O-ring joint. The mixture was heated and then the flask was connected to the main apparatus on a vacuum line. The acetic anhydride solution was frozen and the space above it was evacuated. After the acetic anhydride had thawed, the solution was filtered through a fritted filter and the anhydrous stannous chloride collected. The solid was washed with previously degassed anhydrous benzene (distilled from sodium). The crystals were then vacuum dried to remove all remaining solvent.

Octaethylporphyrin (3 mg) was dissolved in freshly distilled anhydrous pyridine (30 ml). The solution was degassed by three cycles of freeze–pump–thaw. The degassed porphyrin solution was back-filtered onto the anhydrous stannous chloride and the mixture heated. The resulting brown solution was filtered into a receiving flask and subsequently passed through the distributing outlet into appropriate sample tubes for spectral analysis or further reaction.

**Acknowledgments.** We thank the U. S. Public Health Service for support of this work (Grant No. GM 15,238). We are grateful to Professors E. W. Baker, M. S. Brookhart, and T. J. Meyer for valuable discussion and to Mr. P. D. Wildes for assistance with the flash experiments.

# Studies on Transfer Ribonucleic Acids and Related Compounds. III.[1] Synthesis of Hexanucleotide Having the Sequence of the Yeast Alanine Transfer Ribonucleic Acid 3′ End

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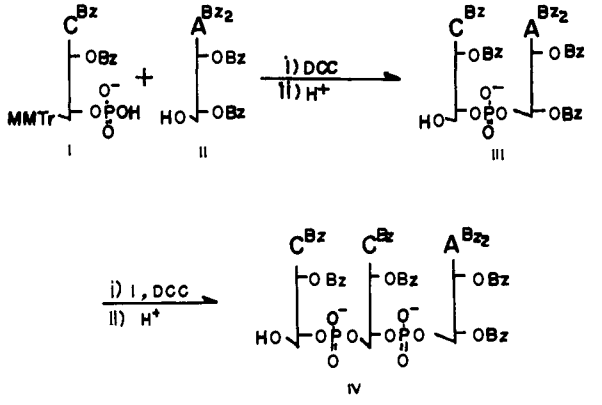
*Contribution from the Faculty of Pharmaceutical Sciences, Osaka University, Toyonaka, Osaka, Japan.* *Received July 18, 1970*

**Abstract:** A hexanucleotide with the base sequence of the 3′ end in some tRNA's, CpCpApCpCpA, was synthesized by condensation of the trinucleotide blocks, MMTr-$C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$(OBz)-*p* and $C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz_2}$(OBz)$_2$ using triisopropylbenzenesulfonyl chloride as the condensing reagent. The hexanucleotide was isolated in protected form as $C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz_2}$(OBz)$_2$ and can be further condensed with oligonucleotide blocks containing a 3′-phosphate end. A polymerization of $C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$(OBz)-*p* yielded the hexanucleotide with the 3′-terminal phosphate. The circular dichroism spectra of CpCpA and CpCpApCpCpA were measured.

The synthesis of ribopolynucleotides may be facilitated by using preformed oligonucleotide blocks. In a previous paper we reported the synthesis of protected ribooligonucleotides with 3′-phosphate end groups.[1,2] In this report we present the synthesis of the hexanucleotide MMTr-$C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz_2}$(OBz)$_2$ (VIa) by condensation of the trinucleotides $C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz_2}$(OBz)$_2$ (IV) and MMTr-$C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$(OBz)-*p* (V).[3] A hexanucleotide of this sequence constitutes the 3′ end of yeast alanine tRNA,[4] *E. coli* tyrosine tRNA,[5] methionine tRNA,[6] valine tRNA,[7] and *Torulopsis utilis* isoleucine tRNA.[8] Selective removal of the 5′-*O*-methoxytrityl group of compound VIa gave the properly protected hexanucleotide VIb for further elongation of the chain.

**Synthesis of $C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz_2}$(OBz)$_2$.** The terminal trinucleoside diphosphate was synthesized by stepwise condensation of the suitably protected mononucleotides as shown in Chart I. *N*,*N*′,2′,3′-*O*-

Chart I




Tetrabenzoyladenosine (II) and 5′-*O*-monomethoxytrityl-*N*,2′-*O*-dibenzoylcytidine 3′-phosphate (I) were condensed by a procedure similar to that used for the 5′-*O*-dimethoxytrityl derivatives.[9] After removal of the trityl group and ion exchange chromatography of the reaction mixture, III was obtained in a yield of 58%.

(1) Part II of this series: E. Ohtsuka, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, **92**, 3445 (1970).

(2) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *ibid.*, **92**, 3441 (1970).

(3) The system of abbreviations is principally as has been suggested by the IUPAC–IUB combined commission: *J. Biol. Chem.*, **241**, 531 (1966). For the protected ribonucleotide essentially the same system is used as described in ref 1 and 2. MMTr$C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$(OBz)-*p* refers to 5′-*O*-monomethoxytrityl-*N*,2′-dibenzoylcytidylyl-(3′–5′)-*N*,2′-dibenzoylcytidylyl-(3′–5′)-*N*,2′-dibenzoyladenosine 3′-phosphate.

(4) R. W. Holley, J. Apgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merril, J. R. Penswick, and A. Zamir, *Science*, **147**, 1462 (1965).

(5) H. M. Goodman, J. Abelson, A. Landy, S. Brenner, and J. D. Smith, *Nature (London)*, **217**, 1019 (1968).

(6) S. Cory, K. A. Marcker, S. K. Dube, and B. F. C. Crick, *ibid.*, **220**, 1039 (1968).

(7) M. Yaniv and B. G. Barell, *ibid.*, **222**, 278 (1969).

(8) S. Takemura, M. Murakami, and M. Miyazaki, *J. Biochem. (Tokyo)*, **65**, 489 (1969).

(9) R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H. G. Khorana, *J. Amer. Chem. Soc.*, **88**, 819 (1966).

For the synthesis of the trinucleotide IV, III was allowed to react with I using dicyclohexylcarbodiimide (DCC) as the condensing agent. Treatment with 80% acetic acid gave the protected trinucleotide IV, which was purified by ion exchange chromatography on TEAE-cellulose at pH 6.5 (see Figure 1 and Table I).

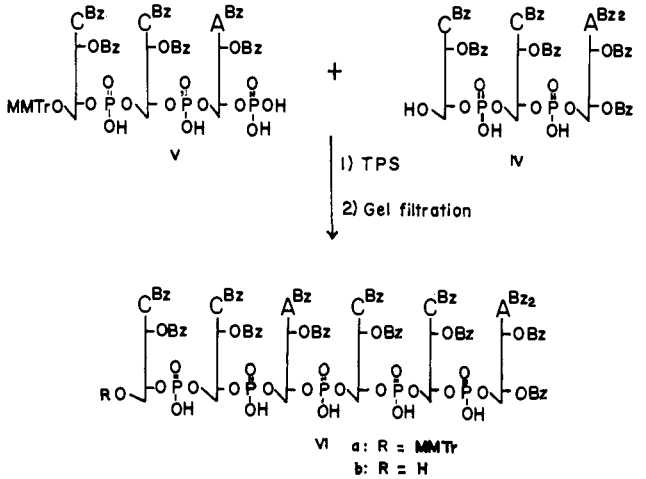
**Table I.** Identification of the Product in the Synthesis of $C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ (Figure 1)

| Peak No. | Fractions pooled | $TOD_{304}$ | Identification |
|---|---|---|---|
| I | 40–55 | 628 | Unidentified |
| II | 61–88 | 1,668 | $C^{Bz}(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ |
| III | 120–140 | 2,770 | $C^{Bz}(OBz)$-*p* |
| IV | 161–180 | 635 | Unidentified |
| V | 200–240 | 3,610 | $C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ |
| VI | 251–280 | 720 | Pyrophosphate between CpCpA and Cp |
| VII | 294–303 | 898 | Unidentified |

Although the principle of chromatography was similar to that used for III, it is interesting to observe that this trinucleotide (peak V) was separated from the mononucleotide I (peak III) at pH 6.5. The isolated yield of IV based on III was 34%. The product was characterized by thin-layer chromatography. $R_f$ values and mobility in paper electrophoresis of debenzoylated product, CpCpA, are shown in Table II. The enzymatic hydrolysis of CpCpA with pancreatic RNase gave the expected product in the correct ratio.

**Synthesis of $C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ (VIb).** The two nucleotide blocks IV and V were condensed with triisopropylbenzenesulfonyl chloride (TPS) to give the hexanucleotide VIa (Chart II). Arylsulfonyl chlorides have been

Chart II




known to give faster rates in the synthesis of phosphodiester linkages than carbodiimides and are used for the condensation of preformed deoxynucleotide blocks.[10] A preliminary experiment with DCC to condense IV and V did not give any detectable amount of VIa. TPS was chosen as the sterically hindered sulfonyl chloride,[11] which would not sulfonylate the primary hydroxyl group of IV. The composition of the condensation mixture was analyzed by paper chromatography in solvent C (Table II). Besides the desired product and starting materials, CpA, a degradation product and an unidentified compound ($R_{Cp}$ 0.71 in solvent C, $R_{fCp}$ 0.36 in electrophoresis) were detected. The hexanucleotide VIa was isolated by gel filtration over Sephadex LH-20 in 95% ethanol. As seen from Figure 2 the resolution of peaks is not satisfactory. The first peak, however, contained the almost pure hexanucleotide VIa. The yield of VIa was 18%. The removal of the 5′-trityl group gave the partially protected hexanucleotide VIb, which is useful for the condensation with another oligonucleotide block.[12]

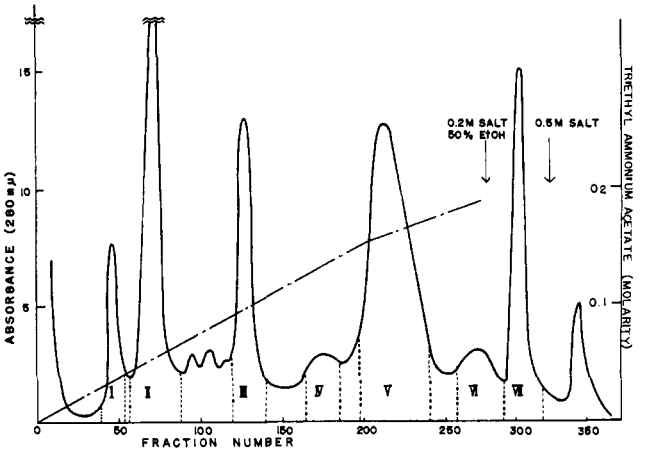



Figure 1. Chromatography of the product obtained in the synthesis of $C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ on a column (2.7 × 50 cm) of TEAE-cellulose (acetate) in 95% ethanol. Elution was performed by a linear gradient of triethylammonium acetate (pH 6.5). The mixing chamber contained 2 l. of 95% ethanol and the reservoir contained an equal volume of 0.15 *M* acetic acid and 0.075 *M* triethylamine in 95% ethanol. The elution was followed by another gradient of 0.15 *M* (1 l.) and 0.20 *M* (1 l.). Fractions of 20 ml were collected every 15 min. The identification of peaks is shown in Table I.

Compound VIb was slightly soluble in water and showed some mobility in paper electrophoresis, whereas VIa showed no mobility (Table II). The completely unprotected hexanucleotide CpCpApCpCpA was analyzed by digesting with RNase M[13] and gave the correct products.

In another run of the block condensation, the partially resolved first peak in gel filtration was chromatographed on TEAE-cellulose ($Cl^-$ form) in 75% ethanol. The elution pattern is shown in Figure 3. The last peak, which contained the hexanucleotide VIa and the pyrophosphate of the trinucleotide V, was rechromatographed on the same column using a shallower gradient. As shown in Figure 4 the main peak was separated from the minor peak which contained the pyrophosphate of the trinucleotide block. This chromatography demonstrated the usefulness of ion exchange chromatography for the separation of protected polynucleotides.

**Polymerization of $C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz}(OBz)$-*p*.** A polymerization of the trinucleotide containing a 5′-hydroxyl and a 3′-phosphate end group was at-

(10) H. G. Khorana, *Pure Appl. Chem.*, 17, 349 (1968); H. Kössel, M. W. Moon, and H. G. Khorana, *J. Amer. Chem. Soc.*, **89**, 2148 (1967); J. Hachmann and H. G. Khorana, *ibid.*, **91**, 2749 (1969).

(11) R. Lohrmann and H. G. Khorana, *ibid.*, **88**, 829 (1966).

(12) A preliminary condensation experiment of IVb with MMTr-$C^{Bz}$-(OBz)-*p*-$G^{Ac}(OAc)$-*p*-U(OAc)-*p* gave the nonanucleotide CpGpUp-CpCpApCpCpA (unpublished experiments by E. Ohtsuka, M. Ubasawa, and M. Ikehara).

(13) M. Irie, *J. Biochem. (Tokyo)*, **62**, 509 (1967).

Table II. Properties of Different Nucleotides in Paper Chromatography and Electrophoresis

| Compound | Paper chromatography, Solvent A[a] | Paper chromatography, Solvent B[a] | Paper chromatography, Solvent C[b] | Paper electrophoresis[b] |
|---|---|---|---|---|
| Cp | 0.14 | | 1.00 | 1.00 |
| Cp> | 0.42 | | 1.77 | 0.69 |
| CpA | 0.28 | | 1.30 | 0.42 |
| CpCpA | 0.12 | | 0.90 | 0.70 |
| CpCpAp | 0.06 | | 0.40 | 1.10 |
| CpCpApCpCpA | 0.014 | | 0.14 | 0.84 |
| CpCpApCpCpAp | | | 0.07 | |
| MMTr-$C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$(OBz)-*p* | | 0.87 | | |
| $C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$-OBz-*p* | | 0.79 | | 0.81 |
| $C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}{}_2$(OBz)$_2$ | | | | 0.12 |
| MMTrCp> | 0.77 | | | |
| MMTrCp | 0.61 | | | |
| MMTrCpCp | 0.27 | | | |
| MMTrCpCpAp | 0.14 | | 1.45 | |
| MMTrCpCpApCpCpA | | | 0.95 | |

[a] $R_f$ values are given. [b] $R_{Cp}$ values are given.

tempted in order to yield a polynucleotide with a repeating sequence. This nonanucleotide with the repeating trinucleotide sequence CpCpAp is contained in *E. coli* tyrosine tRNA[5] at the acceptor end. A preliminary experiment to polymerize $C^{Bz}$(OBz)-*p*-$C^{Bz}$(OBz)-*p*-$A^{Bz}$(OBz)-*p* using TPS gave the hexanucleotide with 3′-phosphate. The polymerization mixture was first subjected to gel filtration on a column of Sephadex G-50 in 50% ethanol. The resolution of protected oligonucleotides, however, was not satisfactory by this procedure. Aqueous ethanol probably gave insufficient swelling of the gel. Therefore the unprotected oligonucleotides were chromatographed on DEAE-cellulose in 7 *M* urea. The hexanucleotide CpCpAp-CpCpAp was isolated in a yield of 2.5% and identified by paper chromatography before and after removal of the phosphomonoester group. The nonanucleotide with the repeating trinucleotide sequences, however, was not detected in the reaction product.

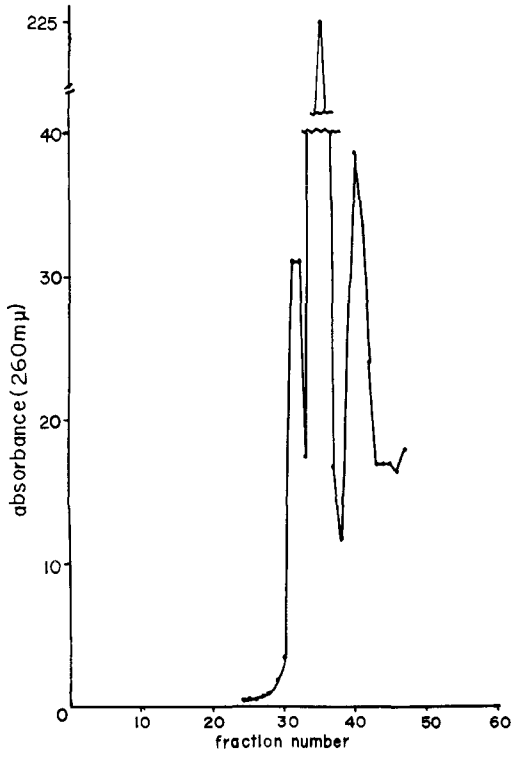



Figure 2. Gel filtration of the product obtained in the synthesis of hexanucleotide VIa on a column (1.2 × 100 cm) of Sephadex LH-20 preequilibrated with 95% ethanol. Elution was carried out with 95% ethanol. Fractions of 1.65 ml werec ollected every 20 min. Fractions 31 and 32 contained VIa; 34 and 35, V; and 39–42, TPS. Pyridine was eluted after fraction 45.

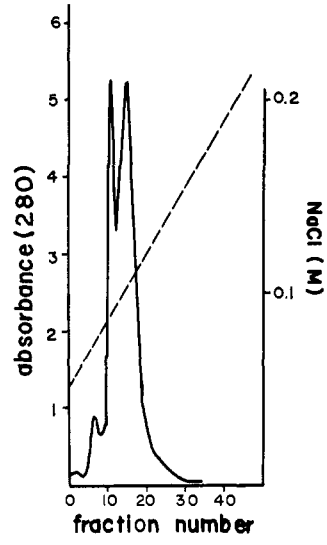



Figure 3. Chromatography of the product obtained in the synthesis of the hexanucleotide VIa on a column (1.2 × 45 cm) of TEAE-cellulose ($Cl^-$) preequilibrated with 75% ethanol containing 0.02 *M* sodium acetate, pH 6.0, after rough separation by gel filtration with Sephadex LH-20. The condition of gel filtration was as described in Figure 2. Elution was performed by a linear gradient of sodium chloride using 0.05 *M* salt (500 ml) and 0.4 *M* salt (500 ml) in the equilibration buffer. Fractions of 10.6 ml were collected every 40 min. The last peak contained the hexanucleotide VIa but contaminated with the pyrophosphate of the trinucleotide V. This part was rendered to rechromatography as shown in Figure 4. Fractions 11 and 12 contained the trinucleotide V.

**Circular Dichroism of CpCpA and CpCpApCpCpA.** Studies on the physical properties of the oligonucleotides of defined sequence are of importance for the elucidation of the structure of polynucleotides. Recent optical

Table III. The CD Spectra of CpCpA and CpCpApCpCpA

| Compd | $\lambda_{max}$, nm | $[\theta]$ | $\lambda_c$, nm[a] | $\lambda_{min}$, nm | $[\theta]$ | $\lambda_{max}$, nm | $[\theta]$ |
|---|---|---|---|---|---|---|---|
| CpCpA | 280 | $2.6 \times 10^4$ | 253 | 235 | $-0.57 \times 10^4$ | 222 | 0 |
| CpCpApCpCpA | 275 | $3.1 \times 10^4$ | 255 | 237 | $-0.77 \times 10^4$ | 222 | $1.5 \times 10^4$ |

[a] $\lambda_c$ stands for wavelength of cross-over point.

studies of the conformation of single-stranded oligonucleotides have shown that bases of oligonucleotides in neutral aqueous solution are oriented in a vertically stacked conformation.[14-17] Brahms, *et al.*,[18] found a unique conformation for trinucleotides with a guanosine residue next to uridine. Since CpCpA is the common sequence at the 3′ end of tRNA, it might be interesting to measure the CD spectrum of the trinucleotide to find any unique base stacking and to compare it with that of the hexanucleotide with the repeating sequence. The CD spectra of these two oligonucleotides are shown in Figure 5 and the results summarized in Table III. The CD spectrum of CpCpA showed a strong positive band at 280 nm, which is in agreement with the calculated optical rotatory dispersion.[19] The hexamer had a spectrum with similar profile. However, the rotational strength of the positive band of the 260–280-nm region increased markedly in amplitude and shifted to shorter wavelengths. This difference probably arises from the different helical nature of these two oligonucleotides. $(Cp)_6$ in neutral pH solution showed very little increase in the intensity of the Cotton band compared with the trimer.[20] The heteropolymer CpCpApCpCpA seems to have different base stacking from that of $(Cp)_6$. A comparison of the spectrum of CpCpA with that of CpC and CpA[17] showed that the spectrum of the triplet depended upon the influence of the nearest residue. This might mean that the triplet exists as a single-stranded ordered structure.[15]

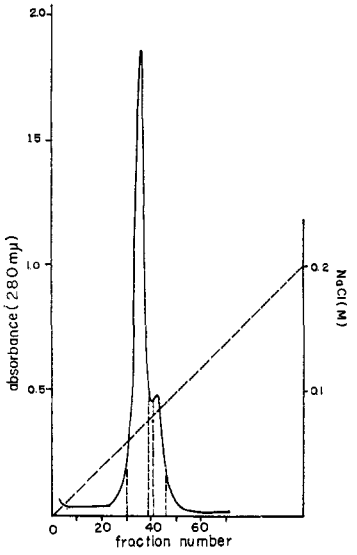



Figure 4. Rechromatography of the hexanucleotide VIa obtained from the chromatography shown in Figure 3. A more shallow salt gradient (0–0.22 *M*) was used. Other conditions were the same as described in Figure 3. The main peak contained the hexanucleotide VIa and the latter minor peak contained the pyrophosphate of the trinucleotide V.

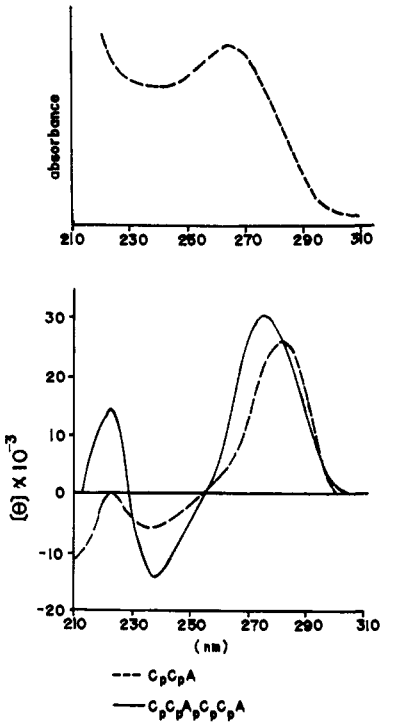
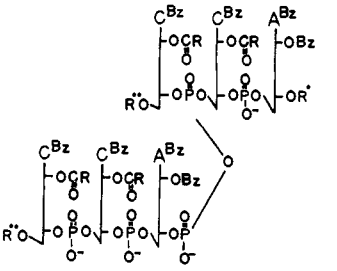



Figure 5. The CD spectra of CpCpA (broken line) and CpCpApCpCpA (solid line). The condition is described in the Experimental Section. The uv spectrum of CpCpA is shown (top).

**General Comments.** The block condensation of two ribotrinucleotides IV and V yields the hexanucleotide with defined sequence, CpCpApCpCpA. The yield of the condensation was not satisfactory. A preferential cleavage to give CpA seems to occur in the condensation of IV and V. The mechanism of this cleavage was not solved in the present study. Phosphotriesters or substituted pyrophosphates of internucleotidic phosphodiester linkages (see Chart III) could be intermediates in

Chart III



(14) M. M. Warshaw and I. Tinoco, Jr., *J. Mol. Biol.*, 13, 54 (1965).
(15) C. R. Cantor and I. Tinoco, Jr., *ibid.*, 13, 65 (1965).
(16) C. A. Bush and J. Brahms, *J. Chem. Phys.*, 46, 79 (1967).
(17) J. Brahms, J. C. Maurizot, and A. M. Michelson, *J. Mol. Biol.*, 25, 481 (1967).
(18) J. Brahms, A. M. Aubertin, G. Dirheimer, and M. Grunberg-Manago, *Biochemistry*, 8, 3269 (1969).
(19) C. R. Cantor and I. Tinoco, Jr., *Biopolymers*, 5, 821 (1967).
(20) J. Brahms, J. C. Maurizot, and A. M. Michelson, *J. Mol. Biol.*, 25, 465 (1967).

this cleavage reaction. If any tetrasubstituted pyrophosphates of phosphodiesters survived in aqueous pyridine, ammonia treatment to remove acyl protections of the 2′-hydroxyl group would cleave the P–O linkage at the 5′ position. As condensation products were identified mainly after removal of the protecting groups, it was not clear whether the cleavage occurred during the condensation reaction or the subsequent ammonia treatment. The presence of the 2′-hydroxyl group and its possible participation during hydrolysis of activated phosphates are the only differences to the corresponding condensation reaction with deoxyribonucleotides. The preferential cleavage of the phosphodiester linkage between two cytidylyl residues is interesting in the light of highest susceptibility to cleavage of this linkage to nonenzymatic hydrolyses.[21] Systematic studies on the block condensation of this type of ribooligonucleotides are necessary. Further elongation of the 3′-terminal sequence and condensation of ribotrinucleotides to give oligonucleotides with sequences found at the 5′ end of tRNA's are under investigation.

The polymerization of ribotrinucleotides should be useful to obtain oligonucleotides with repeating sequences. Although the present experiment gave only dimerization of the trinucleotide in low yield, polymerization could be improved by investigating different conditions.

The availability of CpCpA and CpCpApCpCpA provided the unique opportunity for comparison of the circular dichroism spectra of oligonucleotides with a repeating base sequence. The calculation of optical rotatory dispersion of the 64 ribotrinucleotides predicted the possibility of identification of base sequences of oligonucleotides and some of the trinucleotides were used to confirm the predictions. CpCpApCpCpA is the longest chemically synthesized ribooligonucleotide containing more than one base. Annealing of this oligonucleotide or longer fragments corresponding to the 3′ end of the tRNA with complementary 5′ end oligonucleotides would give double helical compounds. The investigation of the nature of such compounds may be interesting from biochemical and biophysical standpoints. Chemical aminoacylation of the hexanucleotide CpCpApCpCpA and the properties of such aminoacylated oligonucleotides in protein biosynthesis are under investigation.

## Experimental Section

**General Methods.** Paper chromatography was performed by the descending technique in the following solvents: A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); B, ethanol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v); C, *n*-propyl alcohol–concentrated ammonia–water (55:10:35, v/v); D, saturated ammonium sulfate–water–isopropyl alcohol (79:19:2, v/v). Paper electrophoresis was performed at 900 V/60 cm using 0.05 *M* triethylammonium bicarbonate, pH 7.5. Other general methods are mostly as described previously.[2]

**Optical Measurements.** Circular dichroism measurements were performed at −5° using a JASCO ORD/UV-5 spectropolarimeter with attachment for CD measurement. The buffer used was 0.01 *M* potassium cacodylate pH 7.0 in 4 *M* potassium fluoride. Ultraviolet spectra were taken by a Hitachi EPS-3T spectrophotometer. All results are expressed as residue extinction or residue ellipticity. An extinction coefficient of 9350/residue at 260 nm was used for CpCpA and CpCpApCpCpA according to the calculation.[19] The samples were measured in 0.5- and 1-cm pathlength cells at the concentration of $0.5–1.0 \times 10^{-4}$ *M*.

(21) H. Witzel, *Justus Liebigs Ann. Chem.*, **635**, 182 (1960).

$C^{Bz}(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ (III). The compound III was synthesized by condensing 5′-*O*-monoethoxytrityl-*N*,2′-*O*-dibenzoylcytidine 3′-phosphate (I) and *N*,*N*′,2′,3′-*O*-tetrabenzoyladenosine (II) similarly to the procedure described for condensation of II with 5′-dimethoxytrityl derivative instead of I.[9] The yield was 58%.

$C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ (IV). Pyridinium $C^{Bz}(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ (III) (6120 $OD_{304}$ units, 0.39 mmol) and pyridinium MMTr-$C^{Bz}$ (OBz)-*p* (I) (8490 $OD_{304}$ units, 0.7 mmol) were passed through a column (1 × 10 cm) of pyridinium Dowex 50-X2 ion-exchange resin. The effluent and washings were evaporated with pyridine and the anhydrous pyridine solution (7 ml) was added to a mixture of ether and *n*-pentane (3:2) (300 ml). The precipitate was collected by centrifugation and washed with ether. The nucleotides were dissolved in pyridine and evaporated three times with pyridinium Dowex 50-X2 resin (300 mg). The dried mixture was treated with DCC (2.06 g, 10 mmol) in pyridine (5 ml) for 5 days at 20°. Water (5 ml) was added and the solution was extracted with *n*-pentene. After 6 hr the solution was filtered and evaporated to dryness. The residue was dissolved in pyridine and added to a mixture of ether and *n*-pentane (3:2, v/v). The precipitate was treated with 80% acetic acid for 1 hr at 20°. Thin-layer chromatography on silica gel (chloroform–methanol, 9:1) at this stage showed no trityl-containing nucleotide. Acetic acid was removed by evaporation with ethanol and the residue was dissolved in 95% ethanol (100 ml). The solution was applied to a column (2.7 × 50 cm) of TEAE–cellulose (acetate) preequilibrated with 95% ethanol. The elution conditions and the pattern are shown in Figure 1. Peak V contained the product III (3610 $OD_{304}$ units, 0.13 mmol, 34%). Spectral properties of III in 95% ethanol were $\lambda_{max}$ 260, 282 (shoulder), and 304 nm (shoulder); $\lambda_{min}$ 250 nm ($\epsilon_{304/280}$ 0.50). The unprotected product CpCpA (*ca.* 2 $OD_{260}$ units) was digested with pancreatic RNase. The products $C_p$ (0.19 μmol) and adenosine (0.092 μmol) were separated by paper electrophoresis. The ratio was 2.07:1.00.

MMTr-$C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$C^{Bz}$-$(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ (VIa). The triethylammonium salt of $C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz_2}(OBz)_2$ (IV) (40 mg, *ca.* 20 μmol) and MMTr-$C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz}(OBz)$-*p*[2] (360 $OD_{304}$ units, *ca.* 17 μmol) were combined and precipitated in a mixture of ether and *n*-pentane from their pyridine solution (1 ml). The precipitate was washed three times with ether and rendered anhydrous by coevaporation of added pyridine three times. The residue was dissolved in pyridine (0.3 ml) and allowed to react with TPS (25 mg, 83 μmol) for 6 hr at 18°. Aqueous pyridine (50%, 0.3 ml) and triethylamine (25 μl) were added to the solution in an ice bath. The mixture was kept for 16 hr at 4° and concentrated to a small volume. The solution was applied to a column (1.2 × 100 cm) of Sephadex LH-20 preequilibrated with 95% ethanol. The elution pattern is shown in Figure 2. Fractions 31 and 32 contained almost pure hexanucleotide VIa (128 $OD_{280}$ units, 18%). Fractions 34 and 35 contained the trinucleotide V (580 $OD_{280}$ units). For characterization, an aliquot of the protected hexanucleotide was treated with 80% acetic acid for 1 hr at room temperature and then with 15 *N* methanolic ammonia for 16 hr. The mixture was applied to paper chromatography in solvent C for 40 hr. The unprotected hexanucleotide was eluted with water by centrifugation. The hexanucleotide CpCpApCpCpA (*ca.* 3 $OD_{260}$ units) was digested with RNase M[13] (40 μg) in 0.1 *M* ammonium acetate (pH 6.9, 0.1 ml) for 4 hr at 37°. The product was chromatographed first in solvent A to separate adenosine and in solvent D (second dimension). The spots were eluted in 0.1 *N* HCl (3 ml). The ratio of adenosine (0.021 μmol):Ap (0.0225 μmol):Cp (0.090 μmol) was 0.93:1.0:4.0 (theoretical, 1:1:4). The hexanucleotide CpCpApCpCpA had $\lambda_{max}^{H_2O}$ 264 nm, $\lambda_{min}^{H_2O}$ 238 nm ($\epsilon_{280/260}$ 0.61 in water) and $\lambda_{max}^{pH\ 1}$ 270 nm, $\lambda_{min}^{pH\ 1}$ 237 nm ($\epsilon_{280/160}$ 0.97 at pH 1).

**Polymerization of $C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz}(OBz)$-*p*.** The triethylammonium salt of $C^{Bz}(OBz)$-*p*-$C^{Bz}(OBz)$-*p*-$A^{Bz}(OBz)$-*p* (180 $OD_{304}$ units 8.5 μmol) was reprecipitated with ether from its pyridine solution and rendered anhydrous by coevaporation of added pyridine. TPS (40 μmol) was added to the pyridine solution (*ca.* 0.6 ml) and pyridine was evaporated to about half the volume. After 2 hr, 50% aqueous pyridine (0.5 ml) and triethylamine (0.1 mmol) were added under cooling. An aliquot was treated with methanolic ammonia and chromatographed in solvent C. Degraded compounds including CpA, Cp, and CpCp were detected besides slower traveling oligomers. The total mixture was applied to a column (1.2 × 100 cm) of Sephadex G-50 preequilibrated with 50% ethanol. The first peak (99 $OD_{304}$ units) was treated with 15 *N* methanolic ammonia for 16 hr at room temperature and chro-

matographed on a DEAE-cellulos ecolumn (0.5 × 80 cm) preequilibrated with 7 *M* containing 0.02 *M* Tris–HCl, pH 7.5. The elution was carried out using a linear gradient of NaCl (0.03–0.3 *M*) of total 700 ml. The hexanucleotide CpCpApCpCpAp was eluted at a salt concentration of 0.22 *M*. The combined fractions were desalted by gel filtration technique using Bio-Gel P-2 column (2 × 80 cm). $R_f$ values of the hexanucleotide are shown in Table II. Bacterial alkaline phosphatase treatment of the hexanucleotide gave the dephosphorylated compound, which was shown to have the same mobility with the compound derived from VIb in solvent C.

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# The Oxygen-Mediated Reaction between 4-Thiouracil Derivatives and Bisulfite. Isolation and Characterization of 1-Methyluracil 4-Thiosulfate as an Intermediate in the Formation of 1-Methyluracil-4-sulfonate

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**Abstract:** The titled reaction was investigated in detail in order to clarify its mechanism. 1-Methyl-4-thiouracil was treated with [$^{35}$S]bisulfite and oxygen at pH 7 and room temperature, and the reaction mixture was analyzed by paper electrophoresis. It was found that the radioactive sulfur was incorporated into the reaction product, 1-methyluracil-4-sulfonate. Another anionic compound, also radioactive, was observed in the electrophoresis. This compound is an intermediate of the reaction, and was identified as 1-methyluracil-4-thiosulfate (I). The thiosulfate I, which can also be prepared by sulfitolysis of bis(1-methyl-4-thiouracil) disulfide, is readily hydrolyzed by acid to give 1-methyl-4-thiouracil. On treatment with bisulfite at pH 7, I yields 1-methyluracil-4-sulfonate. Compound I is attacked by glycine at pH 10.4, giving rise to 1-methyl-$N^4$-carboxymethylcytosine. A powder of the sodium salt of I is rapidly decomposed by sunlight, whereas its aqueous solution is stable. An aqueous solution of the light-exposed powder of I gives a single spot corresponding to 1-methyl-4-thiouracil in paper chromatography. In analogy to the adduct formation between bisulfite and uracil, both 1-methyl-4-thiouracil and 1-methyluracil-4-sulfonate appear to add bisulfite at their 5,6-double bonds. Nmr spectroscopy supports the 5,6-dihydro-6-sulfonate structure of these addition compounds. Since hydroquinone inhibits the oxygen-mediated reaction between 4-thiouridine and bisulfite, it is proposed that the sulfite radical is participating in the reaction. A procedure to transform 1-methyl-4-thiouracil into 1-methyluracil on a preparative scale is described.

Following the discovery of 4-thiouridine in tRNA of *E. coli*,[1] many methods have been developed for the chemical transformation of the thio base. These methods may be of value for the elucidation of the biochemical role of this minor nucleoside as a constituent of tRNA. Oxidation of 4-thiouridine with iodine results in the formation of bis(4-thiouridine) disulfide.[1] Treatment of 4-thiouridine with cyanogen bromide brings about the formation of uridine 4-thiocyanate,[2] which in turn can be converted into uridine under certain mild conditions.[3] Photochemical oxidation converts thiouridine into uridine.[4] Oxidation with hydrogen peroxide[5] or with osmium tetroxide followed by treatment with acid[6] yields uridine. In these oxidations, the intermediate oxygenated sulfur compounds have not been identified. In the course of our studies on the permanganate oxidation of nucleosides,[7,8] we have become aware that 4-thiouridine undergoes a facile reaction with permanganate, giving rise to an oxygenated sulfur compound in a quantitative fashion. This compound has been identified as uridine-4-sulfonate.[9] Ziff and Fresco have independently shown that 2′,3′-isopropylideneuridine-4-sulfonate is produced by the oxidation of 2′,3′-isopropylidene-4-thiouridine with periodate.[10]

When the time course of the permanganate oxidation of 4-thiouridine was determined, bisulfite was added to the reaction mixture in order to destroy the permanganate. It was found that the bisulfite itself reacts with 4-thiouridine. In a previous note, we described briefly the nature of this reaction.[11] The present paper reports the detailed investigation of this reaction, including the isolation and characterization of an intermediate, uracil-4-thiosulfate derivative, and the discussions on the mechanism of the reaction.

As for the reaction of bisulfite with nucleotide bases, it should be noted that bisulfite undergoes ionic addi-

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