Volume 11 Number 5 1983 Nucleic Acids Research

Studies on transfer ribonucleic acids and related compounds. XLV. Block condensation of ribooligonucleotides containing 2'-O-tetrahydrofuranyl-5'-O-dimethoxytritylnucleosides

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Received 4 January 1983; Accepted 2 February 1983

ABSTRACT

2'-O-Tetrahydrofuranyl-5'-O-dimethoxytrityl-N-protected nucleosides were phosphorylated to give the 3'-(o-chlorophenyl) phosphates which were then condensed with 3',5'-unprotected nucleosides to elongate the chain in the 3'-direction. The 5'-dimethoxytrityl group of these oligonucleotides was selectively deblocked by treatment with zinc bromide. The rate of removal of the dimethoxytrityl group differed in each nucleotide. A dodecamer containing a termination codon UAG, $U(AGU)_3AG$, was synthesized by elongating the chain in the 5'-direction using the selective dedimethoxytritylation followed by condensation of protected oligonucleotide blocks.

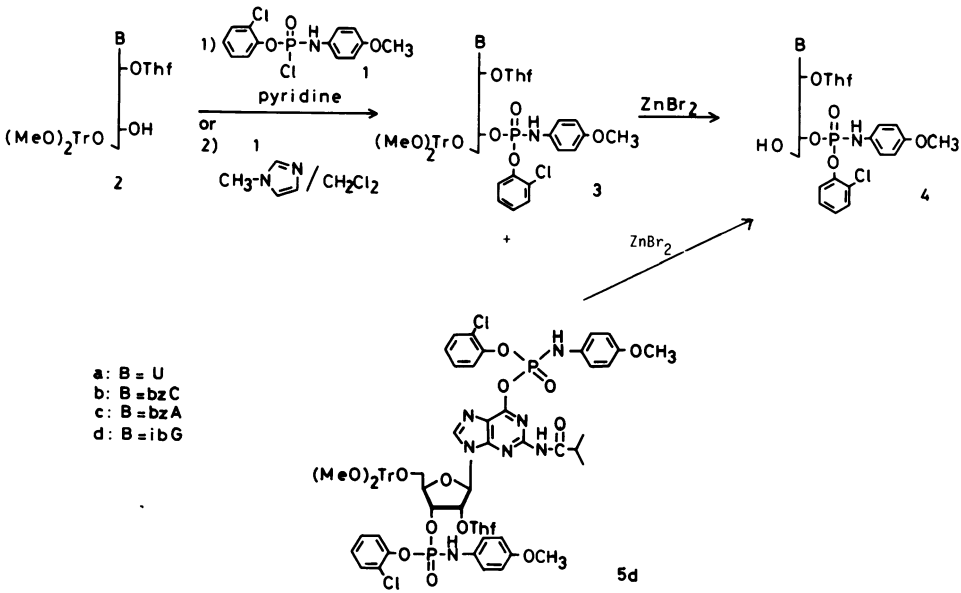
INTRODUCTION

Combination of protecting groups for the 2' and 5'-hydroxyl groups is a crucial problem in ribooligonucleotide synthesis. In the phosphotriester synthesis the 5'-O-protecting group has to be removed selectively in the presence of protecting groups for heterocyclic amino, 2'-hydroxyl and internucleotide phosphate groups.[2,3] We have previously reported a use of 2'-(o-nitrobenzyl) group and 5'-monomethoxytrityl group in the synthesis of ribooligonucleotides of chain length up to twenty.[4] The 2'-tetrahydrofuranyl protection was also employed in the phosphotriester synthesis using selective phosphorylation of the 5'-hydroxyl group.[1,5] In the present paper we describe selective 5'-dedimethoxytritylation with zinc bromide without hydrolyzing acid labile 2'-O-tetrahydrofuranyl functions. A dodecamer containing a termination codon UAG, $U(AGU)_3AG$ was synthesized to prove that the method could be suitable for ribooligonucleotide synthesis. Since the termination codon UAG is suppressed by tRNA having CUA anticodon, the dedecamer $U(AGU)_3AG$ may serve as a messenger RNA in protein synthesizing systems to give tetrapeptides e.g. $(Met)_4$ when modified $tRNA_f^{Met}$ containing CUA anticodon[6] is used.

Phosphorylation of 2'-O-Tetrahydrofuranyl-5'-O-dimethoxytrityl-N-protected nucleosides and Their Dedimethoxytritylation

2'-O-Tetrahydrofuranyl-5'-O-dimethoxytrityl-N-protected nucleosides (2) were phosphorylated with o-chlorophenyl p-anisidophosphorochloridate[7] (1) to yield nucleotides (3) (Chart 1). In the presence of pyridine, cytidine and adenosine derivatives (3b and 3c) were obtained in high yield. Uridine and guanosine derivatives (3a and 3d) were obtained in yields of less than 50% and contaminated with more polar compounds. Using methylimidazole in dichloromethane, 3a was obtained in 98 % yield. However, in the case of guanosine derivative a bis-phosphorylated compound (5d) was found together with 3d. 5d was characterized by ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H-NMR) by quantitation of H-N^1 and protons in phosphate protecting groups. The extra phosphate on the O^6 position was later found to be removed by prolonged treatment with zinc bromide which was used for removal of the 5'-dimethoxytrityl groups.

Dedimethoxytritylation of 3 was investigated by applying zinc bromide treatment under conditions used for deoxyribooligonucleotides. In solutions such as nitromethane[8], nitromethane-water[9], and dichloromethane-isopropanol[10], the 5'-O-dimethoxytrityl group was removed. However, in the presence of moisture the 2'-O-tetrahydrofuranyl group was also hydrolyzed. Zinc



bromide solution in dichloromethane-isopropanol which was kept over molecular sieves was finally employed to prepare compounds 4. Overall yields and time required to convert 3 to 4 are summarized in Table I. As indicated in Table I the 5'-O-dimethoxytrityl groups of ribonucleotides especially in pyrimidine derivaties, were more stable compared to those of deoxynucleotides. The existence of the 2'-O-tetrahydrofuranyl group in this medium was confirmed after treatment for 2 hr. The guanosine derivative (4d) required longer treatment to remove the 6-O-phosphorylated group of the bis-phosphorylated compound (5d).

Synthesis of Dodecaribonucleotide $U(AGU)_3AG$ (19)

Considering above properties, synthetic strategy for $U(AGU)_3AG$ was planned to include dedimethoxytritylation of the adenosine derivative. An abbreviated scheme for the synthesis is shown in Chart 2.

The key intermediate 6 was prepared from 2c by phosphorylation with o-chlorophenyl phosphoroditriazolide[11] followed by elongation of the chain in the 3'-direction as described previously.[1,5] The 3'-terminal dimer (13) was synthesized by using 2',3'-ethoxymethylidene-N-isobutyrylguanosine which was prepared by the similar procedure described for the corresponding methoxymethylidene[12]. Removal of the dimethoxytrityl group in 6 was performed by treatment of powdered 6 with zinc bromide in solution to give 8 in a yield of 76% and two other reactions to give 15 and 17 were performed under the similar conditions except that the compounds were dried by evaporation with pyridine and toluene before treatments with zinc bromide. The yields of 15 and 17 were 89 and 88% respectively after isolation by reversed phase chromatography on alkylated silica gel.

Table I Syntheses and Properties of the 3'-Phosphorylated Nucleosides (4)

Substrate	Step 1 Phosphorylation						Step 2 Removal of $(MeO)_2Tr$ Group		Product			
Compd	1 mol eq	Me-Im mol eq	Pyridine ml/mmol	CH_2Cl_2 ml/mmol	Time h	Temp °C	$ZnBr_2$ mol eq	Time min	Compd	Yield %	Rf A^a	Rf B^b
2a	1.9	2.0		5	4	-10	50	90	4a	66	0.32 0.36	0.69
2b	2.2		5		24	20	50	60	4b	81	0.44 0.48	0.60
2c	2.2		5		16	20	50	15	4c	88	0.46 0.48	0.62
2d	4.0	4.0		5	4	-10	50	240	4d	67	0.33 0.36	0.62

a : Merck $60F_{254}$, $CHCl_3$: MeOH = 10 : 1

b : Merck $60F_{254}$ Silanisiert, CH_3COCH_3 : H_2O = 6.5 : 3.5

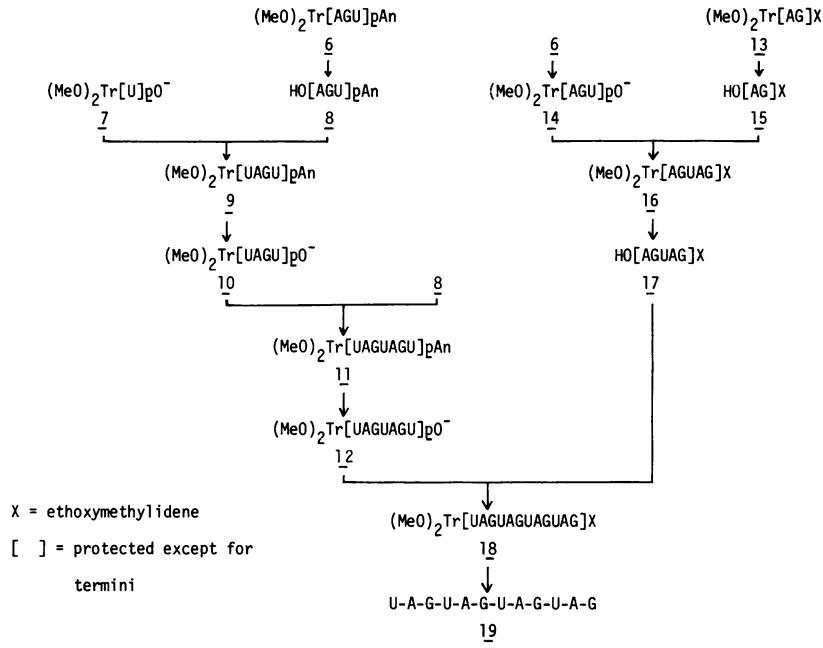


Table II summarizes the yields and reaction conditions for the preparation of of protected oligonucleotides. The fully protected dodecamer (18) was deblocked by treatment with oximate,[12] ammonia and acid under the similar conditions described previously.[5] The deblocked mixture was roughly purified by adsorption with DEAE-cellulose and analyzed by high pressure liquid chromatography (HPLC) on C_{18}-silica gel then subjected to anion-exchange chromatography on DEAE-TOYOPEARL 650 M to yield the

Table II Conditions for Block Condensation

3'-Phospho-diester Component (mmol)		5'-OH Component (mmol)		MSTe (mmol)	Time (min)	Product	Yield (%)
7	(0.28)	8	(0.23)	0.48	35	9	79
10	(0.18)	8	(0.15)	0.37	40	11	60
14	(0.25)	15	(0.24)	0.49	30	16	73
12	(0.075)	17	(0.096)	0.20	35	18	66

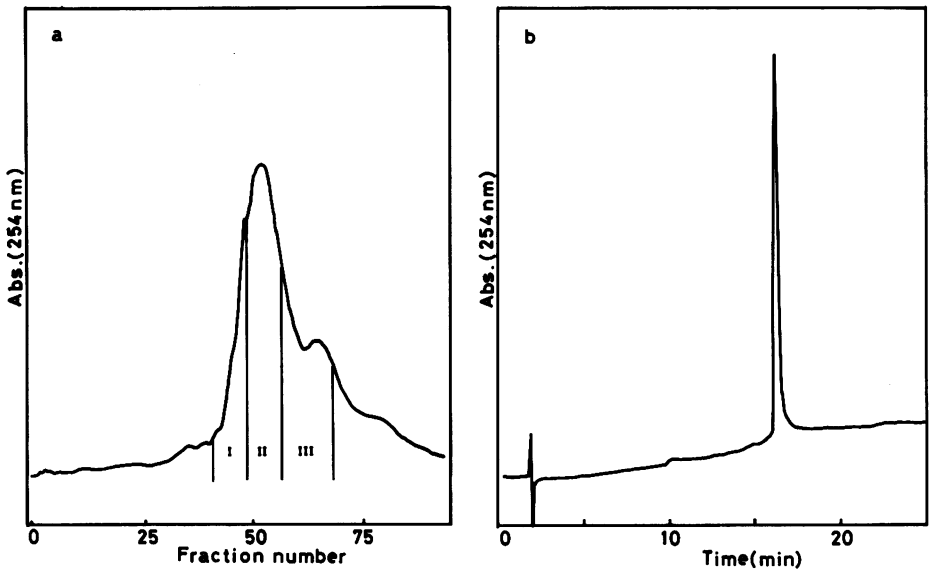


Fig.1 Chromatography of the dodecanucleotide $U(AGU)_3AG$. a) A column (0.8 x 12.5cm) of C-18 silica gel (Waters) and mixture of methanol (10 and 35 %)-1/15 M KH_2PO_4 - Na_2HPO_4 (100 ml each) were used. b) A column of C_{18}-silica gel (TSK-LS410, Toyosoda) and a linear gradient of acetonitrile (5-25 %) in 0.1 M triethylammonium acetate.

unprotected dodecamer (19)(508 A_{260} units). The product was analyzed by reversed phase HPLC using either phosphate or acetate buffer. It was found that impurities eluted later than the dedecamer (19) were separated in phosphate buffer and those eluted earlier than 19 were resolved in acetate buffer. The product (19) was first subjected to a column of C-18 silica gel in phosphate (Fig. 1a). The three parts were analyzed by HPLC on C-18 silica gel in acetate buffer to find the product in each part. The dodecamer (19) was collected and analyzed by HPLC as shown in Fig. 1b. The yield of deblocking after these treatments was 32%. The purified dodecamer $U(AGU)_3AG$ (19) was characterized by mobility shift analysis as shown in Fig. 2.

EXPERIMENTAL

TLC was performed on plates of silica gel (Kieselgel $60HF_{254}$, Merck) using a mixture of chloroform and methanol. For RTLC, silanized silica gel

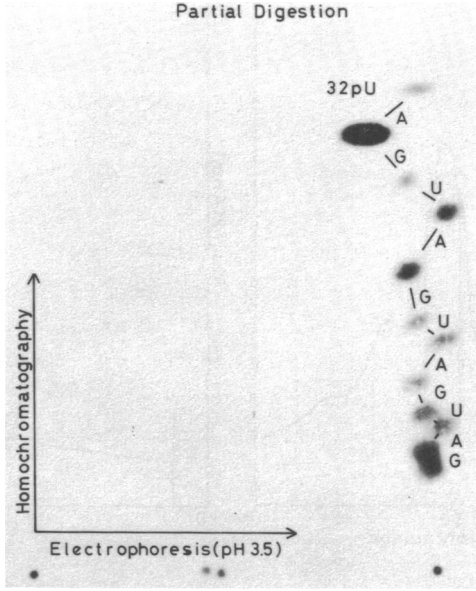


Fig.2 Mobility shift analysis[14] of the dodecamer. The 5'-labeled dodecamer was partially digested with nuclease P1 as described previously.[15] Homo-mix I[16] was used at the second d mension.

(Kieselgel 60 HF_{254} Silanisiert, Merck), HPLC RP-2 F_{254} or RP-8 F_{254} (Merck) were used with a mixture of acetone-water. For columns, silica gel (type 60H, Merck) was used with a mixture of chloroform-methanol. For preparative reversed phase chromatography, alkylated silica gel (C-18, 35-105 μ, Waters) was packed with 60-70 % acetone.

Triethylammonium bicarbonate (TEAB) buffer (pH 7.5) was used to wash organic layers containing protected nucleotides. Other general methods for condensations of nucleotides and for characterization of oligonucleotides were as described previously.[1,4,5]

5'-O-Dimethoxytrityl-2'-O-tetrahydrofuranyl-N-acylnucleosides (2)

2'-O-Tetrahydrofuranyl-N-acylnucleosides[1] (lower isomers) were treated with dimethoxytrityl chloride in pyridine and worked up as described for monomethoxytritylation of nucleosides.[5] Rf values in TLC (10:1) and yields were 2a (0.50, 83%), 2b (0.51, 99%), 2c(0.60, 83%) and 2d(0.46, 93%), respectively.

2'-O-Tetrahydrofuranyl-3'-O-(o-chlorophenyl)phosphoro-p-anisido-N-acylnucleosides (4)

i) 2c (0.303 mmol) was dried by evaporation of pyridine and phosphorylated with o-chlorophenyl phosphoro-p-anisidochloridate[7] (1, 0.663 mmol) in pyridine (1.5 ml) at 15-20° for 16 hr. Water was added with cooling and the product (3a) was extracted with chloroform (25 ml). The organic layer was concentrated and the residue was applied to a column (3.2 x 3.4 cm) of silica gel (10 g). Elution was performed with a gradient of methanol (0-2 %) in chloroform and 3a was precipitated in pentane from its solution in chloroform in a yield of 92% (289 mg, 0.278 mmol). 3c (0.252 mmol) was treated with $ZnBr_2$ (in dichloromethane-isopropanol, 85:15) at room temperature for 15 min and stirred with 1 M ammonium acetate (40 ml). The product (4c) was extracted with chloroform and passed through a column of silica gel. 4c was crystallized from its solution in chloroform. ^{1}H-NMR δ(ppm) in DMSO-d_6 : 11.1 (brs, 1H, NH), 8.73 (s, 1H, H-8 or H-2), 8.68(s, 1H, H-2 or H-8), 8.48(d, 1H, NH-P, J=10.5 Hz), 8.1-7.9 (m, 2H, H-Ar), 7.6-7.1 (m, 7H, H-Ar), 7.12(d, 2H, H-3,5-Anis, J=9Hz), 6.78(d, 2H, H-2,6-Anis, J=9 Hz), 6.13(d, 1H, H-1', J=6.5 Hz), 5.4-4.9 (m, 4H, OH, H-2',3',4'), 4.3(m, 1H, H-1-Thf), 3.67(s, 3H, OCH_3), 1.6(brs, 4H, H-2,3-Thf).

ii) 2d (0.500 mmol) was dissolved in dichloromethane (2.5 ml) in the presence of 1-methylimidazole (1.50 mmol) and stirred with one third of 1 (1.51 mmol) at -10°. After 1 hr another one third was added and the rest was added after 2 hr stirring. The reaction was checked by TLC and RTLC 1-methylimidazole and 1 (0.5 mmol each) were added. The reaction was ceased by addition of water (2 ml). The product (3d and 5) was extracted with dichloromethane (40 ml), washed three times with sat. sodium bicarbonate, concentrated and roughly purified by silica gel column chromatography. The mixture was treated with 1 M $ZnBr_2$ (25 ml) at 18° for 4 hr and added with 1 M ammonium acetate (100 ml). 4d was extracted with dichloromethane, concentrated in the presence of pyridine and applied to a column (3.2 x 3.5 cm) of silica gel (12 g). Elution was performed with a gradient of methanol (0-5 %) in chloroform. 4d (240 mg, 0.335 mmol) was obtained in a yield of 67%. The dedimethoxytritylated product of 5 (48 mg, 0.047 mmol) was obtained in a yield of 9%. NMR δ(ppm) of 4d in DMSO-d_6 : 12.1 (brs, 1H, NH), 11.6 (brs, 1H, NH), 8.54 (d, 1H, NH-P, J=10 Hz), 8.23 (s, 1H, H-8), 7.6-7.0 (m, 4H, H-Ar), 7.12 (d, 2H, H-3,5-Anis, J=9 Hz), 6.82 (d, 2H, H-2,6-Anis, J=9Hz), 5.8-6.0 (m, 1H, H-1'), 5.5-5.0 (m, 3H, OH, H-2',3'), 5.0-4.7 (m, 1H, H-4'), 4.4 (brs, 1H, H-Thf), 3.70 (s, 3H, OCH_3), 2.8 (m, 1H,

t-H), 1.7 (brs, 4H, H-2,3-Thf), 1.13 (d, 6H, CH_3, J=7 Hz). 6 : 11.1 (brs, 1H, NH), 8.9 (br, 1H, NH-p), 8.66 (s, 1H, H-8), 8.51 (d, 1H, NH-P, J=10 Hz), 7.6-6.7 (m, 16H, H-Ar), 6.2-6.0 (m, 1H, H-1'), 5.5-4.8 (m, 4H, OH, H-2',3',4'), 4.3 (br, 1H, H-1-Thf), 3.69 (s, 6H, OCH_3), 2.8 (m, 1H, t-H), 1.6 (brs, 4H, H-2,3-Thf), 1.12 (d, 6H, CH_3, J=7 Hz).

$(MeO)_2Tr(AGU)_pAn$ (6)

2c (2.02 mmol) was phosphorylated with o-chlorophenyl phosphoroditriazolide[8] (3.00 mmol) at 30° for 50 min. The phosphorylated adenosine derivative was extracted with chloroform and washed twice with 0.1 M TEAB (100 ml). Chloroform was evaporated and the residue was mixed with 2'-o-tetrahydrofuranyl-N-isobutyrylguanosine (higher isomer, 2.59 mmol). The mixture was dried by evaporation of pyridine three times and treated with MSTe (3.99 mmol) in pyridine (10 ml) at 30°. After 30 min, 0.1 M TEAB (2 ml) was added with cooling and sat. sodium bicarbonate (100 ml) was added. The product was extracted with chloroform (100 ml) and concentrated to dryness. The residue was applied to a column (6 x 4.7 cm) of silica gel (Kieselgel 60H, 50 g) with a gradient of methanol (0-6 %) in chloroform. The dimer was precipitated with pentane in a yield of 88 % (2.37 g: 1.77 mmol). The Rf values in TLC and RTLC were (0.53, in chloroform-methanol, 10:1) and (0.49, acetone-water, 7:3).

The dimer (1.00 mmol) was phosphorylated with o-chlorophenyl phosphoroditriazolide (1.50 mmol) by the procedure described above and condensed with 4a (1.00 mmol) using MSTe (2.00 mmol) at 30° for 27 min. The trimer (6) was separated by a column (6 x 4.7 cm) of silica gel (Kieselgel 60H, 50 g) with a gradient of methanol (0-5 %) in chloroform. The yield was 82 % (1.66 g, 0.815 mmol). The Rf values in TLC (10:1) and RTLC (7:3) were 0.42 and 0.46 respectively.

Dedimethoxytritylation of 6, preparation of $(AGU)_pAn$ (8)

6 (1.066 g, 0.503 mmol) was treated with 1M $ZnBr_2$ (isopropanol-CH_2Cl_2-=15:85, 25 ml) at room temperature for 15 min and added with 1M ammonium acetate (75 ml). The aqueous layer was extracted three times with dichloromethane (25 ml) and the organic solutions were concentrated. The product was purified by chromatography on a column (4 x 2.1 cm) of silica gel (type 60H, 10 g) with a gradient of methanol (0-7 %) in chloroform. The yield was 76 %, 692 mg, 0.381 mmol. The Rf values in TLC (10:1) and RTLC (7:3) were 0.35 and 0.60, respectively.

2',3'-Ethoxymethylidene-N-isobutyrylguanosine

N-Isobutyrylguanosine (3.31 g, 9.37 mmol) was dissolved in DMF (20 ml)

and stirred with ethylorthoformate (8.23 ml, 50 mmol) in the presence of p-toluenesulfonic acid (177 mg, 0.93 mmol) at room temperature. After 5 hr conc. ammonia (0.1 ml) was added and the mixture was concentrated. The residue was dissolved in chloroform-pyridine (3:2, 80 ml), washed 3 times with sat. sodium bicarbonate (60 ml), concentrated and applied to a column (5 x 5.5 cm) of silica gel (60H, 40 g) with a gradient of methanol (0-7 %) in chloroform. The product was recrystallized with ethyl acetate. The yield was 54 %, 2.06 g, 5.03 mmol.

$(MeO)_2$Tr(UAGU)pAn (9)

2a (175 mg, 0.28 mmol) was phosphorylated as described for the synthesis of 6 and the product (7) was condensed with 8 (418 mg, 0.230 mmol) as summarized in Table II. The product was purified by reversed phase chromatography on a column (3 x 13 cm) of C-18 silica gel with a gradient of acetone (60-80 %) in 0.2 % pyridine. The Rf values in TLC (10:1) and RTLC (7:3) were 0.54 and 0.40, respectively.

Preparation of the 3'-diester (10)

The fully protected tetramer (9)(0.181 mmol) was treated with isoamyl nitrite (1.21 ml, 9.02 mmol) in pyridine-acetic acid (5;4, 5 ml) at 30° for 4.5 hr. The reaction was checked by TLC and a mixture of 0.2 M TEAB (40 ml) and pyridine (20 ml) was added. The aqueous layer was washed twice with ether-pentane (1:1, 50 ml). The product was extracted with chloroform (40 ml), washed three times with 0.2 M TEAB (50 ml) and coevaporated with pyridine.

$(MeO)_2$Tr(UAGUAGU)pAn (11)

The 3'-diester (10) and the trimer (8) were condensed as shown in Table II. The product was purified by reversed phase chromatography on a column (3 x 13 cm) of C-18 silica gel with a gradient of acetone (60-80 %) in 0.2 %. pyridine. The Rf values in TLC (10:1) and RTLC (7:3) were 0.36 and 0.35, respectively.

$(MeO)_2$Tr(AG)X (13)

The dimer was prepared by the similar procedures described for the synthesis of 6 expect that 2',3'-ethoxymethylidene-N-isobutyrylguanosine (0.40 mmol) was condensed with the phosphorylated 2c (0.41 mmol). The product was purified by a column (3.2 x 3.4 cm) of silica gel (60 H, 10 g) with a gradient of methanol (0-3 %) in chloroform. The yield was 84 % (445 mg, 0.336 mmol). The Rf values in TLC (10:1) and RTLC (7:3) were 0.56 and 0.36, respectively.

$(MeO)_2Tr(AGUAG)X$ (16)

14 and 15 were obtained by treatment of 6 and 13 as described for the preparation of 10 and 8, respectively. Condensations for those oligonucleotides are summarized in Table II. The fully protected pentamer (16) was purified by chromatography on a column (4 x 2.1 cm) of silica gel (H, 10 g) with a gradient of methanol (0-7 %) in chloroform. The yield was 73 %, 531 mg, 0.176 mmol. Rf values in TLC (10:1) and RTLC (7:3) were 0.51 and 0.36, respectively.

The fully protected dodecamer (18)

12 and 17 were obtained from 11 and 16 by treatments described for the preparation of 10 and 8, respectively. Condensation was performed as summarized in Table II. The product was isolated by reversed phase chromatography on a column (3 x 13 cm) of C-18 silica gel with a gradient of acetone (60-85 %) in 0.2 % pyridine. The yield was 66 %, 338 mg, 0.049 mmol. Rf values in TLC (10:1) and RTLC (7:3) were 0.3 & and 0.26, respectively.

Deblocking and Purification

18 (36 mg, 5.2 μmol) was stirred with 0.5 M N^1, N^1, N^3, N^3-tetramethylguanidinium syn-pyridine-2-carboxaldoximate (in 50 % aqueous dioxane 4.4 ml) at room temperature for 3 days and diluted with water (15 ml). The mixture was passed through a column (8 ml) of Dowex 50W x 2 (pyridinium) and the column was washed with 30 % aqueous pyridine (120 ml). The combined solution was concentrated and heated with conc. ammonia (5 ml) at 55° for 5.5 hr. Ammonia was removed and the residue was dissolved in 30 % aqueous pyridine and washed with ethyl acetate (50 ml). The aqueous layer was dried by evaporation, added with 0.01 N HCl (10 ml), adjusted to pH 2 by addition of 0.1 N HCl and kept at room temperature for 3.5 hr.

Then, the solution was neutralized with 0.1 N ammonia and the solution was washed twice with ethyl acetate. The aqueous layer was concentrated and applied to a column (1.7 x 5 cm) of DEAE-cellulose (bicarbonate). The column was washed with 0.05 M TEAB (300 ml) and the dodecamer (583 A_{260} units) was eluted with 1 M TEAB (150 ml). The product was purified by anion-exchange chromatography on a column (1.6 x 21 cm) of DEAE-TOYOPEARL 650 M equilibrated with 0.1 M NaCl containing 0.02 M tris-HCl, pH 7.5 and 7 M urea. Elution was performed with linear gradient of NaCl (0.1-0.4 M, total 800 ml). The main peak which contained 508 A_{260} units was further purified by reverse-phase chromatography on a column (0.8 x 12.5 cm) of C-18 silica gel. Elution was performed with a gradient of methanol (10 %-35 %)

in 1/15 M phosphate KH_2PO_4-Na_2HPO_4 (pH 7.5). Aliquots of the three parts (Fig. 1a, I:60A_{260}, II:159A_{260}, III:104A_{260}) were finally purified by HPLC on alkylated silica (TSK-LS410, 10μ, 0.46 x 25 cm) using acetate buffer. The purity of I, II and III were 64%, 79% and 33%, respectively.

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