Tetrahedron Vol. 40, No. 1, pp 47 to 57, 1984
Printed in Great Britain.

0040-4020/84 $3.00 + .00
Pergamon Press Ltd.

CHEMICAL SYNTHESIS OF THE 5′-HALF MOLECULE OF *E.coli* $tRNA_2^{Gly}$

EIKO OHTSUKA, AKIO YAMANE, TAKEFUMI DOI and MORIO IKEHARA
Faculty of Pharmaceutical Sciences, Osaka University, 1–6 Yamadaoka, Suita, Osaka 565, Japan

(*Received in UK 6 April* 1983)

Abstract—A tritriacontanucleotide which has the sequence of the 5′-half molecule of *E.coli* glycine $tRNA_2$, was synthesized by the phosphotriester method involving *p*-anisidate protection for the 3′-phosphate ends. Di- and trinucleotide units were prepared from 5′-dimethoxytrityl-2′-O-tetrahydrofuranyl-3′-O-(*o*-chlorophenyl)phosphoryl derivatives of uridine, N-benzoylcytidine, N-benzolyadenosine and N-isobutyrylguanosine by condensation with 3′,5′-unprotected nucleosides followed by phosphorylation to give 3′-phosphodiester blocks. The 3′-terminal dimers and trimers were synthesized by using 3′-(*o*-chlorophenyl)phosphoro-*p*-anisidates instead of 3′,5′-unprotected nucleosides. The 3′-phosphodiesters of oligonucleotides with a chain length of larger than 5 were obtained by removal of the 3′-phosphoro-*p*-anisidate with isoamyl nitrite. The 5′-dimethoxytrityl group was removed by treatment with zinc bromide under anhydrous conditions. Fragments were designed to use common dimer blocks and to reduce the step for 5′-deblocking of larger fragments. Finally a 3′-phosphodiester block with a chain length of 20 was condensed with a 5′-OH component (tridecanucleotide). The fully protected 33 mer was deblocked and purified by chromatography. The structural integrity of the product was confirmed by mobility shift analysis and complete digestion with RNase T2.

Chemical synthesis of ribooligonucleotides is an important subject in organic chemistry. It also provides a useful approach for studies on biological and physico-chemical properties of ribonucleic acids. In the early 1960s short oligomers such as all possible ribotriplets were synthesized by the phosphodiester method and used to elucidate the genetic code.[1] Later phosphodiester ribooligonucleotide blocks were prepared for the synthesis of tRNA fragments.[2] For the synthesis of larger oligonucleotides, the phosphotriester method becomes a method of choice with introduction of phenyl derivatives as protecting groups for internucleotidic phosphates and arenesulfonyl azolides as activating reagents for phosphodiester groups.[3] We have been synthesizing ribooligonucleotides either by the phosphodi- or triester method, and have performed the synthesis of *E.coli* formyl methionine tRNA by enzymatic joining of the chemically synthesized fragments with RNA ligase.[4] The methodology used in this study can be applied to replacement of functional parts of the tRNA molecule with synthetic oligonucleotides. Synthesis of larger fragments is advantageous in reducing enzymatic joining steps to construct modified tRNAs. Larger quantities of oligonucleotides, which can only be obtained chemically, are very useful for studies on interaction of nucleic acids with proteins, such as aminoacyl-tRNA synthetases.[5] Completely chemical synthesis of RNA of the size of tRNA is a challenging subject in chemistry and has its own value. In the present paper we report a synthesis of a tritriacontanucleotide having the sequence of the 5′-half molecule (1–33) of *E.coli* $tRNA_2^{Gly}$.[6] The synthesis involved phosphotriester block condensations using a combination of tetrahydrofuranyl[7] and dimethoxytrityl[8] groups for the 2′- and 5′-OH functions, respectively.[9] The abbreviated scheme of the synthesis is shown in Fig. 1. The 5′-dimethoxytrityl group was shown to be removed selectively in the presence of the 2′-O-tetrahydrofuranyl group on treatment with zinc bromide,[9] although this reaction required anhydrous conditions, in contrast to the conditions used with deoxyoligonucleotides.[10] In the phosphotriester synthesis, protection of the 2′-OH group is an essential problems and various groups have been used in combination with selectivity removable 5′-O-protecting groups. Tetrahydropyranyl,[8] 4-methoxytetrahydropyranyl,[11] tert-butyldimethylsilyl,[12] and *o*-nitrobenzyl[13] groups have been used for synthesis of larger ribooligonucleotides: octadecamer,[14] nonadecamer[15] and eicosamer.[16] The present tetrahydrofuranyl group has previously been used and shown to have the properties required for a 2′-O-protecting groups, e.g. facile introduction, stability during synthesis and complete removal at the final stage.[9,17–19]

Preparation of di- and trinucleotide blocks. For the synthesis of larger oligonucleotides, condensation of protected oligonucleotides has obvious advantages. Preparation of oligonucleotides with phosphotriester internucleotidic phosphate requires two kinds of protecting groups for phosphates. One of those has to be removed selectively. A variety of combinations has been reported for the phosphotriester synthesis. Phenyl derivatives,[20] 2-cyanoethyl,[21] phenylethyl derivatives[22] and 5′-chloroquinolinyl[23] have been used for protection for internucleotide phosphates. As removable protecting groups, 2-cyanoethyl,[24] 2,2,2,-trichloroethyl,[25] anilido,[26a] and anisido[16,26b] groups are used at the terminal phosphate. In the present synthesis 3′-(*o*-chlorophenyl)-*p*-anisido phosphoryl derivatives (**5**) were synthesized by phosphorylation of 5′-dimethoxytrityl-2′-O-tetrahydrofuranylnucleosides (**2**) with *o*-chlorophenyl *p*-anisidophosphorochloridate[26b] (**3**) as the phosphorylating reagent followed by removal of the 5′-dimethoxytrityl group with zinc bromide as illustrated in Fig. 2 using the condensations described

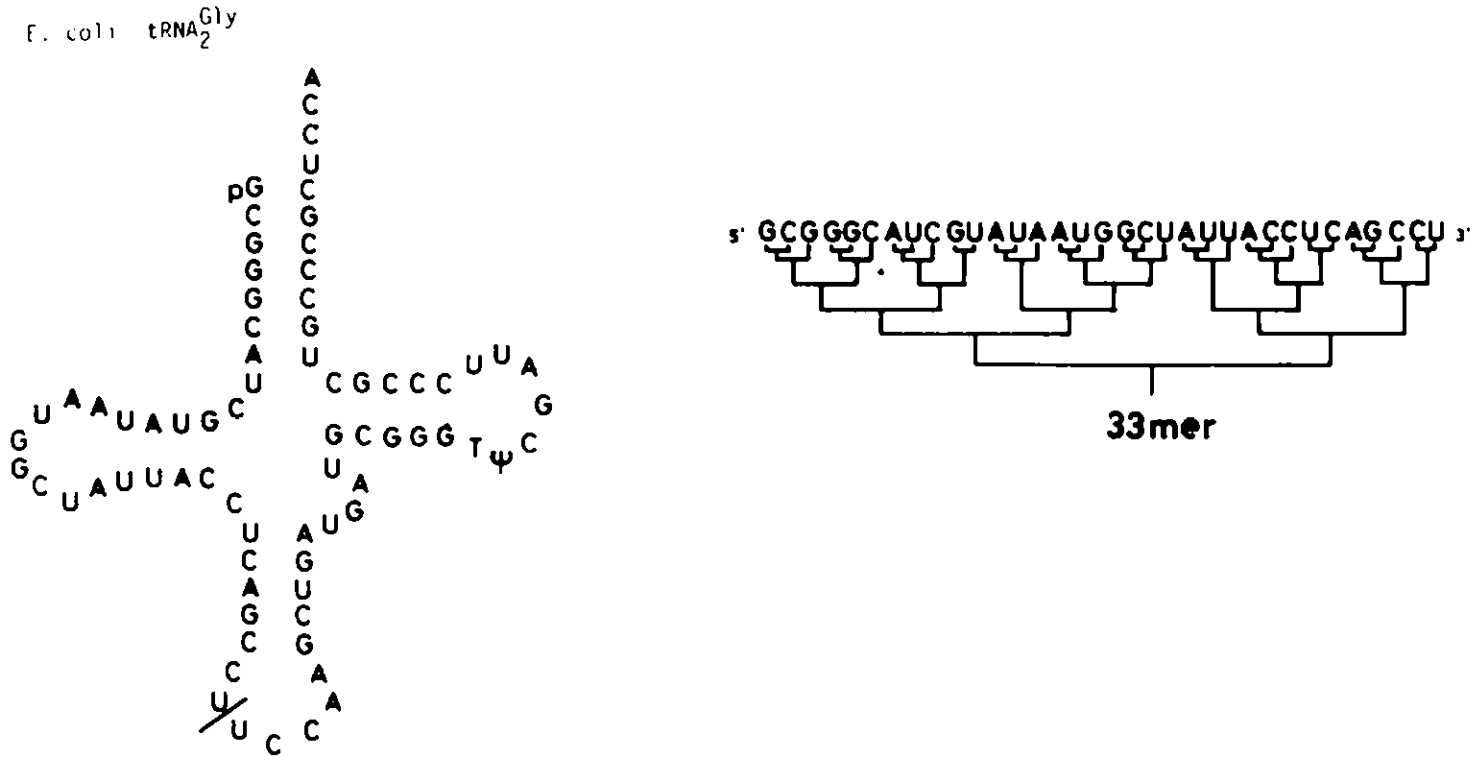


Fig. 1. Structure of the *E.coli* $tRNA_2^{Gly}$ and the 5′-half sequence (1–33).

previously.[9] Dimer units (**7**) were prepared by condensation of 3′,5′-unprotected nucleoside (**1**) with 5′-dimethoxytrityl-2′-O-tetrahydrofuranyl-nucleoside 3′-(*o*-chlorophenyl)phosphates (**6**), which in turn were prepared by phosphorylation of **2** with *o*-chlorophenyl bis-(1H-1,2,4-triazol-1-yl) phosphate[27] using mesitylenesulfonyl tetrazolide (MSTe, 1-(2,4,6-trimethylbenzenesulfonyl)-1H-tetrazole).[28] For further elongation in the 3′-direction, **7** was phosphorylated. The terminal dimer blocks (**8**) were obtained by condensation of the 3′-phosphodiesters (**6**) with the 5′-free nucleotides (**5**). Reaction conditions for the preparation of dimers are summarized in Table 1. The dimers were isolated by chromatography on silica gel or alkylated silica gel.

Trimers were prepared from the above dimers by

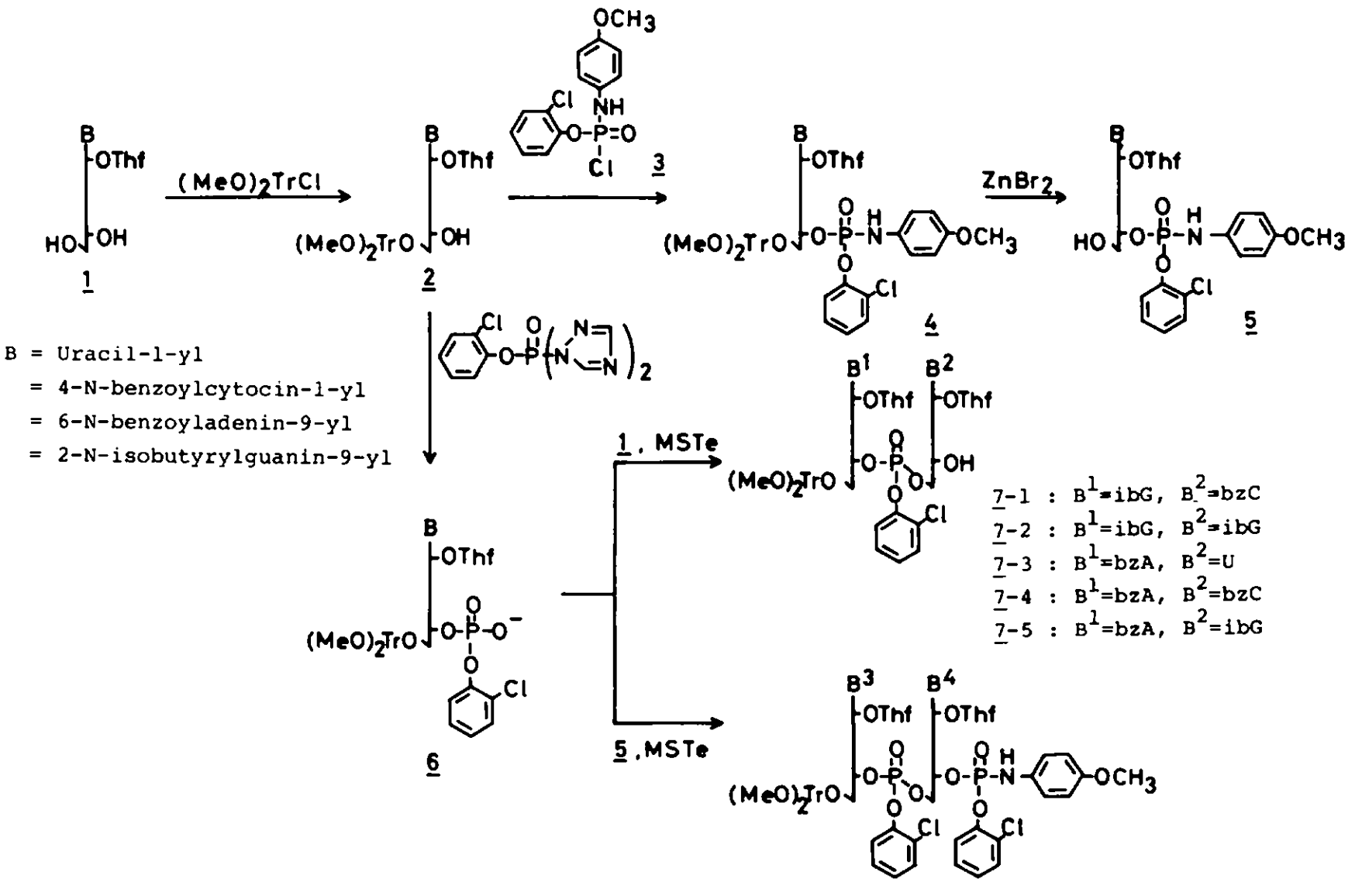


Fig. 2. Preparation of dimers.

Table 1. Reaction conditions for the synthesis of dimers

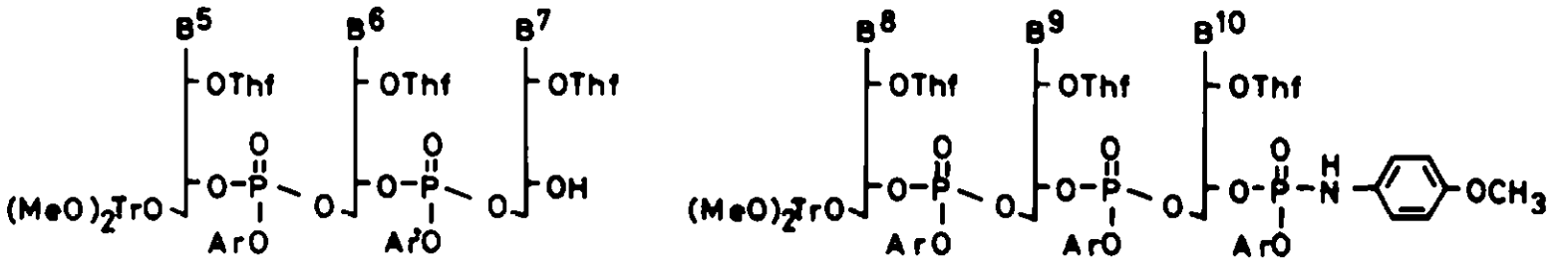
3'-Phospho-diester Component* (mmol)		5'-OH Component (mmol)		MSTe (mmol)	Time (min)	Product		Yield (%)
DT[G]OH	(8.00)	HO[C]OH	(11.23)	15.90	25	DT[GC]OH	(7-1)	62
DT[G]OH	(2.81)	HO[G]OH	(3.93)	5.60	25	DT[GG]OH	(7-2)	72
DT[A]OH	(10.13)	HO[U]OH	(13.99)	19.91	25	DT[AU]OH	(7-3)	75
DT[A]OH	(2.45)	HO[C]OH	(3.29)	4.71	35	DT[AC]OH	(7-4)	64
DT[A]OH	(2.02)	HO[G]OH	(2.59)	3.99	35	DT[AG]OH	(7-5)	88
DT[G]OH	(2.00)	HO[U]pAn	(1.70)	3.40	25	DT[GU]pAn	(8-1)	78
DT[U]OH	(1.50)	HO[C]pAn	(1.40)	2.96	25	DT[UC]pAn	(8-2)	85
DT[C]OH	(0.60)	HO[U]pAn	(0.50)	1.01	30	DT[CU]pAn	(8-3)	74

DT = $(MeO)_2Tr$, An = NHϕ-p-OCH_3, p = o-chlorophenyl phosphate, [ ] = protected except for 3' and 5' termini, * : DT[N]OH was converted to 3'-phosphodiester component by phosphorylation with o-chlorophenyl phosphoroditriazolide followed by treatment with H_2O.

essentially the same procedure. Structures of the trimers are shown in Fig. 3 and reaction conditions are summarized in Table 2. The trimers were separated by reversed phase chromatography on alkylated silica gel. These dimers and trimers were designed to have purines at the 5′-terminal position, since removal of the dimethoxytrityl group of purine nucleosides was found to be easier.

Synthesis of tritriacontemer by condensation of oligonucleotide blocks. All 3′-diesterified trinucleotide intermediates used in this synthesis were prepared by phosphorylation of trinucleoside diphosphates listed in Table 2. As shown in Fig. 4 the 5′-OH components were derived by removal of the 5′-dimethoxytrityl groups. Yields and conditions of these conversions are summarized in Table 3. Reaction conditions of these nucleotide blocks are given in Table 4. The 3′-phosphoro-*p*-anisidate of penta- and hexanucleotides were converted to the phosphate by treatment with isoamyl nitrite when elongation was in the 3′-direction. The eicosamer (**48**) was obtained as the 3′-phosphodiester form by elongating the chain in the 3′-direction. It was activated by the condensing reagent to react with the tridecamer (**49**). Removal of the 5′-dimethoxytrityl of larger oligonucleotides was avoided as far as possible. Complete removal of the 5′-protecting group of the hexamers and tridecamer was not intended. Unchanged dimethoxytritylated oligonucleotides were recovered when the reaction had been slow. Yields listed in Table 4 varied mainly due to decomposition during chromatography on reversed-phase support. The eicosamer (**46**) was partially lost by conversion to polar compounds. The fully protected tritriacontamer (**50**) was not purified by chromatography. It was collected by preparation and isolated after deblocking.

Deblocking of the product was performed by a procedure similar to that reported previously,[9] by treatment with: (1) isoamyl nitrite in pyridine-acetic acid (5:4), (2) 0.5 M 1,1, 3,3-tetramethylguanidinium *syn*-pyridine-2-carboaldoximate (TMG-PAO),[29] (3) ammonium hydroxide, (4) anion-exchange resin Dowex 50 W × 2 (pyridinium form), and (5) dilute hydrochloric acid (pH 2). The deblocked product was



9 : B^5=ibG, B^6=bzC, B^7=ibG
10 : B^5=bzA, B^6=U, B^7=bzC
11 : B^5=bzA, B^6=U, B^7=bzA
12 : B^5=bzA, B^6=U, B^7=ibG
13 : B^5=bzA, B^6=U, B^7=U
14 : B^5=bzA, B^6=bzC, B^7=bzC
15 : B^5=bzA, B^6=ibG, B^7=bzC

16 : B^8=ibG, B^9=ibG, B^{10}=bzC
17 : B^8=ibG, B^9=bzC, B^{10}=U

3

Fig. 3. Structure of trimers.

Table 2. Reaction conditions for the synthesis of trimers

3'-Phospho-diester Component*	(mmol)	5'-OH Component	(mmol)	MSTe (mmol)	Time (min)	Product		Yield (%)
DT[GC]OH	(1.72)	HO[G]OH	(2.43)	3.45	30	DT[GCG]OH	(9)	76
DT[AU]OH	(1.72)	HO[C]OH	(2.42)	3.23	30	DT[AUC]OH	(10)	72
DT[AU]OH	(1.08)	HO[A]OH	(1.43)	2.04	30	DT[AUA]OH	(11)	69
DT[AU]OH	(2.92)	HO[G]OH	(4.21)	6.03	25	DT[AUG]OH	(12)	73
DT[AU]OH	(0.66)	HO[U]OH	(0.93)	1.30	30	DT[AUU]OH	(13)	59
DT[AC]OH	(1.35)	HO[C]OH	(1.89)	2.71	30	DT[ACC]OH	(14)	58
DT[AG]OH	(0.56)	HO[C]OH	(0.79)	1.23	25	DT[AGC]OH	(15)	70
DT[GG]OH	(1.66)	HO[C]pAn	(1.67)	3.34	25	DT[GGC]pAn	(16)	73
DT[GC]OH	(3.04)	HO[U]pAn	(3.05)	6.02	30	DT[GCU]pAn	(17)	76

DT = $(MeO)_2Tr$, An = NHϕ-p-OCH_3, p = o-chlorophenyl phosphate, [ ] = protected except for 3' and 5' termini, * : DT[NN]OH was converted to 3'-phosphodiester component by phosphorylation with o-chlorophenyl phosphoroditriazolide followed by treatment with H_2O.

separated by gel filtration on Sephadex G-50. Figure 5 shows profiles of gel filtration of the eicosamer (20 mer) and tritriacontamer (33 mer). The product were analyzed by reversed-phase high pressure liquid chromatography (HPLC) and the 33 mer was found to be contaminated with partially protected compounds. Acid treatment for removal of the 2'-O-tetrahydrofuranyl group was repeated and the product was fractionated by reversed-phase HPLC after gel filtration as shown in Fig. 6. The fractionated products were found to be homogeneous (Fig. 7).

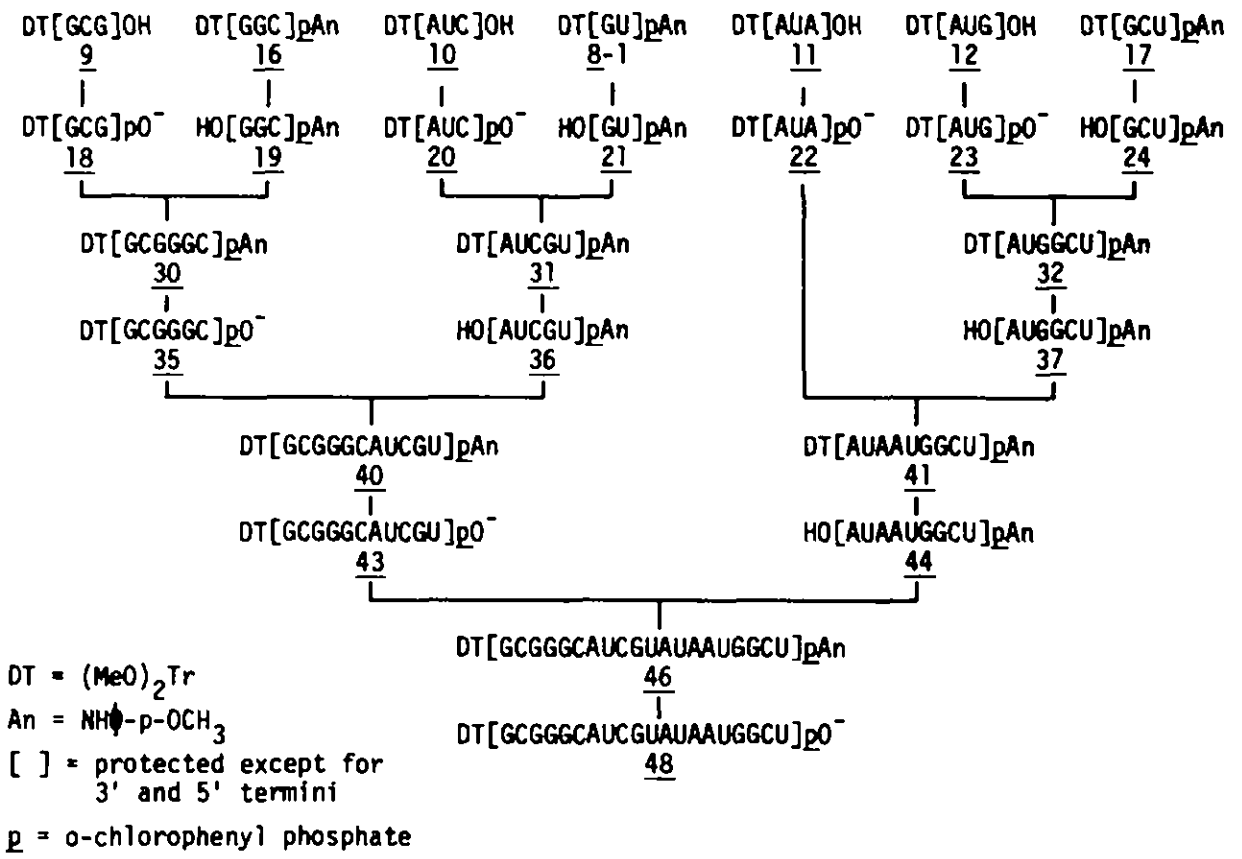
The 20 and 33 mer were identified by analysis of the chain length (Fig. 8) and mobility shift method (Fig. 9). Figure 8 shows a radio-autograph of a 20% polyacrylamide gel electrophoresis[30] of the 5'-labeled products. For mobility shift analysis[31] and polymers were labeled at either end with polynucleotide kinase[32] and [γ-^{32}P]ATP or with RNA ligase plus 5'-labeled pCp.[33]

Complete removal of the protecting groups and maintaining of the 3'-5' internucleotide linkages were confirmed by complete digestion with RNase T2[34] followed by labeling with polynucleotide kinase and [γ-^{32}p] ATP. The results of two dimensional thin layer chromatography on cellulose[35] of the mixture of 5'-labeled pNp are shown in Fig. 10. This test indicated that the 20 mer and 33 mer were digested with RNase T2 to give nucleoside 3'-phosphates.

Table 3. Removal of the 5'-dimethoxytrityl group

Substrate (mmol)		1M $ZnBr_2$* (ml)	Time (min)	Product	Yield (%)
16	(1.195)	40	2.5	HO[GGC]pAn (19)	67
8-1	(1.289)	40	3	HO[GU]pAn (21)	84
17	(2.250)	70	2.5	HO[GCU]pAn (24)	68
8-2	(1.146)	30	20	HO[UC]pAn (27)	73
8-3	(0.354)	10	12	HO[CU]pAn (29)	76
31	(0.791)	35	2	HO[AUCGU]pAn (36)	70
32	(1.129)	50	1	HO[AUGGCU]pAn (37)	57
33	(0.603)	30	5	HO[ACCUC]pAn (38)	81
34	(0.221)	11	5	HO[AGCCU]pAn (39)	67
41	(0.380)	25	2	HO[AUAAUGGCU]pAn (44)	56
47	(0.064)	10	2	HO[AUUACCUCAGCCU]pAn (49)	66

An = NHϕ-p-OCH_3, p = o-chlorophenyl phospate, [ ] = protected except for 3' and 5' termini, * ; CH_2Cl_2:iso-PrOH = 85:15.



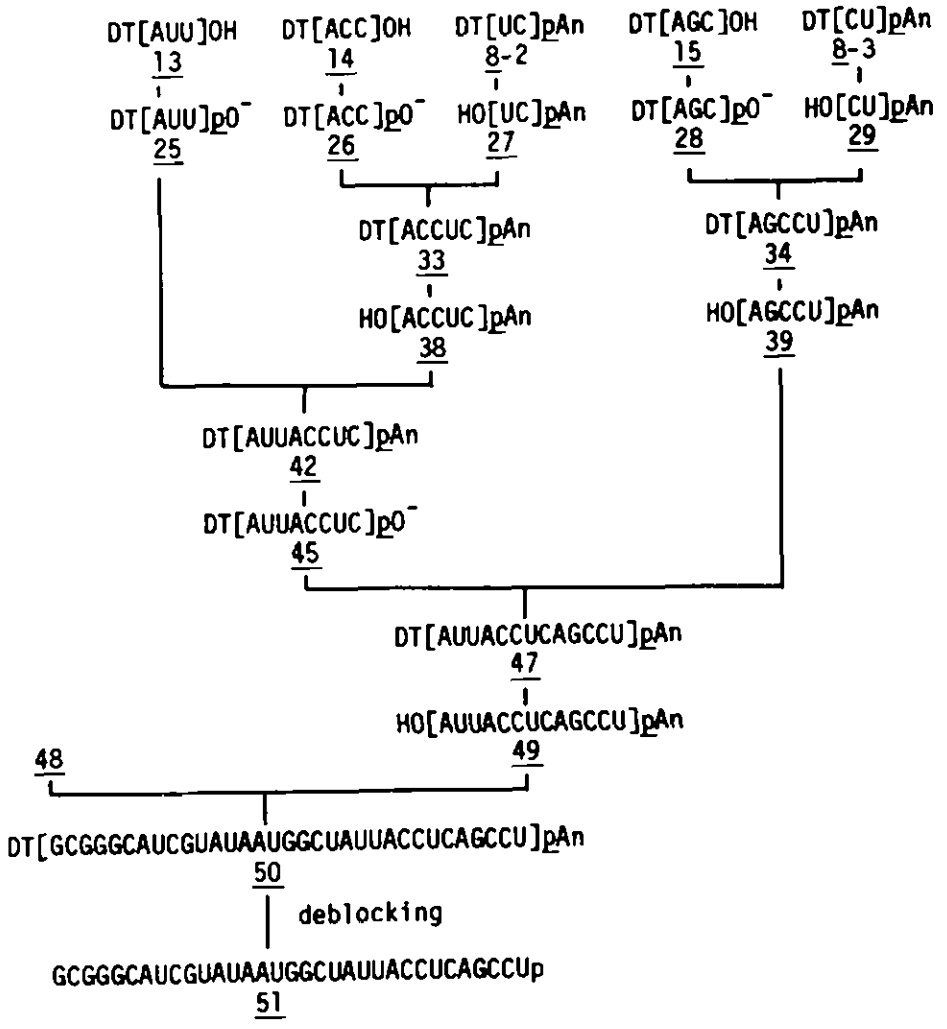


Fig. 4. Synthesis of the 33 mer.

EXPERIMENTAL

TLC was performed on plates of silica gel (Kieselgel 60 HF_{254}, Merck) using a mixtures of $CHCl_3$ and MeOH. For reversed phase TLC (RPTLC), silanized silica gel (Kieselgel 60 HF_{250} Silanisiert, Merck) was used with a mixture of acetone–water. For columns, silica gel (type 60 or 60 H, Merck) was used with a mixtures of $CHCl_3$–MeOH. For preparative reversed phase chromatography, alkylated silica gel (C-18, 35–105 μ, Waters) was packed with 60–70% acetone and elution was performed with a gradient of acetone (60–80%) in 0.2% aqueous pyridine. HPLC was carried out on an Altex 332 MP apparatus using a reversed phase column (TSK-LS410, Toyosoda).

Two dimensional homochromatography[31] was performed as described previously.[36]

Triethylammonium bicarbonate (TEAB) buffer (pH 7.5)

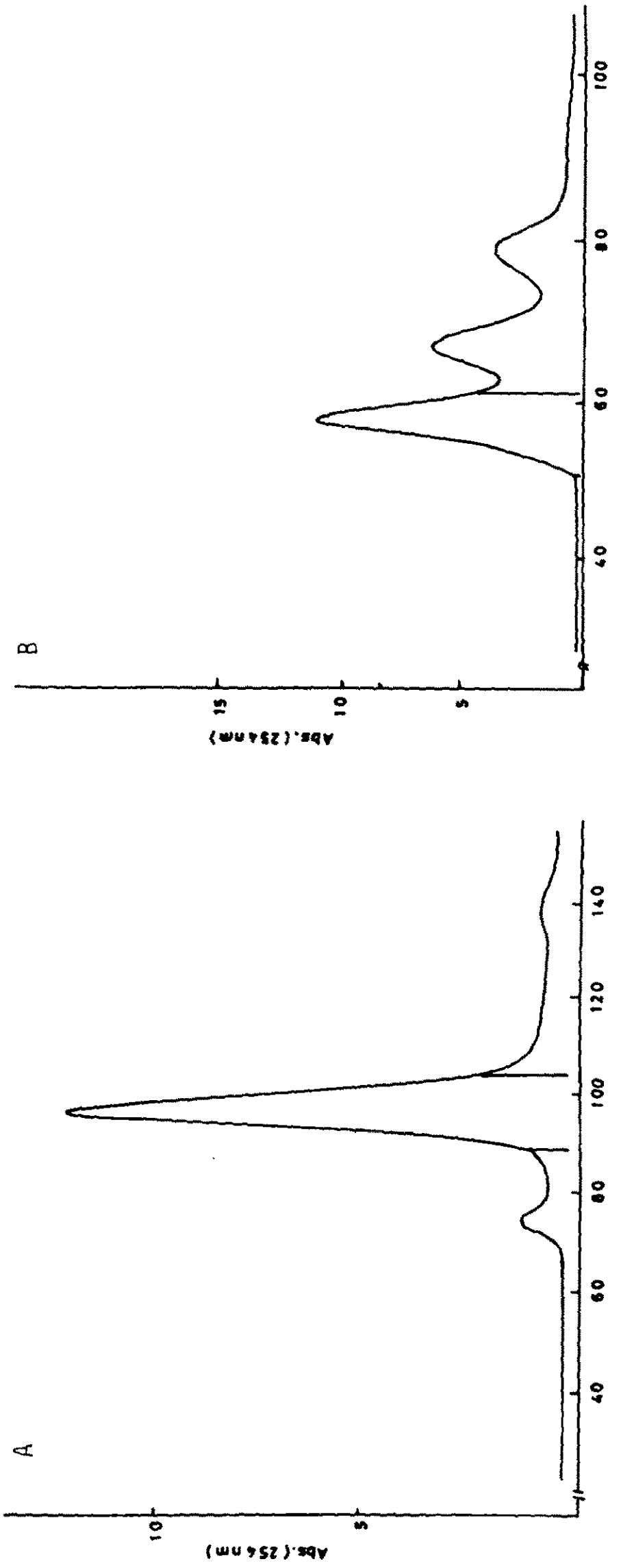


Fig. 5. Gel filtration of the 22 mer (A) and 33 mer (B) on a column (2.8 × 117 cm) of Sephadex G-50 equilibrated with 0.1 M TEAB. A, Fractions of 2.7 ml were collected every 5 min. B, Fractions of 3.9 ml were collected every 6 min.

Table 4. Conditions for block condensation

3'-Phospho-diester Component (mmol)		5'-OH Component (mmol)		MSTe (mmol)	Time (min)	Product (Chain length)	Yield (%)
9 a)	(0.903)	19	(0.791)	1.988	40	30 (6 mer)	59
10 a)	(1.061)	21	(1.060)	2.620	40	31 (5 mer)	79
12 a)	(1.702)	24	(1.492)	3.702	35	32 (6 mer)	77
14 a)	(0.778)	27	(0.794)	1.660	25	33 (5 mer)	84
15 a)	(0.365)	29	(0.277)	0.650	35	34 (5 mer)	82
30 b)	(0.456)	36	(0.466)	1.008	40	40 (11 mer)	49
11 a)	(0.675)	37	(0.646)	1.693	40	41 (9 mer)	74
13 a)	(0.352)	38	(0.356)	1.062	55	42 (8 mer)	64
40 b)	(0.222)	44	(0.183)	0.720	50	46 (20 mer)	22
45	(0.155)	39	(0.146)	0.464	55	47 (13 mer)	63
46 b)	(0.020)	49	(0.020)	0.187	90	50 (33 mer)	

a) : DT[NNN]OH was converted to 3'-phosphodiester component by phosphorylation with o-chlorophenyl phosphoroditriazolide followed by treatment with H_2O.
b) : Fully protected ribooligonucleotide was converted to 3'-phosphodiester component by treatment with isoamyl nitrite.

was used to wash organic layers containing protected nucleotides.

Dinucleoside monophosphates **7**; Table 1, Example (7-1)

General methods for phosphorylation and condensation. 5′-Dimethoxytrityl-2′-O-tetrahydrofuranyl-N-isobutyrylguanosine (**2**, B = ibG; 5.805 g, 7.998 mmol) was dried by evaporation of pyridine and dissolved in pyridine (3 ml).

o-Chlorophenyl phosphoroditriazolide (12.0 mmol in 40 ml of dioxane) was added and the mixture was shaken for 20 min at 30°. TLC and RPTLC showed disappearance of the starting material. After 25 min, 0.1 M TEAB (150 ml) and pyridine (70 ml) were added. The product (**6**) was extracted with $CHCl_3$ (150 ml). The aqueous phase was reextracted with $CHCl_3$-pyridine (3:1, 40 ml) and the combined organic layer was washed twice with TEAB (150 ml). After evaporation of organic solvents the residue was dried by evaporation of pyridine 3 times and mixed with **2** (B = bzC) (higher isomer, 4.686 g, 10.15 mmol). The mixture was dried as above and treated with MSTe (4.012 g, 15.90 mmol) in pyridine (40 ml) at 30° for 15 min. Completion of the reaction was confirmed by TLC and RPTLC. After 25 min, water (3 ml) was added and evaporated. The residue was dissolved in $CHCl_3$ and washed with sat $NaHCO_3$ aq. The product (7-1) was separated by chromatography on silica gel (Kieselgel 60 H, 150 g, ϕ 10 × 5.4 cm) using a gradient MeOH in $CHCl_3$, and precipated with hexane from its soln in $CHCl_3$, yield was 62%, 6.512 g, 4.95 mmol.

Dinucleotide **8**. Table 1, Example (8-2). Compound **2** (B = U) (0.927 g, 1.50 mmol) was phosphorylated as described above and **6** (B = U) was condensed with **5** (B = BzC) (0.996 g, 1.40 mmol) in the presence of MSTe

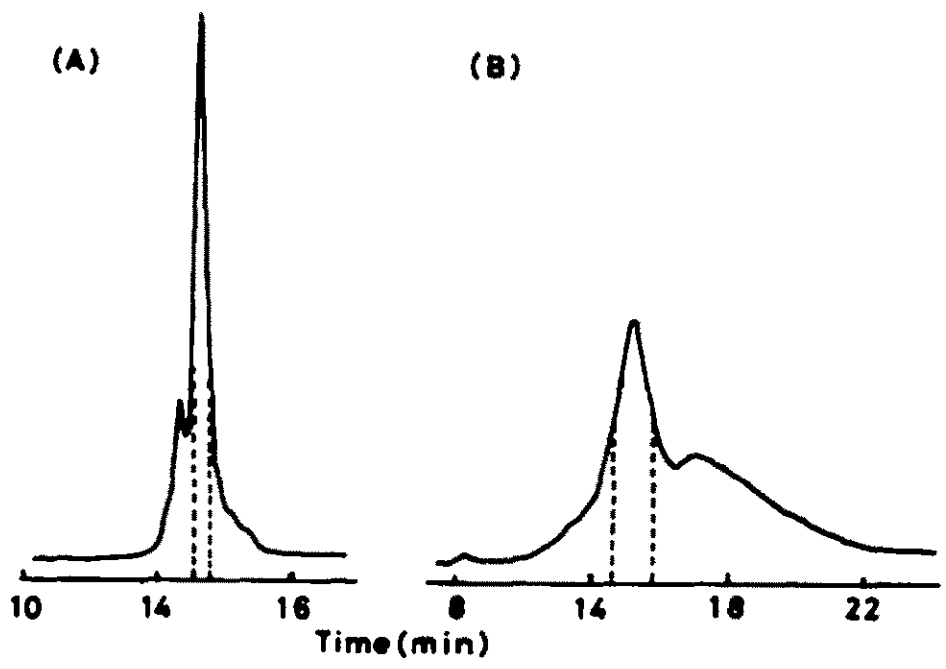


Fig. 6. Purification of the 22 mer (A) and 33 mer (B) by HPLC on silica gel (TSK-LS410) with a flow rate of 2 ml/min. A, a linear gradient of acetonitrile (5–25% during 30 min) in 0.1 M triethylammonium acetate. B, a linear gradient of acetonitrile (11–15% during 03 min) in 0.1 M triethylammonium acetate.

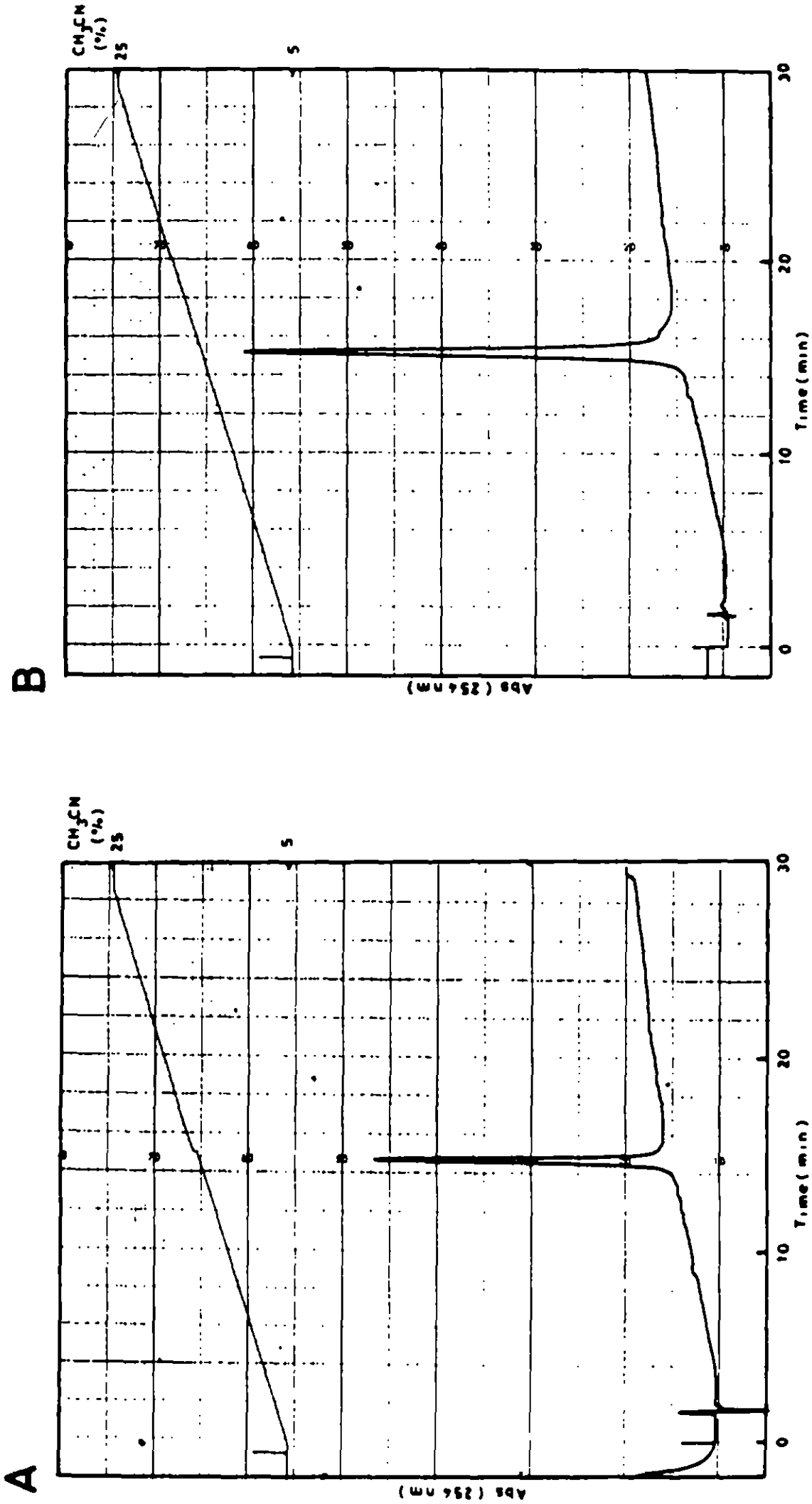


Fig. 7. Analysis of the purified 20 and 33 mer by HPLC on C-18 silica gel (TSK-LS410) in 0.1 M *triethylammonium* acetate.

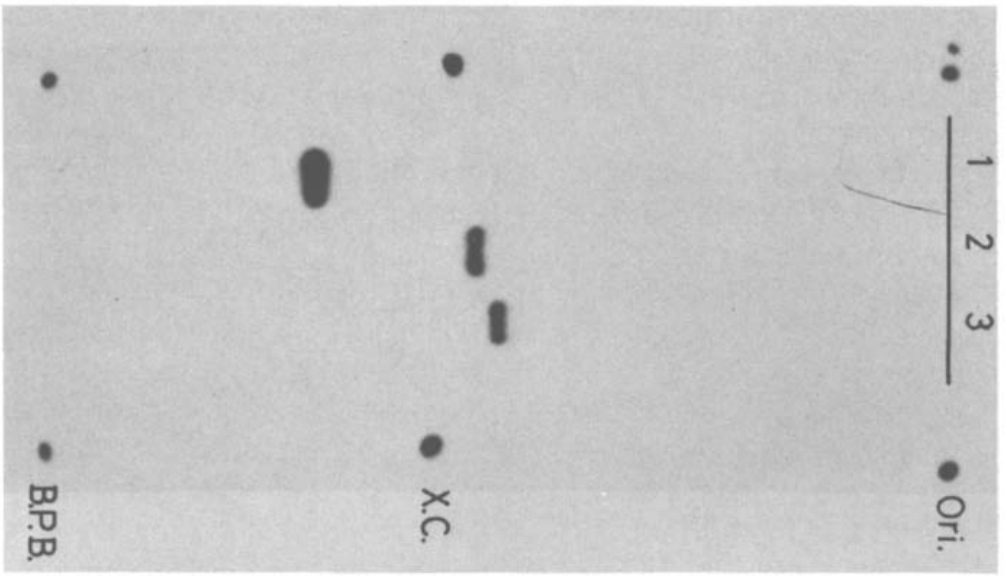


Fig. 8. Gel electrophoresis of the 20 mer (1), 33 mer (2) and a marker (3, 34 mer from the *Ecoli* $tRNA_f^{Met}$) on 10% polyacrylamide.

(0.746 g, 2.96 mmol) at 30° for 15 min. The reaction was checked by TLC and RPTLC. After 25 min water (2 ml) was added and the mixture was concentrated. The residue was dissolved in $CHCl_3$ and washed twice with sat $NaHCO_3$ aq (70 ml). The product (8-2) was isolated by reversed phase chromatography on C-18 silica gel (ϕ 4 × 9.5 cm) using a gradient of acetone in 0.2% pyridine and precipated with hexane from its soln in $CHCl_3$. The yield was 85%, 1.789 g, 1.193 mmol.

Synthesis of the trimers (**9–17**) Table 2. Trimers (**9–15**) were synthesized from **7** by phosphorylation as described for the preparation of **6** followed by condensation N, 2'-protected nucleosides (**1**). For the synthesis of trinucleotides (**16, 17**), nucleotides (**5**) were used. Synthetic procedures were the same described for dimers (**7, 8**) and trimers were isolated by reversed phase chromatography on C-18 silica gel (ϕ 4 × 9 cm) using a gradient of acetone in 0.2% pyridine.

Removal of the 5'-dimethoxytrityl group (Table 3). The trinucleotide (protected GCUp, **17**) (4.723 g, 2.25 mmol) was dried by evaporation three times with pyridine, three times with toluene and shaken with 70 ml of 1 M $ZnBr_2$ in CH_2Cl_2-PrOH (85:15, v/v) for 2.5 min at room temp. A soln of 1 M ammonium acetate (200 ml) was added with shaking and the product was extracted with CH_2Cl_2 (100 ml). The organic soln was washed twice with 1 M ammonium acetate (100 ml) and concentrated. The residue was applied to a column (ϕ 5 × 5.5 cm) of Kieselgel 60 H (40 g). The oligonucleotide was eluted with a gradient of MeOH in $CHCl_3$ and precipated with hexane from its soln in $CHCl_3$. The yield was 68%, 2.728 g, 1.521 mmol.

Condensation of oligonucleotide blocks

(1) *The nonanucleotide* (**41**). The trimer **11** (1.245 g, 0.675 mmol) was phosphorylated by the procedure described for the synthesis of **6**, and condensed with **37**, (3.254 gm 0.646 mmol) using MSTe (4.27 mg, 1.69 mmol) at 30° for 25 min. Completion of the reaction was checked by TLC and RPTLC and the product was isolated by reversed phase chromatography as described for the trimers.

(2) *The undecamer* **40** (*Removal of the anisido group*). The protected **30** (1.817 g, 0.456 mmol) was treated with isoamyl nitrite (3.1 ml, 23 mmol) in pyridine-AcOH (5:4, 14 ml) at 30° for 5 hr. The mixture was added with 0.2 M TEAB (100 ml), pyridine (75 ml), ether–pentane (1:1, 100 ml) on the aqueous phase was washed with ether–pentane (1:1, 100 ml). The product (**35**) was extracted with $CHCl_3$ (140 ml), washed three times with 0.2 M TEAB (100 ml), applied to a column (ϕ 4 × 7 cm) of C-18 silica gel and eluted with a gradient of acetone in 0.2% pyridine. The hexamer **35** was collected, dried by evaporation with pyridine and condensed with **36** (1.348 g, 0.466 mmol) using MSTe (254 mg, 1.01 mmol) at 30° for 40 min. The product was isolated by reversed phase chromatography as above and precipated with pentane. The yield was 49% 1.505 g, 0.223 mmol. The R_f values in TLC (10:1) and RPTLC (7:3) were 0.29 and 0.23, respectively.

(3) *The tridecamer* (**47**). The octamer **45** (0.756 g, 0.155 mmol) and **39** (0.437 g, 0.146 mmol) were condensed using MSTe (0.117 g, 0.464 mmol) at 30° for 55 min and the product was isolated by reversed phase chromatography on a column (ϕ 3 × 8 cm) of C-18 silica gel. The R_f values in TLC (10:1) and RPTLC (8:2) were 0.43 and 0.70, respectively.

(4) *The eicosamer* (**46**). The undecamer **43** and **39** were condensed using conditions shown in Table 4 and **46** was isolated first by reversed phase chromatography on a column (ϕ 4 × 7 cm) of C-18 silica gel. The product **46** was purified by chromatography on a column (ϕ 3 × 2.8 cm) of silica gel (Kieselgel 60 H, 7 g) using a gradient of MeOH in $CHCl_3$. The yield was 22%, 0.481 g, 0.041 mmol. The R_f values in TLC (10:1) and RPTLC (8:2) were 0.35 and 0.62, respectively.

(5) *The tritriacotamer* (**50**). The eicosamer **46** (239 mg, 0.020 mmol) was treated with isoamyl nitrite (0.15 ml, 1.11 mmol) in pyridine-AcOH (5:4, 1.5 ml) at 30° for 5.5 hr. Completion of the reaction was checked by TLC and RPTLC. The eicosamer **48** was extracted with $CHCl_3$-pyridine (2:1, 45 ml), washed 4 times with 0.2 M TEAB (50 ml) and precipated with ether–pentane (1:4, 50 ml) from its soln in $CHCl_3$ (2.5 ml). The eicosamer **48** was reprecipated, dried by evaporation with pyridine and condensed with **49** (150 mg, 0.020 mmol) in pyridine (0.5 ml) using MSTe (27 mg, 0.11 mmol) at 30° for 50 min. Starting materials were detected after 50 min in TLC and RPTLC. The mixture was treated with MSTe (20 mg, 0.079 mmol) for 40 min and added with water (0.5 ml). The product was extracted with $CHCl_3$-pyridine (5:2, 70 ml), washed twice with 0.1 M TEAB (50 ml) and precipated with pentane from its soln in $CHCl_3$. The yield of the crude product was 463 mg.

The deblocked 20 mer. The protected **46** (25 mg, 0.002 mmol) was treated with isoamyl nitrite (0.027 ml, 0.2 ml) in pyridine–AcOH (5:4, 0.3 ml) at 30° for 5 hr and the product was extracted with $CHCl_3$–pyridine (2:1, 30 ml). The eicosamer was precipated with ether–pentane (1:4, 50 ml), treated with 1 M TMG-PAO in dioxane (2 ml) and water (2 ml) at 30° for 60 hr. The volatile materials were removed and the residue was dissolved in pyridine (2 ml). The mixture was treated with conc ammonia (10 ml) at 55°

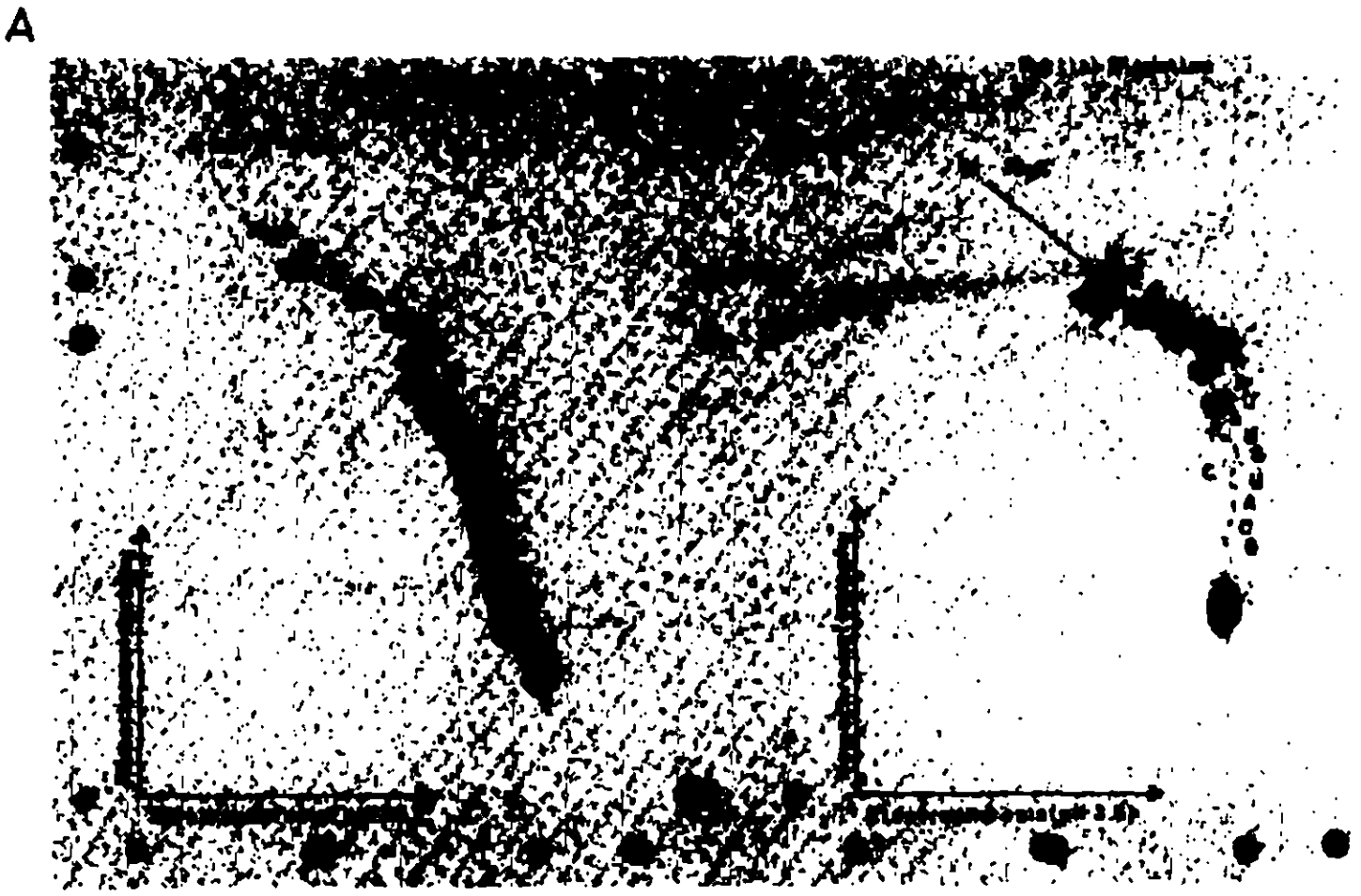


Fig. 9. Mobility shift analysis of the 20 mer (A) and 33 mer (B) using Homo-mix I.[31b]

for 6 hr and concentrated. The product was dissolved in aqueous pyridine (30%, 20 ml) and passed through a column (5 ml) of Dowex 50 W × 2 (pyridinium form). The column was washed with 30% pyridine (100 ml) and combined solns were concentrated. The residue was dissolved in 0.1 M TEAB (50 ml), washed twice with ether (40 ml), evaporated three times with added toluene and mixed with 0.1 N HCl (15 m). The solution was adjusted to pH 2 with 0.1 N HCl, kept at 25° for 9 hr, neutralized with 0.1 M ammonium hydroxide, washed twice with ether (40 ml) and concentrated. The residue was applied to a column of Sephadex G-50 (Fig. 5a) and the product in peak I (272 A_{260}) was

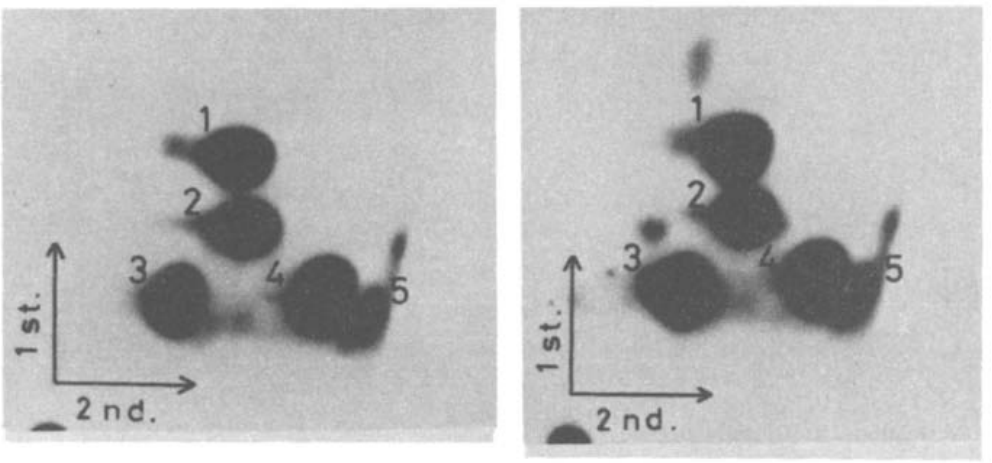


Fig. 10. Two dimensional TLC of mononucleotides obtained by digestion with RNase T2 of the 20 mer (A) and 33 mer (B) Spots: 1, Ap, 2, Cp; 3, Gp; 4, Up; 5, Pi. Solvents: 1st dimension, isobutyric acid-0.5 M ammonium hydroxide (5:3, v/v); 2nd dimension, isopropanol-conc. hydrochloric acid-water (70:15:15, v/v).

collected. An aliquot (6.8 A_{260}) was subjectected to HPLC (TSK LS 410) (Fig. 6) and 1.7 A_{260} units of the pure eicosamer was obtained. The estimated yield from 46 was 16% assuming ϵ of the eicosamer being 20×10^4.

The deblocked 33 *mer* (**51**). The protected 33 mer **50** (crude, 77 mg) was treated with isoamyl nitrite (0.018 ml, 0.6 mmol) in pyridine–AcOH (1:1, 1 ml) at 30° for 6 hr and treated with 0.5 M TMG-PAO (16 ml) using procedures described for the deblocking of the 20 mer. The 33 mer was then treated with conc ammonia (20 ml) at 55° for 6 hr, concentrated and passed through a column of Dowex 50 W × 2 (10 ml, pyridinium form). Acid treatment was performed as described for the 20 mer using 0.1 N HCl (30 ml) and 0.01 N HCl. The product was applied to gel filtration (Fig. 5b) and fractions containing the 33 mer were combined (234 A_{260}). HPLC analysis showed incomplete removal of protecting groups. The product (220 A_{260}) was retreated at pH in HCl (15 ml) at 25° for 8 hr and neutralized. The 33 mer was subjected to gel filtration and a part of the product (fraction No. 65, 18 A_{260}) was further purified by HPLC (TSK-LS 410) as shown in Fig. 6.

REFERENCES

[1]R. Lohrman, D. Soll, H. Hayatsu, E. Ohtsuka and H. G. Khorana, *J. Am. Chem. Soc.* **88**, 819 (1966).

[2]E. Ohtsuka, K. Murao, M. Ubasawa and M. Ikehara, *Ibid. Am. Chem. Soc.* 3445 (1970).

[3]C. B. Reese, *Tetrahedron* **34**, 3143 (1978); E. Ohtsuka, M. Ikehara and D. Soll, *Nucleic Acids Res.* 6553 (1982).

[4]E. Ohtsuka, S. Tanaka, T. Tanaka, T. Miyake, A. F. Markham, E. Nakagawa, T. Wakabayashi, Y. Taniyama, S. Nishikawa, R. Fukumoto, H. Uemura, T. Doi, T. Tokunaga and M. Ikehara, *Proc. Natl Acad. Sci. U.S.A.* **78**, 5493(1981).

[5]D. Soll and P. R. Schimmel, *The Enzymes* **10**, 489 (1974).

[6]J. W. Roberts and J. J. Carbon, *J. Biol. Chem.* **250**, 5530 (1975).

[7]C. G. Kurse, N. L. J. M. Kroekhof and A. van der Gen, *Tetrahedron Letters* 1725 (1976); C. G. Kurse, F. L. Jonkers, V. Dert and A. van der Gen, *Rect. Trav. Chim. Pays-Bas* **98**, 371 (1979).

[8]M. Smith, D. H. Rammler, I. H. Goldberg and H. G. Khorana, *J. Am. Chem. Soc.* **84**, 430 (1962).

[9]E. Ohtsuka, A. Yamane and M. Ikehara, *Nucleic Acids Res.* **11**, 1325 (1983).

[10]M. D. Matteucci and M. H. Caruthers, *Tetrahedron Letters* 3243 (1980). F. Chow, T. Klupe and G. Palm, *Nucleic Acids Res.* **9**, 2807 (1981). R. Kierzek, H. Ito, R. Blatt and K. Itakura, *Tetrahedron Letters* 3761 (1981).

[11]C. B. Reese and J. E. Sulston, *J. Am. Chem. Soc.* **89**, 3366 (1967); C. B. Reese, R. Saffhill and J. E. Sulston, *Tetrahedron*, **26**, 1023 (1970).

[12]K. K. Ogilvie and M. J. Mener, *Can J. Chem.* **58**, 1389 (1980).

[13]E. Ohtsuka, S. Tanaka and M. Ikahara, *Nucleic Acids Res.* **1**, 1351 (1974). Idem *Chem. Pharm. Bull.* **25**, 949 (1977). Idem *Synthesis* 453 (1977).

[14]J. A. J. den Hartog, G. Wille, R. A. Schueblin and J. H. van Boom, *Biochemistry* 21, 1009 (1982).

[15]S. S. Jones, C. B. Reese, S. Sibanda and A. Ubasawa, *Tetrahedron Letters* 4755 (1981).

[16]E. Ohtsuka, K. Fujiyama and M. Ikahara, *Nucleic Acids Res.* **9**, 3503 (1981).

[17]E. Ohtsuka, A. Yamane and M. Ikehara, *Chem. Pharm. Bull.* **30**, 376 (1982).

[18]E. Ohtsuka, A. Yamane and M. Ikahara, *Ibid.* **31**,1534 (1983).

[19]E. Ohtsuka, M. Ohkubo, A. Yamane and M. Ikehara, *Ibid.* **31**, 1910 (1983).

[20]J. H. van Boom, P. M. J. Burgers, P. H. van Deursen, R. Arenzen and C. B. Reese, *Tetrahedron Letters* 3785 (1974).

[21]G. M. Tener, *J. Am. Chem. Soc.* **83**, 159 (1961). R. L. Letsinger and K. K. Ogilvie, *Ibid.* **89**, 4801 (1967).

[22]E. Uhlmann and W. Pfleiderer, *Tetrahedron Letters* 1181 (1980). W. Pfleiderer, E. Uhlmann, R. Charubala, D. Flocerzi, G. Silber and R. S. Varma, *Nucleic Acids Res.* Symp. Ser. **7**, 61 (1980).

[23]H. Takaku, M. Kato and T. Hata, *Chem. Letters* **8**, 873 (1975).

[24]K. Itakura, N. Katagiri, C. P. Bahl and S. A. Narang, *J. Am. Chem. Soc.* **97**, 7327 (1975).

[25]J. C. Catlin and F. Cramer, *J. Org. Chem.* **38**, 245 (1973); J. H. van Boom and P. M. J. Burgers, *Recl. Trav. Chim. Pays-Bas*, **97**, 73 (1978).

[26a]E. Ohtsuka, K. Fujiyama, T. Tanaka and M. Ikehara, *Chem. Pharm. Bull.* **29**, 2799 (1981); [b]E. Ohtsuka, Y. Taniyama, R. Marumoto, H. Sato, H. Hirosaki and M. Ikehara, *Nucleic Acids Res.* **10**, 2597 (1982).

[27]C. Broka, T. Hozumi and Arenzene, K. Itakura, *Ibid.* **8**, 5461 (1980).

[28]J. Stawinski, T. Hozumi, S. A. Narang, C. B. Bahl and R. Wu, *Ibid.* **4**, 353 (1977).

[29]C. B. Reese, R. Titmas and L. Yau, *Tetrahedron Letters* 2727 (1978).

[30]T. Maniatis, A. Jeffrey and H. van de Sande, *Biochemistry* **14**, 3787 (1975).

[31a]F. Sanger, G. G. Brownlee and B. G. Barrell, *J. Mol. Biol.* **13**, 373 (1965); [b]E. Jay, R. Bambara, R. Padmanabham and R. Wu, *Nucleic Acids Res.* **1**, 331 (1974).

[32]C. C. Richardson, *Proc. Natl Acad. Sci. U.S.* **54**, 158 (1965).

[33]T. E. England and O. C. Uhlenbeck, *Nature* **275**, 561 (1978).

[34]F. Egami, K. Takahashi and T. Uchida, *Progress in Nucleic Acid Res. Mol. Biol.* **3**, 59 (1964).

[35]S. Nishimura, *Ibid.* **12**, 50 (1972).

[36]E. Ohtsuka, S. Tanaka and M. Ikehara, *J. Am. Chem. Soc.* **100**, 8210 (1978).