodiesters (6) were prepared as described.[8]

Comparison of time required for removal of aryl esters on an internucleotide phosphate

The o-chlorophenyl ester on internucleotide linkages should be easier to remove by alkaline hydrolysis or oximate promoted hydrolysis compared to the corresponding p-chlorophenyl ester. To investigate rates of these reactions o-chlorophenyl and p-chlorophenyl esters of 5'-O-monomethoxytrityl-(3'-5')-3'-O-benzoylthymidine were prepared by the procedure described below. The p-methylthiophenyl ester of TpT was for comparison prepared by using p-methylthiophenyl phosphorodichloridate. The p-methylthiophenyl moiety was thought to be a potentially useful protecting group if it could be oxidized to the more acidic phenyl group. Fig. 1 shows the decomposition of the triesters by hydrolysis with 0.3 M and 0.5 M syn-pyridine-2-carboxyaldoxamate. The half lives were calculated assuming the reaction to be pseudo first order. As shown in the legend to Fig. 1 $t_{1/2}$ for removal of the o-chlorophenyl was 6.5 min

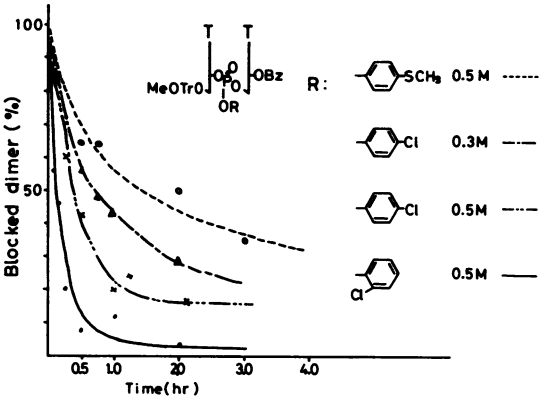

Fig. 1 Oximate promoted hydrolysis of aryl esters of 5'-monomethoxytrityl thymidylyl-(3'-5')3'-benzoylthymidine. o——o, the o-chlorophenyl ester with 0.5 M 1,1,3,3-tetramethylguanidinium pyridine-2-aldoxamate (PAO); X- - - - -X, the p-chlorophenyl ester with 0.5 M PAO: Δ——·——Δ, the p-chlorophenyl ester with 0.3 M PAO; o- - - - -o; the p-methylthiophenyl ester with 0.5 M PAO. Products were separated by TLC and eluted with $HClO_4$ MeOH (3:1).

and that of the p-derivative was 24.1 min with 0.5 M oxamate. Hydrolysis with 0.3 M reagent was much slower. Although these reaction can vary with composition of nucleosides and sizes of molecules, the o-chlorophenyl group appears to be a suitable protecting group which can be completely removed from phosphotriester linkages.

Synthesis of oligonucleotide blocks and their condensation to yield pentadecanucleotide

The principle for the synthesis of oligonucleotide blocks is illustrated in the preparation of the dinucleotide (7) shown in Chart 2. Activation of mononucleotides (6)(3'-phosphodiester component) was performed with mesitylenesulfonyl triazolide[8] (MST) for overnight reactions and condensation with 5'-hydroxyl components (5) gave fully protected dinucleotides. To elongate the chain in the 5'-direction, the mono- or dimethoxytrityl groups protecting the 5'-hydroxyl group was removed by treatment with benzenesulfonic acid. Condensation with mononucleotides (6) gives trinucleotides. The dimethoxytrityl group was used for protection of the 5'-hydroxyl especially when N, 3'-O-dibenzoyldeoxyadenosine was present in the 3'-terminus of oligonucleotides which were subjected to acid treatment. The trinucleotides used for the synthesis of the two pentadecanucleotides were prepared by this procedure except that the 3'-termnal trimers were synthesized from 3'-O-benzoyl-N-isobutyryldeoxyguanosine[7b] or N, 3'-O-dibenzoyldeoxyadenosine[7a]. The scheme for the synthesis of the pentadecamer, dAGCTTATAATGCTCG (upper strand in Chart 1) is shown in Chart 3. The abbreviated scheme for the other pentadecamer (lower strand) is shown in Chart 4. The 3'-phosphoro-p-anisidate group of oligonucleotides was removed by treatment with isoamyl nitrite[9] to convert to the phosphodiester for condensation. Activation of 3'-phosphodiesters of oligonucleotide blocks was effected by using mesitylenesulfonyl tetrazolide[7c] (Ms-Te) which was found to be a more active condensing reagent than MST. The time required for condensation of mononucleotides with MST was 20 hr and that of oligonucleotides with Ms-Te was 20-60 min. All protected oligonucleotides were isolated by chromatography on silica gel and/or silanized silica gel. Table I summarizes overall yields of tri-

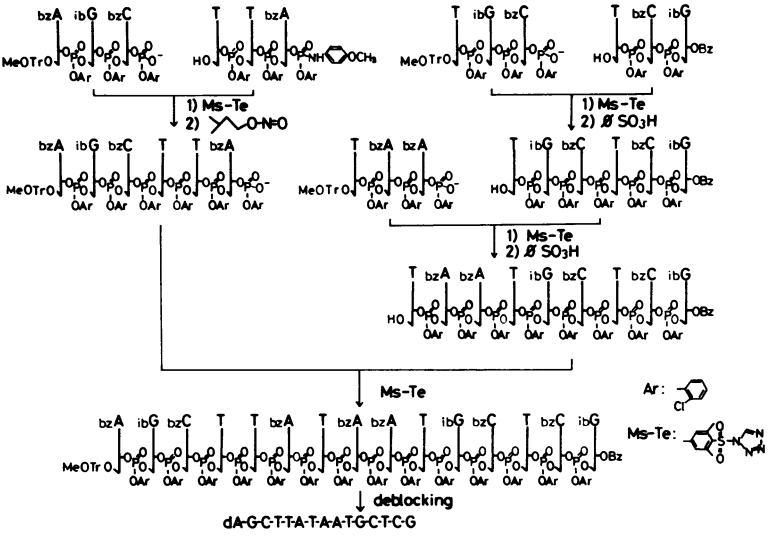

Chart 3

nucleotides and yields in condensations of oligonucleotide blocks. Conditions for elution of products and time required for removal of anisidates or trityl derivatives are also included. The protected pentadecamers were deblocked by treatment with pyridine-2-aldoximate[10] and then ammonia. The monomethoxytrityl or dimethoxytrityl group was removed with 80 % acetic acid. The completely deprotected pentadecamers were subjected to anion-exchange chromatography on DEAE-cellulose in the presence of 7 M urea and purified further by high pressure column chromatography on a reversed phase column (C_{18} silica gel)(Fig. 2). The structure of these pentadecamers was confirmed by base composition analysis[2], mobility shift analysis[11] and determination of the

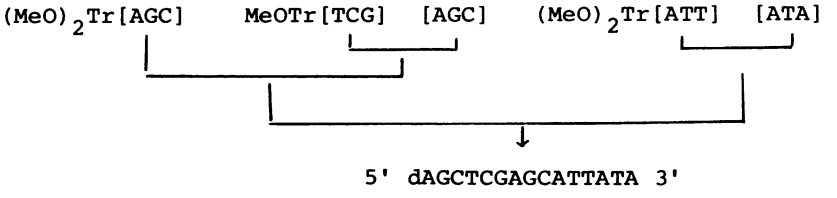

Chart 4

Table I Yield and separation method of oligonucleotides

Product		Overall yield	Column for separation solvent	Acid treatment	Isoamyl nitrite treatment
	(mmol)	(%)		(min)	(hr)
[TCG]Bz	0.60	60	C-18[a] 70:30	13	—
DMTr[TGC]p̊An	0.20	31	H[b] 40:1	—	2.5
DMTr[TAA]p̊An	0.32	45	60[b] 25:1	—	2.5
DMTr[TTA]p̊An	0.49	58	H[b] 50:1 C-18 7:3-8:2	20	—
MMTr[AGC]p̊An	0.83	56	H[b] 60:1-40:1	120	2
[ATA]Bz	0.635	78	60[b] 50:1-40:1	3	—
DMTr[ATT]p̊An	0.611	76	60[b] 30:1	—	2
MMTr[TCG]p̊An	0.80	64	C-18[a] 75:25	—	2
MMTr[AGCTTA]p̊An	0.120	89	H[b] 25:1-20:1	—	3
DMTr[ATTATA]Bz	0.051	65	H[b] 50:1-25:1	3.5	—
MMTr[TGCTCG[Bz	0.12	89	H[b] 40:1	100	—
MMTr[TCGAGC]p̊An	0.17	82	C-18[a] 8:2, H 70:1-60:1	150	—
MMTr[TAATGCTCG]Bz	0.074	73	H[b] 20:1-10:1	60	—
DMTr[AACTCGAGC]p̊An	0.052	74	H[b] 20:1	—	3
MMTr[AGCTTATAATGCTCG]Bz	0.039	66	C-18[a] 7:3-8:2	—	—
DMTr[AGCTCGAGCATTATA]Bz	0.019	92	C-18[a] 4:1-9:1	—	—

a, Reverse phase column. The ratio represents acetone to 0.02 M triethylammonium acetate (TEAA).

b, Silica gel column. The ratio represents chloroform to methanol.

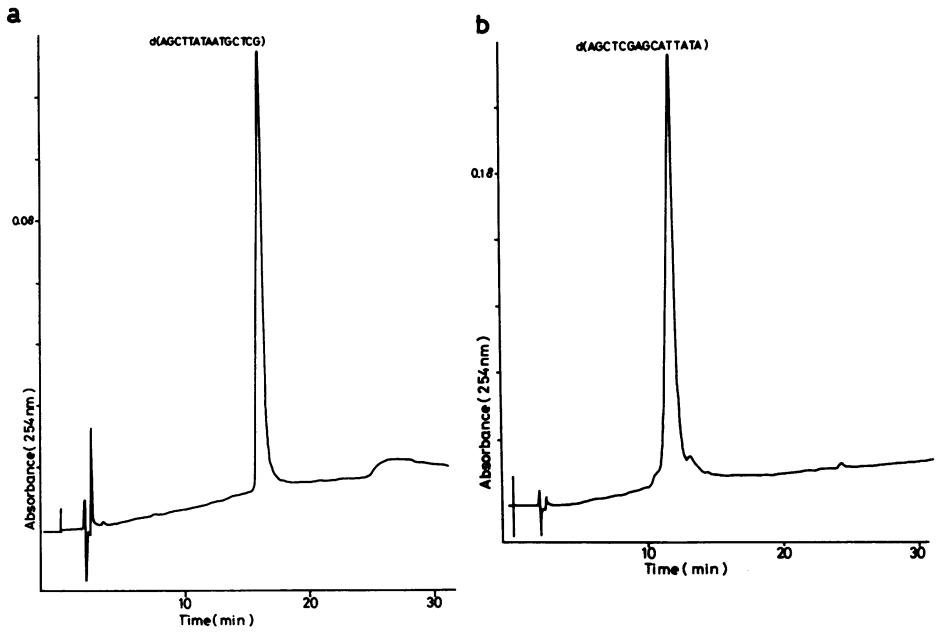

Fig. 2 Reverse-phase chromatography of dAGCTTATAATGCTCG (a) and dAGCTCGAGCATTATA (b) on a column (4.6 x 250 mm) of Hypersil ODS. Elutions was performed with a gradient of acetonitril (5-25 %) in 0.1 M triethylammonium acetate.

5'-nucleotide.[2]

To investigate thermal stability of this duplex in aqueous solution, especially under conditions for DNA ligase reaction, the two pentadecamers were mixed in a low salt buffer in the presence of 10 mM $MgCl_2$ and the temperature-absorption profile was measured to find the mid point (Tm) being 38°C as shown in Fig. 3.

CONCLUSION

The pentadecadeoxyribonucleotides have been synthesized by the triester method by condensing oligonucleotide blocks prepared with the aid of the new phosphorylating reagent o-chlorophenyl phosphoro-p-anisidochloridate (1) which introduces the anisido group as a protecting group for 3'-phosphodiester ends. Phosphorylation of N, 5'-O-protected deoxynucleotides with 1

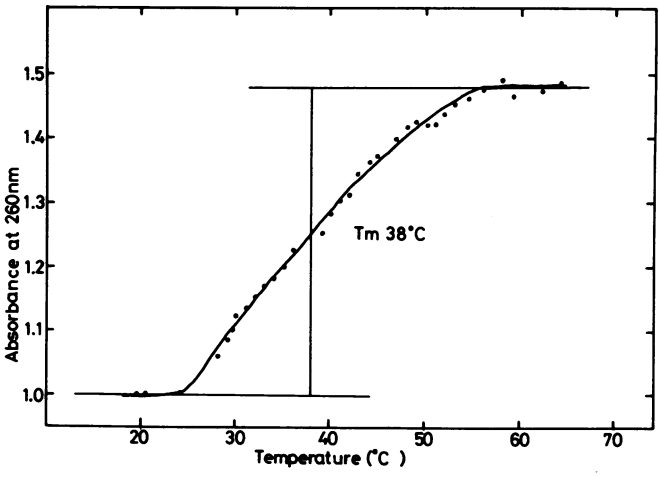

Fig. 3 Absorption-temperature profile of the duplex (Chart 1). The oligonucleotides (1.15 A_{260} in 1.4 ml each) in 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ were heated at 60° for 30 min in a water bath. The mixture was left without heat til room temperature then cooled to 10°.

can be optimized further to increase the yields of 4. This and the physical properties for 4 and 5 will be reported elsewhere. Presumably because of acidity of the o-chlorophenyl group, 1 seems to be more active than the corresponding p-chlorophenyl derivative.[3] Side products were formed with an excess of the phosphorylating reagent (1). A deoxyguanosine derivative with an extra phosphate on the ring was converted to 5 after acid treatment. Isolation of 5 without purification of 4 was performed in the present experiments. The use of o-chlorophenyl as a protection for internucleotide linkages seems to be prefered to that of the p-chlorophenyl group because the former is more easily removed. The present reagent was suitable to attach o-chlorophenyl and p-anisidate groups to the 3'-phosphate. Phosphoro-p-anisidate compounds were found to be stable during isolation and purification by silica gel or alkylated silica gel chromatography.

EXPERIMENTAL

Thin layer chromatography (TLC) was performed on plate of

silica gel (Kieselgel 60 F_{254}, Merck) with a mixture of chloroform and methanol unless otherwise specified. For reversed phase TLC (RTLC), silanized silica gel or HPTLC RP-8 and RP-18 (Merck) were used with a mixture of acetone-20 mM TEAA. For column chromatography, silica gel 60 or 60 H (Merck) and alkylated silica gel (C-18 35-105 μ, Waters) were used. The reaction mixture could be directly applied to a column (6.6 x 24 cm) of C-18 silica gel after removal of pyridine. A concentrated solution of the mixture in acetone was added with 20 mM triethylammonium acetate and the slightly turbid solution was applied to the column. Pyridine could substitute for acetone when compounds were insoluble in acetone. Elution was usually performed with acetone-20 mM TEAA: 65:35 (dimers), 65:35-70:30 (trimers), 70:30-75:25 (tetramer) and 80:20-85:15 (higher oligomers). Pressure or suction could be applied to the column.

o-Chlorophenyl phosphoro-p-anisidochloridate (1)

o-Chlorophenyl phosphorodichloridate (0.56 mol, bp 123°/3 mmHg) was obtained by the published procedure[6] from phosphorus oxychloride (2.66 mol), aluminium chloride (8 mmol) and o-chlorophenol (0.78 mol). p-Anisidine (1.23 g, 10 mmol) in dry ether (10 ml) was added to a ice-cooled solution of o-chlorophenyl phosphorodichloridate (1.05 ml, 5 mmol) in dry ether (5 ml) and the mixture was stirred at room temperature for 4 hr. The product was collected by filtration, washed with cooled water to remove anisidine hydrochloride and recrystallized with benzene. The yield was 1.35 g, 4.05 mmol, 90 %. Anal. Calcd. for $C_{13}H_{12}NO_3Cl_2$: C, 47.01; H, 3.64; N, 4.22; Cl, 21.39. Found: C, 47.24; H, 3.62; N, 4.39; Cl, 21.38. NMR ($CDCl_3$ TMS) δ : 3.78 (3H, s), 6.61-7.61 (9H, m).

General methods for phosphorylation of nucleosides with o-chlorophenyl phosphoro-p-anisidochloridate (1)

5'-O-Dimethoxytrityl-N-benzoyldeoxycytidine (3.25 g, 5 mmol) was dissolved in dioxane (30 ml) and 1-methylimidazole (1.8 ml). 1 (3.6 g, 11 mmol) was added with cooling and the reaction mixture was checked by TLC (chloroform-methanol, 10:1) after the mixture was kept at 25° for 1.5 hr. Two diastereoisomers (Rf 0.61, 0.74) were detected as the major products.

Water (10 ml), methylene chloride (150 ml) and 0.1 M TEAB (pH 7.5, 150 ml) were added with cooling. The organic layer was washed with water, and concentrated. The residue was applied to a column (5 x 7 cm) of silica gel (H, 60 g). The product was eluted with methylene chloride-methanol (70:1-60:1). Rf values of the two diastereomers were 0.61 and 0.74 in TLC (10:1). The yield was 3.7 g, 3.98 mmol, 79 %.

Preparation of trinucleotide blocks

The 3'-phosphodiester component (1.1-1.2 equivalent to the 5'-hydroxyl component) and the 5'-hydroxyl component were dried by coevaporation with pyridine and dissolved in pyridine (7-10 ml/mmol). The mixture was treated with MST (2-3 equivalent to the phosphodiester component) at 31° for 40 hr. Pyridine was removed by evaporation and the residue was coevaporated with toluene. The mixture was immediately applied to a column of silica gel (15 fold excess of nucleotides by weight). Elution was performed with suction (silica gel H) or N_2 (air) pressure (silica gel 60). Overall yields of trimers are listed in Table I.

Removal of the anisidate and 5'-protecting groups

Deanisidation of MMTr[AGC]$\overset{o}{p}$An (0.2 mmol, 387 mg) was performed by treatment with isoamyl nitrite (12 mmol, 1.6 ml) in pyridine-acetic acid (6:5, v/v, 8 ml) at 30° for 2 hr. The reaction was checked by RTLC and terminated by addition of 7M urea (7.5 ml) with cooling followed by stirring for 1 hr at room temperature. The product was extracted 3 times with chloroform (10 ml), washed 5 times with 0.1 M triethylammonium bicarbonate (20 ml) and concentrated. The residue was dried by evaporation, precipitated with ether-pentane (1:1, v/v, 50 ml) from its solution in chloroform (5 ml) and washed with the same mixture (50 ml) 3 times. The yield was 376 mg, ca. 0.194 mmol. DMTr[ATT]$\overset{s}{p}$An (0.114 mmol, 204 mg) was converted to the diester by the same procedure in a yield of 86 %. The product was contaminated with the detritylated compound and treated with dimethoxytrityl chloride (0.1 mmol) for 20 hr to obtain the pure product. The overall yield was 81 %.

Removal of the dimethoxytrityl or monomethoxytrityl group was performed by treatment of nucleotides (1 mmol) in chloro-

form (31 ml) with benzenesulfonic acid (0.379 M in methanol, 13.2 ml) at 0° for various period as shown in Table I.

Synthesis of dAGCTCGAGCATTATA

Condensations of protected oligonucleotide blocks were performed by essentially the same procedure as above except for MSTe was used for 1 hr. The yield and amounts of products are summarized in Table I. An aliquot of the protected pentadecamer (75.64 mg, 10 μmol) was treated with 0.5 M tetramethylguanidinium pyridine-2-carboxaldoximate for 48 hr at 25°C and the solution was then added with concentrated ammonia (30 ml) and pyridine (3 ml). The mixture was shaken at 55° for 5 hr and volatile materials were removed by evaporation. The residue was passed through a column (5 ml) of Dowex 50 x 2 (pyridinium form) in 30 % pyridine and combined washings were concentrated. The residue was dissolved in water, washed with ethyl acetate and treated with 80 % acetic acid (1 ml) at 25° for 30 min. Acetic acid was removed by coevaporation with toluene and the product was dissolved in aqueous pyridine. Dimethoxytritanol and the oxime were removed by extraction with chloroform. The aqueous layer (4100 A_{260} units) was applied to a column (1.5 x 64 cm) of DEAE-cellulose in 20 mM Tris-HCl (pH 7.5) and 7 M urea. Elution was performed with a linear gradient of NaCl (0.05-0.5 M, total 800 ml). The pentadecamer was eluted with 0.275 M salt. Fractions (800 A_{260}) center of the peak was desalted by adsorption to DEAE-cellulose (bicarbonate form) (40 ml), removal of salt with 0.03 M triethylammonium bicarbonate and elution of the product with 1 M triethylammonium bicarbonate. The yield was 655 A_{260} units.

Synthesis of dAGCTTATAATGCTCG

Conditions for condensations and deblocking were the same as the other strand except for the treatment with 80 % acetic acid was 1 hr. The completely deblocked pentadecamer (335 A_{260} units) was obtained from 47 mg (6 μmol) of the protected material. An aliquot of the deblocked product (40 A_{260}) was further purified by reverse phase chromatography using HPLC on a column (2 x 250 mm) of Hypersil ODS, 5 μ to give 19 A_{260} units of the pentadecamer.

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