Modified phosphotriester method for chemical synthesis of ribooligonucleotides. Part I. Synthesis of riboundecaadenylate and two fragments constituting the sequence of R-17 translation control signal

WING L. SUNG AND SARAN A. NARANG

Division of Biological Sciences,[1] National Research Council of Canada, Ottawa, Ont., Canada K1A 0R6

Received August 4, 1981

WING L. SUNG and SARAN A. NARANG. Can. J. Chem. **60**, 111 (1982).

A modified phosphotriester method has been successfully applied for the chemical synthesis of ribooligonucleotides. The starting material is a fully protected ribomononucleoside containing a 3′-phosphotriester group **5**. The coupling reaction is performed using mesitylenesulfonyl tetrazole and purification of the product achieved using reversed phase column chromatography. The effectiveness of this method has been demonstrated by achieving an efficient and rapid synthesis of r-A_7, r-A_{11}, r5′-AAACAUGAGGA-3′, and r5′-UUACCCAUGU-3′ (R-17, translation control sequence).

WING L. SUNG et SARAN A. NARANG. Can. J. Chem. **60**, 111 (1982).

On a appliqué avec succès une méthode de phosphotriester modifié à la synthèse chimique des ribooligonucléotides. On utilise comme produit de départ un ribonucléoside entièrement protégé contenant un groupe phosphotriester en position 3′(**5**). On a réalisé le couplage en utilisant le mésitylène sulfonyle tétrazole et on a purifié le produit par chromatographie sur colonne en phase inversée. On démontre l'efficacité de cette méthode en réalisant une synthèse rapide et efficace du r-A_7, r-A_{11}, r5′-AAACAUGAGGA-3′ et du r5′-UUACCCAUGU-3′ (R-17, unité de contrôle de translation).

[Traduit par le journal]

Introduction

The availability of ribooligonucleotides of well-defined sequence is of great importance in the biological studies of various RNA-regulatory signals, understanding the splicing mechanism of introns and the synthesis of biologically active RNA molecules such as t-RNA or 5S RNA. The recent discovery that T_4-RNA ligase enzyme (1) can join small fragments of RNA end to end without their complementary strand offers an additional impetus for the development of an efficient and rapid method of chemical synthesis. In spite of these incentives, progress in chemical synthesis of ribooligonucleotides has lagged behind deoxy synthesis (2). Although the phosphodiester approach was successfully applied by Khorana and coworkers (3) to synthesize all 64 triplet RNA codons, its application to the synthesis of longer sequences is quite laborious (4). Even use of the phosphotriester approach (5), the phosphite modification (6), or solid phase techniques (7) have only very recently proved capable of synthesizing defined sequences of oligoribonucleotides (8–11) with length of 9–16 units, that are of biological interest.

The main difficulty is the presence of an additional 2′-hydroxyl group, the protection of which presents the most critical problems at all stages of ribooligonucleotide synthesis. At the monomer stage, specificity between the 2′- and 3′-hydroxyls must be achieved; any protecting group must be stable enough to remain intact throughout an extended synthesis yet be complementary in its chemical behaviour, i.e. for selective removal to other protecting groups. Increasingly effective phosphorylation/condensation conditions involving the 3′-hydroxyl must be devised to overcome the additional steric hindrance from the protected 2′-hydroxyl group. Finally deprotection must occur under conditions which prevent any 3′ to 2′ migration of the internucleotidic phosphodiester linkages. The most commonly utilized 2′-hydroxyl protecting groups are substituted tetrahydropyran (9) and *O*-nitrobenzyl (12), but their introduction requires multiple chemical steps, whereas *tert*-butyldimethyl silyl (TBDMS), a bulkier protecting group, has been employed by Ogilvie *et al* (13). The main advantages of this protecting group are its ease of protecting the 2′-hydroxyl function and also stability towards acidic and basic conditions in order to avoid migration and cleavage of 3′ → 5′ phosphodiester linkages.

In this study we present a practical solution to the ribopolynucleotide synthesis by employing the modified phosphotriester approach (14) developed in our laboratory for the deoxy series. The basic feature of our approach is to start the synthesis of an oligonucleotide from the fully protected ribomononucleoside containing the 3′-phosphotriester group **5**. Since each intermediate oligonucleotide synthesized thus contained the fully masked 3′-phosphate group, the necessity for a phosphorylation step at each condensation stage was eliminated.

[1]NRCC No. 19384.

0008-4042/82/020111-10$01.00/0

Rapid condensations and simple chromatographic purification of intermediates make this approach comparable in speed to polymer support or phosphite techniques with additional advantages of simplicity in handling the large-scale synthesis. In this paper we wish to demonstrate its application in the efficient synthesis of r-A_7, r-A_{11}, r-AAACAUGAGGA, and r-UUAACCAUGU fragments. The heterosequence fragments constitute a natural sequence of the R-17 translation control signal (15) (Fig. 1). The sequence of each synthetic fragment has been analyzed by the mobility shift method (16) and the radioaudiograms are shown in Fig. 5.

Discussion

Large-scale preparation of fully protected ribomononucleoside 3′-phosphotriester **5**

In our present studies we chose the *tert*-butyldimethyl silyl protecting group (TBDMS) for the 2′-hydroxyl which was easily prepared by treating **1** with *tert*-butyldimethyl silyl chloride and imidazole (13); a mixture of 2′- and 3′-monoprotected isomers was produced. Previously, these isomers were purified by preparative thin layer chromatography (13) which would not be suitable for any large-scale synthesis because of economic and time factors. We have effectively solved this problem using short silica gel column chromatography to collect a pure fraction containing the faster moving 2′-protected isomer. The remaining mixture was eluted from the column and further equilibrated to a mixture of 2′- and 3′-isomers with triethylamine–methanol. On subsequent chromatography, more of the pure 2′-protected isomer was isolated. Repetition of this sequence three times produced pure 2′-protected isomer (25–30 g, 45–64% isolated yield) in less than 7 h. The general method of preparation is outlined in Fig. 2.

The presence of *tert*-butyldimethyl silyl, a bulkier protecting group, at the 2′-position makes the 3′-hydroxyl function less accessible to the phosphorylation reaction due to steric hindrance. Consequently, phosphorylation with *p*-chlorophenyl phosphoryl ditriazolide (17) in dioxane with a stoichiometric amount of triethylamine was found to be slow (overnight) and migration of the 2′-protecting group to the 3′-hydroxyl was also observed under these conditions, possibly due to the presence of residual triethylamine. Obviously, a more powerful phosphorylating reagent was required to phosphorylate the 3′-position quantitatively in a minimum time period to avoid possible migration of the 2′-silyl protecting group. We next attempted phosphorylation with *p*-chlorophenyl phosphoryl ditetrazolide (Fig. 3). As expected, the phosphorylation reaction was over in less than 5 min without any migration and on subsequent addition of β-

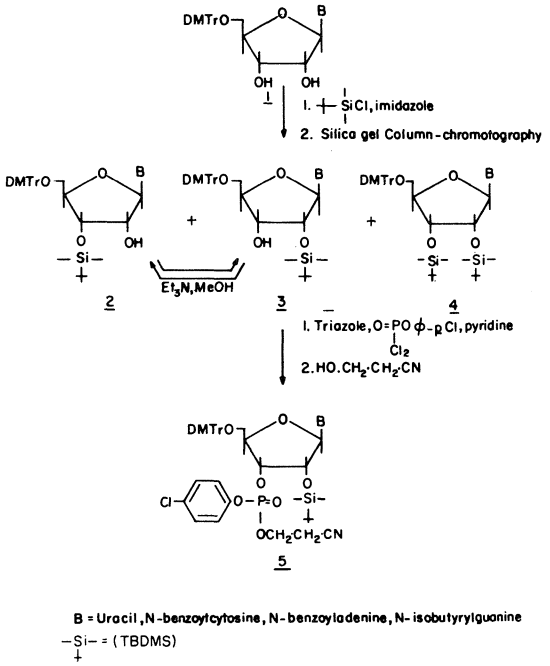

FIG. 2. General method of preparation of fully protected ribomononucleoside containing 3′-phosphotriester group.

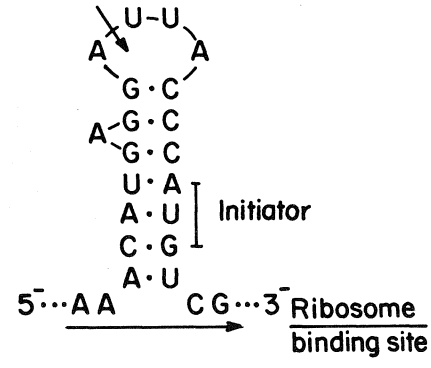

FIG. 1. Sequence of R-17 translational control region.

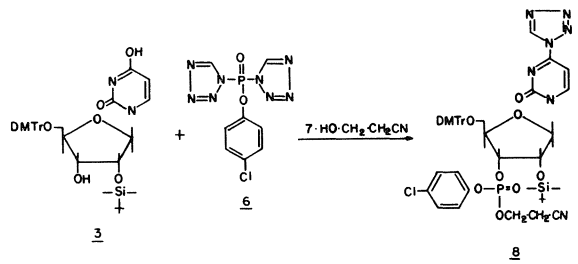

FIG. 3. Modification of uridine moiety.

cyanoethanol, a fully protected ribomononucleotide was prepared and isolated by short silica gel column chromatography in about 70–80% yield. However, in the case of 5′,2′-protected uridine, we observed an unexpected maxima of 324 nm in uv spectra analysis in addition to the regular maximum at 259 nm. Mass spectrometric analysis by the plasma absorption technique (18) also showed an unexpected increase of 52 units in the mass value, which is equivalent to addition of a tetrazole moiety.[2] On complete deblocking the unprotected nucleotidic material had a uv spectra identical to cytidine. Obviously, *p*-chlorophenyl phosphoryl ditetrazolide reagent has introduced a tetrazole moiety at the 4-position of uridine as shown in Fig 3. Subsequently, we discovered that Reese and Ubasawa (19) have also observed a similar modification on prolonged treatment with mesitylenesulfonyl tetrazolide and diphenylphosphate. We overcame this problem by modifying our original phosphorylation procedure by treating 5′-dimethoxytrityl ribomononucleoside 2′-*tert*-butyldimethyl silyl protected compound **3** with *p*-chlorophenyl phosphodichloridate in the presence of excess triazole in pyridine[3] solution *in situ*. Under these conditions, the phosphorylation reaction at the 3′-position was complete in less than 5 min and on subsequent treatment with an excess of β-cyanoethanol the fully protected desired mononucleotide **5** was prepared in 70–80% yield. Under these reaction conditions, no modification and migration were observed. Each of the four fully protected monoribonucleotides in 20–30 g amounts was easily purified on a short reversed phase silanized silica gel column and stored as a dry foamy material at 4°C.

Modified procedure for amino group protection of cytidine

Generally, *N*-protection in the case of cytidine, adenosine, and guanosine involves the complete acylation of all the hydroxyl as well as amino functions followed by partial deacylation with sodium hydroxide to generate *N*-protected compound. In the case of cytidine, we have developed a "one-pot" method in which the hydroxyl groups are first silylated on treatment with trimethylsilylchloride followed by the addition of benzoyl chloride *in situ* to benzoylate the amino function. The reaction mixture was decomposed with water–dichloromethane followed by evaporation *in vacuo*. The *O*-silyl groups are hydrolyzed by the benzoic acid produced *in situ* to yield a pure *N*-benzoylated cytidine in quantitative yield. Unfortunately, similar approaches to produce *N*-protected adenosine and guanosine were not successful.

Di-isopropylamine as a more efficient reagent for the selective removal of the β-cyanoethyl group from **5**

In our synthetic studies of deoxyribooligonucleotides, it became apparent that a most time-consuming part is the decyanoethylation of the 3′-phosphotriester group **5** on treatment with triethylamine–pyridine (20) (pyrimidine bases required 4 h and purines 2 h). Even in the case of dimer or trimer blocks, the rate of removal of the β-cyanoethyl group was dependent on the nature of the 3′-terminal base. In order to improve on this aspect we examined a variety of bases and found a more suitable base, di-isopropylamine, with pK_a 11.05 compared to triethylamine (pK_a 10.05) which in anhydrous pyridine removes the β-cyanoethyl group in less than 1 h in both the deoxy- and ribo-series. Under this condition, *N*-acyl and *p*-chlorophenyl at the internucleotide phosphate groups were found to be *completely intact* as checked by tlc. The results of these comparative studies are summarized in Table 1.

Modified phosphotriester approach for oligoribonucleotide synthesis

The basic strategy of this approach is to start the synthesis from a fully protected ribomononucleoside 3′-phosphotriester **5** as outlined in Fig. 4. The chain was elongated from the 3′- towards the 5′-end by using the 2′,3′-diTBDMS derivative as the 3′-end. Mesitylenesulfonyl tetrazole (21) was used routinely for 15 min as a coupling reagent. One of the major problems in the phosphotriester ap-

TABLE 1. Decyanoethylation of 5′-dimethoxytrityl 2′-*tert*-butyldimethyl silyl *N*-protected ribonucleoside 3′-*p*-chlorophenyl β-cyanoethyl phosphate **5** with di*iso*propylamine

Mononucleotide[a]	Triethylamine (h)	Di*iso*propylamine (h)
[$(MeO)_2$]Tr-dbzA∓CE	3	0.6
[$(MeO)_2$]Tr-dIsoG∓CE	2	0.5
[$(MeO)_2$]Tr-dbzC∓CE	4	0.6
[$(MeO)_2$]Tr-dT∓CE	4	0.6
[$(MeO)_2$]Tr-rbzASi∓CE	4	0.8
[$(MeO)_2$]Tr-rIsoGSi∓CE	4	0.6
[$(MeO)_2$]Tr-rbzCSi∓CE	4	0.8
[$(MeO)_2$]Tr-USi∓CE	4	0.8

[a]Concentration of mononucleotide (0.1 mmol) in an appropriate amine (0.5 mL) and pyridine (2 mL). The course of reactions was followed by tlc on silanized silica gel using acetone containing 30% water as solvent.

[2]Thanks to C. J. McNeal, Texas A & M University, for performing this experiment.

[3]Gait *et al.* (ref. 2, p. 251) have reported a variation of this reaction condition in which *p*-chlorophenyl phosphorodichloridate was mixed with anhydrous triazole in pyridine solution for 20 min separately and then treated with 5-O′-protected deoxynucleoside.

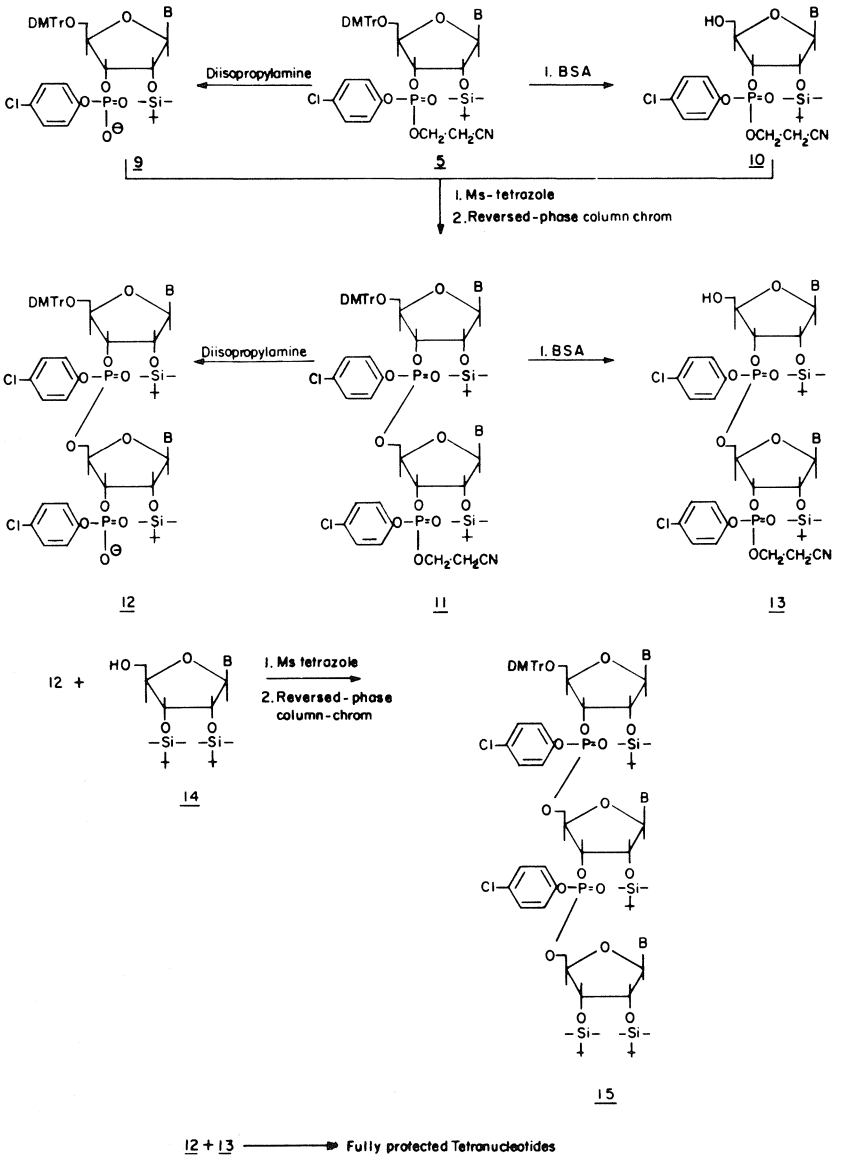

FIG. 4. General method of synthesis by the modified phosphotriester approach.

proach of synthesis is isolation of the pure desired product. In the case of ribooligonucleotide synthesis, the addition of the TBDMS protecting group at each 2′-position causes serious streaking problems in silica gel chromatography. We overcame this problem by developing a short reversed phase column chromatography using an acetone–water solvent system which has the following advantages: (*i*) separation of reaction mixture components is achieved according to the size, i.e. desired product, usually the largest size fragment is the slowest moving component; (*ii*) a 50 mg–10 g amount can be purified easily in less than 1 h time period; and, finally, (*iii*) recovery is quantitative. The reaction conditions and their isolated yields are given in Table 2.

Final deblocking steps

The sequential removal of all protecting groups is one of the most difficult as well as critical steps in the successful development of a chemical synthetic approach. Our strategy included initial removal of the 5′-dimethoxytrityl group with 2% benzenesulfonic acid in chloroform–methanol (22), followed by a two-step concentrated ammonia treatment (23) to remove *N*-protecting groups as well as

TABLE 2. Reaction conditions and isolated yield of various ribooligonucleotides synthesized by the modified phosphotriester approach

5′-Protected component[a] (mmol)	3′-Hydroxyl component[b] (mmol)	Condensing[c] reagent (mmol)	Time (min)	Product[d,e] (isolated yield %)
	A. r-A_{11}			
$[(MeO)_2]$rbzA∓bzA-ClPh (0.25)	rbzA-$(OSi)_2$ (0.20)	0.50	15	$[(MeO)_2]$rbzA∓bzA∓bzA-$(OSi)_2$ (70)
$[(MeO)_2]$rbzA$(∓bzA)_2$∓A-ClPh (0.20)	rbzA∓bzA∓bzA-$(OSi)_2$ (0.13)	0.50	15	$[(MeO)_2]$rbzA$(∓bzA)_5$∓bzA-$(OSi)_2$ (45)
$[(MeO)_2]$rbzA$(∓bzA)_2$∓A-ClPh (0.08)	rbzA$(∓bzA)_5$∓A-$(OSi)_2$ (0.04)	0.20	15	$[(MeO)_2]$rbzA$(∓bzA)_9$∓A-$(OSi)_2$ (35)
	B. r-AAACAUGAGGA (11-mer)			
$[(MeO)_2]$rIsoG∓IsoG-ClPh (0.24)	rbzA-$(OSi)_2$ (0.20)	0.50	15	$[(MeO)_2]$rIsoG∓IsoG∓bzA-$(OSi)_2$ (55)
$[(MeO)_2]$rbzA∓U∓IsoG∓bzA-ClPh (0.15)	rIsoG∓IsoG∓bzA-$(OSi)_2$ (0.10)	0.30	15	$[(MeO)_2]$rbzA∓U∓IsoG∓bzA∓IsoG∓ IsoG∓bzA-$(OSi)_2$ (45)
$[(MeO)_2]$rbzA∓bzA∓bzA∓bzC-ClPh (0.05)	rbzA∓U∓IsoG∓bzA∓IsoG∓ IsoG∓bzA-$(OSi)_2$ (0.03)	0.15	15	$[(MeO)_2]$rbzA∓bzA∓bzA∓bzC∓bzA∓U∓ IsoG∓bzA∓IsoG∓IsoG∓A-$(OSi)_2$ (30)
	C. r-UUACCCAUGU (10-mer)			
$[(MeO)_2]$rIsoG-ClPh (0.24)	rU-$(OSi)_2$ (0.18)	0.45	15	$[(MeO)_2]$rIsoG∓U-$(OSi)_2$ (60)
$[(MeO)_2]$rbzC∓bzC∓bzA∓U-ClPh (0.13)	rIsoG∓U-$(OSi)_2$ (0.09)	0.28	15	$[(MeO)_2]$rbzC∓bzC∓bzA∓U∓IsoG∓U-$(OSi)_2$ (50)
$[(MeO)_2]$rU∓U∓bzA∓bzC-ClPh (0.06)	rbzC∓bzC∓bzA∓U∓IsoG∓U-$(OSi)_2$ (0.04)	0.14	15	$[(MeO)_2]$rU∓U∓bzA∓bzC∓bzC∓bzC∓ bzA∓U∓IsoG∓U-$(OSi)_2$ (33)

[a]Abbreviations are as suggested by IUPAC-IUB (1970) Biochemistry 9, 4022. A phosphodiester linkage is represented by a hyphen and phosphotriester linkage is represented by a (∓) symbol. Each internucleotidic phosphate is protected by a *p*-chlorophenyl (ClPh) group and each 2′-hydroxyl of each nucleoside is protected by a *tert*-butyldimethyl silyl (TBDMS) group.
[b]The 2′- and 3′-*cis*-hydroxyl groups of the 3′-terminal residue are protected by two *tert*-butyldimethyl silyl (TBDMS) groups.
[c]Mesitylenesulfonyl tetrazole (MS-Tet).
[d]Pure fully protected product was isolated by short column RP-2 chromatography.
[e]Using mesitylenesulfonyl tetrazole, in 2 to 3-fold equivalents, the sulfonated side product was obtained in 5–10% yields.

p-chlorophenyl from the internucleotidic phosphotriester bonds.[4] Under these conditions, the *tert*-butyldimethyl silyl group is still intact at every 2′-position. After washing with ether the partially deblocked material was then evaporated to dryness *in vacuo* and the dry powdery residue was treated with an excess of tetrabutylammonium fluoride in tetrahydrofuran under anhydrous conditions at room temperature. The time required to deblock all the silyl groups is mainly dependent upon the size of the fragment (dimer, 30 min; undecamer, 4 h). After evaporation, the residual material was applied, and excess salt from the applied band was washed with spray of methanol and water. The isolated yield of the desired pure material after total deblocking was 70–80%. The main side products were degradation products formed during the deblocking procedure.

Characterization

Unprotected ribooligonucleotides containing 3′ → 5′ phosphodiester linkages

(a) *Confirmation of the presence of 3′ → 5′ phosphodiester linkages*

Of paramount importance in ribo-synthesis is the proof of the preservation of each internucleotidic phosphodiester linkage as a 3′ → 5′ bond after all chemical reactions. The presence of each 3′ → 5′ phosphodiester linkage was established as follows.

Each ribooligonucleotide was labeled at the 3′-end with [^{32}P]Cp using T_4-RNA ligase (2). The labeled compound was purified on 20% gel electrophoresis which on treatment with spleen phosphodiesterase enzyme completely digested the labeled material to a radioactive monomer thus establishing that each linkage of the phosphodiester band is indeed 3′ → 5′.

(b) *Sequence analysis*

Each of the ribooligomers was labeled with [γ-^{32}P]-ATP with T_4-polynucleotide kinase and the labeled compound was sequenced by the mobility shift method (16) after partial digestion with snake venom phosphodiesterase or nuclease P_1-enzyme (24), and the results of each sequencing experiment are shown in Fig. 5.

[4]We also carried the deblocking procedure as recommended by de Rooij *et al.* (26) which involved, first, treatment with concentrated ammonia to remove the *p*-chlorophenyl group from the internucleotidic phosphotriester and *N*-acyl groups, followed by acidic treatment to remove the 5′-dimethoxytrityl group. After removing all the TBDMS groups from the 2′-positions, we compared the separation patterns on 20% gel electrophoresis with our original procedure (21) and found that these were almost comparable in terms of yields of the desired product and various side products.

Conclusion

In the present studies, we have demonstrated that the rapid and efficient synthesis of ribooligonucleotides can be achieved by a modified phosphotreister approach, comparable to the phosphotriester (5) as well as the phosphite route (6).

Experimental

General methods and materials

Uridine, cytidine, adenosine, and guanosine (Sigma), *tert*-butyldimethyl chlorosilane (TBDMS), diphenyl-*tert*-butyl chlorosilane, triphenyl chlorosilane, tetrabutylammonium fluoride (Aldrich), *p*-chlorophenyl phosphodicloridate (Alfa-Ventron), RP-2 silanized silica gel (Brinkman), T_4-RNA ligase (RNAse free) and nuclease-P_1 enzyme (P-L Biochemical), and [^{32}P]Cp (Amersham) were purchased commercially.

5′-Dimethoxytrityl *N*-benzoyl adenosine, 5′-dimethoxytrityl *N*-isobutyryl guanosine, 5′-dimethoxytrityl uridine, and 5′-dimethoxytrityl *N*-benzoylcytidine were prepared according to the published procedures (3).

Solvent systems

The homogeneity of each fully protected compound was established by reversed phase (RP-2) tlc using acetone containing 20–30% water (v/v).

Modified method of N-*benzoylcytidine preparation*

Chlorotrimethylsilane (420 g, 3.9 mol) was added, with stirring, to a solution of cytidine (100 g, 0.41 mol) in pyridine (1.7 L) and kept for 1 h at room temperature. This was followed by the addition of benzoyl chloride (72 g, 0.52 mol). After 1 h, the reaction mixture was decomposed by adding a mixture of water and dichloromethane (700 mL each). The organic layer was separated and the removal of solvent from the organic extract *in vacuo* gradually yielded white crystals which were resuspended in dichloromethane (500 mL) overnight. The crystals were filtered, washed with acetone, and on drying *in vacuo* yielded pure *N*-benzoylcytidine (133 g) in 93% yield which was identical to an authentic sample (mp 234–236°C, lit. (25) mp 238–239°C).

Large-scale silylation of 5′-dimethoxyribonucleoside

Each of the ribonucleosides was prepared by the modification of the published procedure (13) as described below.

*5′-Dimethoxytrityl 2′-*tert-*butyldimethyl silyl* N-*benzoylcytidine* ***3***

5′-Dimethoxytrityl *N*-benzoylcytidine (50 g, 77 mmol) in pyridine solution (150 mL) was treated with *tert*-butyldimethyl chlorosilane (17 g, 113 mmol) and imidazole (15 g, 220 mmol) at room temperature. After 4 h, the reaction mixture was decomposed with water (200 mL) and dichloromethane (350 mL). The aqueous layer was extracted with dichloromethane (100 mL × 2) which was mixed with the organic layer. Evaporation of solvent *in vacuo* yielded a gum which was dissolved in ether (100 mL) and precipitated by pouring slowly into petroleum ether (40–60°C, 800 mL) with stirring. The precipitate was collected and washed twice with petroleum ether, then was dissolved in 30% petroleum ether – dichloromethane and chromatographed on silica gel (160 g) (short column of 6 cm diameter). Elution of column with (*i*) dichloromethane containing 30% petroleum ether (40–60°C, 400 mL) removed the 2′,3′-disilylated derivative (2.0 g); (*ii*) dichloromethane solvent (500 mL) eluted pure 2′-silylated isomer (30 g); and, finally, (*iii*) dichloromethane containing 8% acetone (2 L) removed the 3′-silylated isomer contaminated with the 2′-isomer (16 g).

The last fraction containing mainly the 3′-*tert*-butyldimethyl

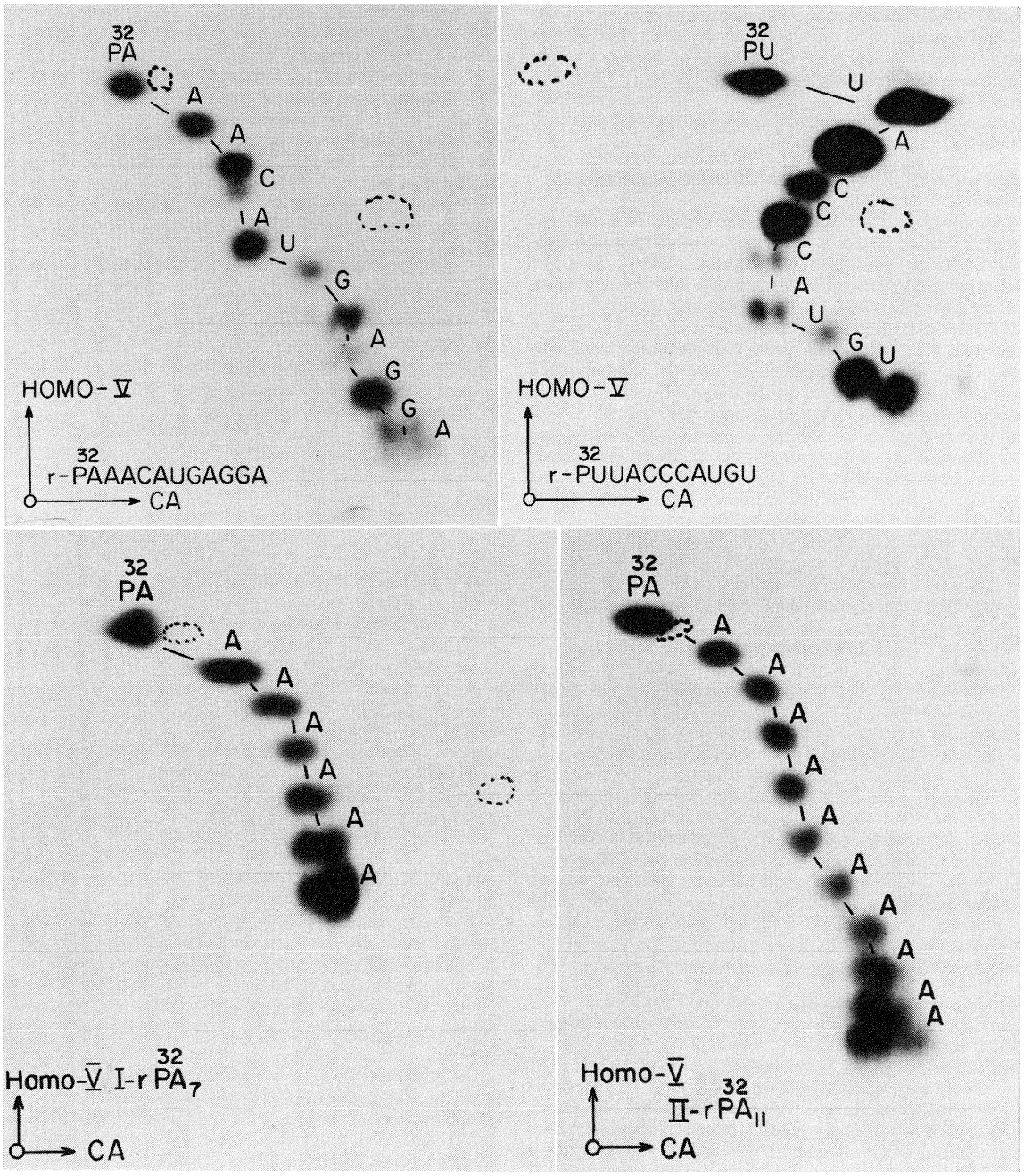

FIG. 5. Two-dimensional chromatographic fingerprints of synthetic ribooligonucleotides after partial digestion with nuclease P_1-enzyme. The first dimension is electrophoresis on cellulose acetate strip of pH 3.5 and the second dimension is homochromatography on 20 cm × 20 cm DEAE-cellulose plate in solvent Homo-V.

silylated derivative (16 g) was treated with triethylamine (0.5 mL) in methanol (200 mL) for 40 min at room temperature. On removing the solvent *in vacuo*, a gum was produced which was again chromatographed on a silica gel column as described above to yield a further 9 g of the 2′-protected isomer. The final isolated yields were the 2′-isomer, 39 g (66%); the 3′-isomer, 4.5 g (8%); and the 2′,3′-disilylated product, 2.0 g (4%). The structure of the 2′-protected isomer was confirmed by comparing its nmr spectral data with the literature values (13).

*5′-Dimethoxytrityl 2′-*tert*-butyldimethyl silyl uridine*

5′-Dimethoxytrityluridine (50 g, 93 mmol) in pyridine solution (150 mL) was treated with *tert*-butyldimethyl chlorosilane in the presence of imidazole according to conditions similar to those

described in ref. 13. Purification by silica gel (160 g) chromatography and subsequent isomerization of the 3′-silylated isomer and repeated chromatography (three times) as described above yielded the pure 2′-*tert*-butyldimethyl silyl isomer, 31 g (50%) and the 3′-isomer, 9 g (15%). The structure of the 2′-protected isomer was confirmed by comparing its nmr spectral data with the published values (13).

*5′-Dimethoxytrityl 2′-*tert*-butyldimethyl silyl N-benzoyladenosine*

5′-Dimethoxytrityl *N*-benzoyladenosine (50 g, 74 mmol) was silylated as described above. The reaction mixture was chromatographed on a silica gel (160 g) short column using the following solvent systems: (*i*) ethyl acetate – dichloromethane – petroleum ether (40–60°C) (1:1:3 v/v) (500 mL); (*ii*) ethyl acetate – dichloromethane – petroleum ether (40–60°C) (1:1:2 v/v) (2 L); (*iii*) acetone (800 mL). The pure 2′-protected isomer was isolated (12 g) in fraction number (*ii*) and the contaminated 2′-protected isomer (6 g) with the 3′-protected isomer (18 g) in fraction number (*iii*). Subsequent isomerization of the 3′-protected isomer fraction containing contaminated 2′-protected isomer followed by rechromatography on silica gel column under the conditions described above yielded further amounts of the pure 2′- and 3′-silylated isomers. After two cycles of isomerization and silica gel column chromatographic purification, the overall yield of the 2′-silylated protected isomer was 26 g (45%) and a slightly contaminated 3′-silylated isomer, 9 g (16%). The structure of the 2′-protected isomer was confirmed by comparing its nmr spectral data with the literature (13).

*5′-Dimethoxytrityl 2′-*tert*-butyldimethyl silyl N-isobutyrylguanosine*

5′-Dimethoxytrityl *N*-isobutyrylguanosine (50 g, 76 mmol) was silylated according to the procedure described above. Purification by column chromatography on silica gel with the following solvent systems: (*i*) petroleum ether – dichloromethane (1:1 v/v) (300 mL); (*ii*) dichloromethane (500 mL); (*iii*) dichloromethane containing 4% acetone (2 L); and, finally, (*iv*) acetone, yielded the pure 2′-silylated isomer (10 g) in fraction number (*ii*) and a mixture of the 2′-protected isomer (6 g) and the 3′-protected isomer (23 g) in fraction number (*iii*). After three cycles of isomerization and purification by silica gel column chromatography under the above conditions, the overall yields were 2′-protected isomer, 24.5 g (42%), and a slightly contaminated 3′-isomer, 10 g (17%). The structure of the 2′-protected isomer was confirmed by comparison with literature values (13).

*Attempted phosphorylation of 5′-dimethoxytrityl 2′-*tert*-butyldimethyl silyl uridine with p-chlorophenyl phosphoryl ditetrazolide*

The 5′-dimethoxytrityl 2′-*tert*-butyldimethyl silyl uridine (1.0 g, 1.5 mmol) and tetrazole (0.30 g, 4.8 mmol) in anhydrous pyridine (3 mL) was treated with *p*-chlorophenyl phosphodichloridate (0.59 g, 2.4 mmol). After 20 min at room temperature, β-cyanoethanol (0.8 g, 12 mmol) was added and kept for a further 2 h to complete the reaction. The reaction mixture was decomposed by adding a mixture of water and dichloromethane (30 mL each), then the organic layer was separated and washed with 5% aqueous sodium bicarbonate (30 mL). Evaporation *in vacuo* yielded a gummy residue which was purified on reversed phase (RP-2) (11 g) short column chromatography using the following solvents: (*i*) acetone–water (50:50 v/v) (250 mL) to remove excess β-cyanoethanol and other non-triester components; (*ii*) acetone–water (58:42 v/v) (150 mL) to elute the fully protected product. Extraction of solvent (*ii*) with dichloromethane (50 mL) and subsequent evaporation gave a mixture of two fully protected phosphotriester nucleotide compounds which were further fractionated on preparative silica gel tlc plates developed in dichloromethane–acetone (9:1 v/v) into two bands. Elution of the faster moving band with methanol–dichloromethane (3:7 v/v) and evaporation *in vacuo* yielded 4-tetrazolo-1-(β-D-5′-dimethoxytrityl-2′-*tert*-butyldimethyl silyl ribofuranosyl)-2(1*H*)-pyrimidinone 3′-*p*-chlorophenyl β-cyanoethyl phosphotriester **8** as a foamy material, 0.42 g (35%). The structure was confirmed by its ultraviolet spectra (methanol), λ_{max}: 324 nm; nmr ($CDCl_3$) δ: 9.44 (1H, s, H(5) of tetrazole), 2.48 (2H, m, $OCH_2CH_2{\cdot}CN$), 0.92 (9H, s, $(CH_3)_3CSi$). The molecular ion (M^+) occurs at 957 m^+/e which is an increase of 52 mass units over the corresponding uridine derivative and corresponds to the addition of the tetrazole moiety (18).

On complete deblocking, the resulting product has uv spectra characteristic of cytosine.

Phosphorylation with p-chlorophenyl phosphodichloridate and triazole

(i) *Preparation of 5′-dimethoxytrityl 2′-*tert*-butyldimethyl silyl N-benzoyl cytidine-3′-p-chlorophenyl β-cyanoethyl phosphotriester 5*

1,2,4-Triazole (10.0 g, 145 mmol) was added to a pyridine solution (65 mL) containing the 2′-*tert*-butyldimethyl silylated cytidine **3** (33.0 g, 43.3 mmol) at room temperature. *p*-Chlorophenyl phosphodichloridate (17.7 g, 72 mmol) was then added immediately and the reaction mixture stirred for 5 min to complete the phosphorylation reaction as checked by tlc on silica gel and RP-2 plates. β-Cyanoethanol (25 g, 356 mmol) was then added and after a further 3 h at room temperature the reaction mixture was decomposed by adding water (200 mL). The solution was extracted with dichloromethane (500 mL) and the organic layer was washed with 5% aqueous sodium bicarbonate solution (100 mL). Evaporation of solvent *in vacuo* gave a gummy residue which was purified by RP-2, short column chromatography (130 g) using solvents (*i*) acetone–water (50:50 v/v) (1.5 L) to remove excess β-cyanoethanol, followed by (*ii*) acetone containing 45% water (2 L) to elute the fully protected desired product. The desired product was recovered by extracting the solvent (*ii*) with dichloromethane (500 mL), drying, and removing the solvent to produce a foamy material, 31.2 g (71%); uv (MeOH) λ_{max}: 262, 305 nm; nmr ($CDCl_3$) δ: 2.62 (2H, m, $CH_2{\cdot}CH_2{\cdot}CN$), 0.97 (9H, s, $(CH_3)_3CSi$—). *Anal.* (foamy material) calcd. for $C_{54}H_{26}N_4O_{11}ClPSi$: C 61.99, H 5.60, N 5.56; found: C 61.90, H 5.57, N 5.42.

The corresponding fully protected ribonucleoside 3′-*p*-chlorophenyl, and the β-cyanoethyl phosphotriester of uridine, *N*-benzoyladenosine, and isobutyrylguanosine were also prepared according to the above reaction conditions. The isolated yields were uridine (61%), *N*-benzoyladenosine (75%), and *N*-isobutyrylguanosine (72%). Their analytical data are given below.

(ii) *5′-Dimethoxytrityl 2′-*tert*-butyldimethyl silyl uridine 3′-p-chlorophenyl β-cyanoethyl phosphotriester*

Ultraviolet (MeOH) λ_{max}: 263 nm; nmr ($CDCl_3$) δ: 2.63 (2H, m, CH_2CH_2CN), 0.91 (9H, s, $(CH_3)_3CSi$). *Anal.* (foamy material) calcd. for $C_{45}H_{51}N_3O_{11}ClPSi$: C 60.25, H 5.68, N 4.64; found: C 60.12, H 5.54, N 4.56.

(iii) *5′-Dimethoxytrityl 2′-*tert*-butyldimethyl silyl N-benzoyladenosine 3′-p-chlorophenyl β-cyanoethyl phosphotriester*

Ultraviolet (MeOH) λ_{max}: 276 nm; nmr ($CDCl_3$) δ: 2.78 (2H, m, CH_2CH_2CN), 0.89 (9H, s, $(CH_3)_3CSi$). *Anal.* (foamy material) calcd. for $C_{53}H_{56}N_6O_{10}ClPSi$: C 61.71, H 5.47, N 8.14; found: C 61.50, H 5.57, N, 8.26.

(iv) *5′-Dimethoxytrityl 2′-*tert*-butyldimethyl silyl N-isobutyryl guanosine 3′-p-chlorophenyl β-cyanoethyl phosphotriester*

Ultraviolet (MeOH) λ_{max}: 274, 281 nm; nmr ($CDCl_3$) δ: 2.75

(2H, m, CH_2CH_2CN), 0.93 (9H, s, $(CH_3)_3Si$). *Anal.* (foamy material) calcd. for $C_{50}H_{58}N_6O_{11}ClPSi$: C 59.25, H 5.76, N 8.29; found: C 59.05, H 5.84, N 8.49.

Selective removal of β-cyanoethyl group from the fully protected ribomononucleotide **5** *on treatment with diisopropylamine – anhydrous pyridine*

Fully protected ribomononucleotide (2.5 mmol) was dissolved in anhydrous pyridine (5 mL) containing diisopropylamine (1.5 mL) and kept at room temperature. The reaction was over within an hour as monitored on reversed phase tlc and the results are documented in Table 1 for individual nucleotides. After completion of the reaction, the solvent was removed *in vacuo* and the foamy residue was reevaporated in pyridine solvent to dryness.

General method of large-scale and rapid synthesis of sixteen fully protected ribodinucleotides **11**

5′-Dimethoxytrityl 2′-*tert*-butyldimethyl silyl *N*-protected ribomononucleoside 3′-*p*-chlorophenylphosphate **9** (4.5 mmol) and 5′-hydroxyl ribomononucleoside, 2′-*tert*-butyldimethyl silyl, 3′-*p*-chlorophenyl, β-cyanoethyl phosphate **10** (4.0 mmol) in anhydrous pyridine (20 mL) were treated with mesitylenesulfonyl tetrazole (9 mmol) at room temperature for 15 min. The reaction mixture was taken up in dichloromethane (100 mL) and 5% aqueous sodium bicarbonate solution (100 mL). The aqueous layer was extracted twice with dichloromethane (50 mL). Evaporation of the organic solvent *in vacuo* yielded a gummy residue which was chromatographed on a reversed phase gel (40 g) packed in a short column (4 cm diameter). The column was eluted with the following solvents: (*i*) acetone containing 40% water (1.0 L) to remove the unreacted 3′-phosphodiester containing material as well as 5′-hydroxyl; (*ii*) acetone containing 25% water (300 mL) eluted the fully protected ribodinucleotide **11**. The desired product was isolated by extracting solvent (*ii*) with an equal volume of dichloromethane. Subsequent evaporation of the dichloromethane solvent *in vacuo* yielded pure fully protected dinucleotide in 50–70% yields. This purification procedure required 90 min.

General method of ribooligonucleotide synthesis

The synthesis of fully protected ribooligonucleotide was achieved by condensing 5′-dimethoxytrityl, 2′-*tert*-butyldimethyl silylated, *N*-protected oligonucleotides containing 3′-*p*-chlorophenylphosphate with the 5′-hydroxyl containing component in anhydrous pyridine solution in the presence of mesitylene tetrazole for 15 min at room temperature. After the usual work-up the desired product was isolated by short column chromatography on reversed phase (RP-2) silica gel as described above. The entire operation of coupling, work-up, purification, and isolation was complete within 3 h. The purity of each intermediate was 80–90% as judged by tlc on reversed phase silica gel plates as was the case also after the complete deblocking step, analysis on PEI plate, and sequence determination. The summary of reaction conditions and isolated yields are documented in Table 2.

Final deblocking and purification step

A fully protected undecamer containing the base sequence (r-AAACAUGAGGA) (15 mg) was treated with 2% benzenesulfonic acid in methanol–dichloromethane (3:7 v/v) (6 mL) for 1 min at room temperature followed by washing with 5% aqueous sodium bicarbonate solution (6 mL). After separating the aqueous layer, the organic solvent was evaporated *in vacuo* to dryness and then dissolved in concentrated ammonia–pyridine (2:3 v/v) (5 mL). After keeping the reaction mixture at room temperature for 48 h, it was heated to 37°C for 24 h. Subsequent evaporation *in vacuo* gave a gummy residue which was coevaporated in pyridine (1 mL) to a glassy product which was next washed with anhydrous ethyl ether (1 mL) for 15 min. Removal of ethyl ether produced a powder which was then treated with 1.0 *M* tetrabutylammonium fluoride in tetrahydrofuran (1 mL) for 4 h at room temperature. Shorter time periods, e.g. 30 min for the dimer, 2 h for the hexamer, are sufficient for the removal of all the 2′-TBDMS protected groups. The reaction mixture was applied directly on the PEI-tlc plate (~ 300 μL per plate) and the application area washed first by spraying with distilled water and then with methanol to remove excess salts and nonnucleotidic materials. The PEI plate was first developed in water (for a height of 3 cm) followed by 0.6 *M* LiCl–7 *M* urea solvent at 60°C. After 2 h, the plate was removed and then soaked in a methanol tank. The desired major band was eluted with 1 *M* triethylammonium bicarbonate pH 7.5 solution.

Characterization

(a) *Confirmation of 3′ → 5′ phosphodiester internucleotidic linkages in snythetic ribooligomers*

The r-AAACAUGAGGA (11-mer) acceptor (1 mmol, 100 μ*M*) and donor [r-5′-^{32}P]PCP (0.5 mmol, 50 μ*M*) in reaction buffer (10 μL) containing 50.0 m*M* HEPES, pH 7.5, 20 m*M* $MgCl_2$, 3.3 m*M* (dithiothreitol), 0.1 m*M* ATP, 0.25 μg bovine serum albumin, 10% v/v dimethyl sulfoxide was incubated with RNA ligase (2) (1.5 units) for 30 min at 37°C. The reaction was terminated by heating at 100°C for 2 min. The labeled compound was purified by electrophoresis on a 20% gel slab. The labeled material was then treated with spleen phosphodiesterase in 10 m*M* EDTA and 100 m*M* potassium phosphate (pH 6.5) for 30 min at 37°C. The digested material was analyzed by homochromatography using Homo-V solvent (15). All the radioactivity was found at the solvent front corresponding to mononucleotide.

(b) *Sequence analysis by mobility shift method*

(i) *5′-Labeling of synthetic ribooligonucleotides*

Each synthetic ribooligonucleotide fragment was labeled using [γ-^{32}P]-ATP and T_4-polynucleotide kinase enzyme. The labeled compound was purified by 20% gel electrophoresis according to the published procedure (22).

(ii) *Partial digest of 5′-^{32}P-labeled ribooligonucleotide with nuclease P_1 enzyme*

The 5′-labeled ribooligonucleotide in 20 μL containing 50 μg of carrier t-RNA in 50 m*M* ammonium acetate (pH 5.3) was incubated with nuclease P_1 enzyme (1 μg) at room temperature. Aliquots (5 μL) were removed at 0, 5, 10, and 20 min and transferred to 5 m*M* EDTA-containing diethyl pyrocarbonate (10 μL/100 mL buffer) (10 μL) and inactivation of the enzyme was accomplished by heating at 100°C for 2 min. An aliquot (1 μL) of each time interval fraction was analyzed in one dimension by homochromatography using solvent V. The appropriate aliquots were mixed, and chromatographed by the two dimensional system. The radioautograms of each oligomer are shown in Fig. 5 which confirms their individual sequences.

Acknowledgments

We are grateful to Professor Olke Uhlenbeck, University of Illinois, Urbana, for suggesting the R-17 sequence for the chemical synthesis, and to Miss Diana Schmidt for her technical assistance.

1. R. Silber, V. G. Malathi, and J. Hurwitz. Proc. Natl. Acad. Sci. U.S.A. **69**, 3009 (1972); G. C. Walker, O. C. Uhlenbeck, E. Bedows, and R. I. Gumport. Proc. Natl. Acad. Sci. U.S.A. **72**, 122 (1975).
2. Nucleic Acids Res., Symposium Series No. 7, 1980.

3. R. LOHRMANN, D. SÖLL, H. HAYATSU, E. OHTSUKA, and H. G. KHORANA. J. Am. Chem. Soc. **88**, 819 (1966).
4. E. OHTSUKA, M. UBASAWA, and M. IKEHARA. J. Am. Chem. Soc. **93**, 2296 (1971).
5. R. L. LETSINGER and K. K. OGILVIE. J. Am. Chem. Soc. **89**, 4801 (1967).
6. R. L. LETSINGER, J. L. FINNAN, G. A. HEAVER and W. B. LUNSFORD. J. Am. Chem. Soc. **97**, 3278 (1975).
7. K. K. OGILVIE and M. J. NEMER. Tetrahedron Lett. **21**, 4159 (1980); E. OHTSUKA, H. TAKASHIMA, and M. IKEHARA. Tetrahedron Lett. **22**, 765 (1981).
8. T. NEILSON and E. S. WERSTIUK. J. Am. Chem. Soc. **96**, 2295 (1974).
9. S. S. JONES, B. RAYNES, C. B. REESE, A. UBASAWA, and M. UBASAWA. Tetrahedron, **36**, 3075 (1980).
10. K. K. OGILVIE and M. J. NEMER. Can. J. Chem. **58**, 1389 (1980).
11. J. H. VAN BOOM. Heterocycles, **7**, 1197 (1977).
12. E. OHTSUKA, S. TANAKA, and M. IKEHARA. J. Am. Chem. Soc. **100**, 8210 (1978).
13. K. K. OGILVIE, K. K. SCHIFMAN, and C. L. PENNY. Can. J. Chem. **57**, 2230 (1979).
14. K. ITAKURA, C. P. BAHL, N. KATAGIRI, J. MICHNIEWICZ, S. A. NARANG, and R. H. WIGHTMAN. Can. J. Chem. **51**, 3649 (1973); T. C. CATLIN and F. CRAMER. J. Org. Chem. **38**, 245 (1973); S. A. NARANG, R. BROUSSEAU, H. M. HSIUNG, and J. J. MICHNIEWICZ. Methods Enzymol. **65**, 610 (1979).
15. J. GRALLA, J. A. STEITZ, and D. M. CROTHERS. Nature, **248**, 204 (1974).
16. C. D. TU, E. JAY, C. P. BAHL, and R. WU. Anal. Biochem. **74**, 73 (1976).
17. N. KARAGIRI, K. ITAKURA, and S. A. NARANG. J. Am. Chem. Soc. **97**, 7332 (1975).
18. C. MCNEAL, S. A. NARANG, R. D. MACFARLANE, H. M. HSIUNG, and R. BROUSSEAU. Proc. Natl. Acad. Sci. U.S.A. **76**, 735 (1979).
19. C. B. REESE and A. UBASAWA. Nucleic Acids Res., Symp. Series No. 7, 1980. p. 5.
20. A. K. SOOD and S. A. NARANG. Nucleic Acids Res. **4**, 2757 (1977); R. W. ADAMIAK, M. Z. BARCISZEWSKA, E. BIALA, K. GRZESKOWIAK, R. KIERZEK, A. KRASZEWSKI, W. T. MARKIEWICZ, and M. WIEWIOROWSKI. Nucleic Acids Res. **3**, 3397 (1976).
21. J. STAWINSKI, T. HOZUMI, and S. A. NARANG. Can. J. Chem. **54**, 670 (1976).
22. J. STAWINSKI, T. HOZUMI, S. A. NARANG, C. P. BAHL, and R. WU. Nucleic Acids Res. **4**, 353 (1977).
23. H. M. HSIUNG, W. L. SUNG, R. BROUSSEAU, R. WU, and S. A. NARANG. Nucleic Acids Res. **8**, 5753 (1980).
24. M. SILBERKLANG, A. M. GILLUM, and U. L. RAJBHANDARY. Nucleic Acids Res. **4**, 4091 (1977).
25. K. A. WATANABE and J. J. FOX. Angew. Chem. Int. Ed. Engl. **5**, 579 (1966).
26. J. F. M. DE ROOIJ, G. WILLE-HAZELEGER, P. M. J. BURGERS, and J. H. VAN BOOM. Nucleic Acids Res. **6**, 2237 (1979).