Scheme IV: Summary

This secoalkylative annelation is particularly suitable for the addition of ring B to a preformed CD unit in steroid synthesis.

Conclusions

Scheme IV summarizes the variety of structural units that can be easily created by the above techniques. Starting with one class of reagents, the cyclopropylides, and two fundamental reactions, cyclopropanation and epoxide formation, creation of four-, five-, and six-membered carbocycles, γ-butyrolactones, and a wide variety of acyclic units are all possible. Clearly, the present work only points the way for future directions.

Carbon–carbon bond-forming reactions are among the oldest and most important to the synthetic chemist. Much new and important research in this area continues. Among the most exciting developments stand those new methods involving stereochemical purity and chemospecificity—the latter eliminating the need for protecting, blocking, or activating groups. The above methods contribute to these aspects.
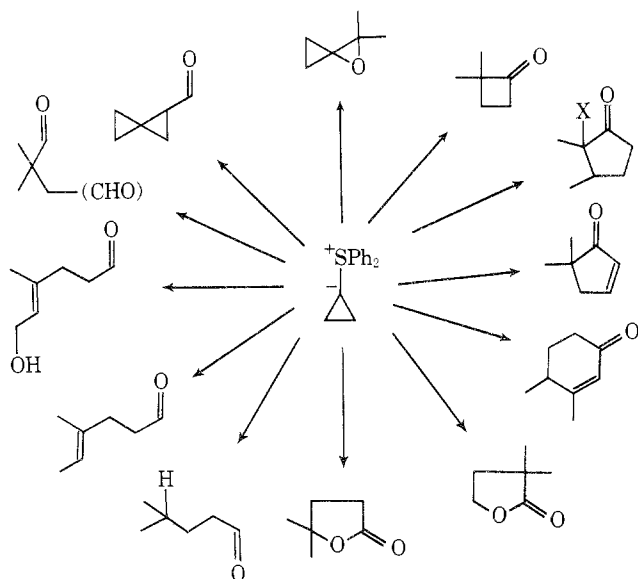
cleavage, it has been termed secoalkylation. A particularly intriguing application is a cyclohexenone annelation sequence which complements the normal Robinson annelation procedure (see Scheme III).

Synthesis of Ribooligonucleotides Having Sequences of Transfer Ribonucleic Acids

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During the recent decade the chemical synthesis of polynucleotides has been explored by many investigators and there has been significant progress in methodology.[1] The most brilliant success in this field is perhaps the synthesis of a gene for yeast alanine tRNA by Khorana and his coworkers.[2]

This and other important syntheses were accomplished by a combination of chemical synthesis of deoxyribooligonucleotides and enzymatic joining of them to each other employing DNA ligase.[3] The methods utilized for the chemical synthesis of *deoxy*-ribooligonucleotides reached the level of deca- (10 units) to icosa- (20 units) nucleotides.

On the other hand, the chemical synthesis of *ri*-booligonucleotides, which have an additional 2'-hydroxyl group in each carbohydrate moiety, is rather difficult, mainly for the following reasons. (1) Selective protection of the 2'-OH while leaving the 3'-OH unprotected usually requires lengthy pathways. (2)

Migration of phosphate groups so as to change a ribose moiety with 2'-OH, 3'-phosphate to 2'-phosphate, 3'-OH, or *vice versa*, occurs rather easily under catalysis by acid or alkali. (3) Yields were relatively low in the condensation steps that join nucleotide units, presumably due to steric hindrance.

Problem 1 may be circumvented by using a nucleoside 3'-phosphate, in which the phosphate group acts both as a selective protecting group for the 3'-OH and as a reactive site for linking to other ribose moieties; also, acyl groups can be introduced successively either in the 2'-OH or in the heterocyclic amino group of a nucleoside 3'-phosphate. The second problem is partially solved by using 2'-O-acylated 3'-nucleotides. Also helpful is the use of trityl derivatives for the protection of the primary 5'-OH, which can later be exposed to further reaction by treating with mild acid. The third problem is the

(1) H. G. Khorana, *Pure Appl. Chem.,* 71, 349 (1968).
(2) K. L. Agarwal, H. Büchi, M. H. Caruthers, N. Gupta, H. G. Khorana, E. Kleppe, A. Kumar, E. Ohtsuka, U. L. RajBhandari, J. H. Van de Sande, V. Sgaramella, H. Weber, and T. Yamada, *Nature (London),* 227, 27 (1970).
(3) B. S. Zimmerman, J. W. Little, C. K. Oshinski, and H. Gellert, *Proc. Nat. Acad. Sci. U. S.,* 57, 184 (1967); B. Weiss and C. C. Richardson, *ibid.,* 57, 1021 (1967).
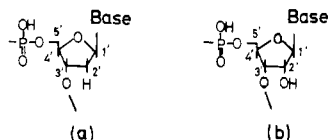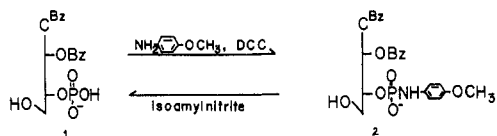
Figure 1. Structure of the repeating units in (a) a deoxyribopolynucleotide and (b) a ribopolynucleotide. The "base" moiety is usually of uracil (U), thymine (T), cytosine (C), adenine (A), or guanine (G).

most difficult; however, the use of an arylsulfonyl chloride[4] instead of the widely used dicyclohexylcarbodiimide (DCC) to effect the linking of two units showed some advantages. These methods for the synthesis of ribooligonucleotides were developed around 1965,[5] and they were employed for the synthesis of relatively short oligonucleotides, notably trinucleoside diphosphates[6] which code for 20 common amino acids in the biosynthesis of proteins.[7]

It is evident, however, that synthesis of longer polymers by the mere repetitive joining of mononucleotide units one by one is likely to be time consuming and difficult because the cumulative yield becomes very small after several steps. There are advantages to employing the block condensation method, which has been proved to be suitable in polypeptide[8] and in deoxyoligonucleotide synthesis.[9] The minimum requirements for a ribooligonucleotide "block" are to have a free phosphate and/or OH group on the chain terminus and suitable protecting groups in the middle nucleotides units on both the heterocyclic amino and 2'-OH groups. Cyanoethyl phosphates,[10] widely used in deoxyoligonucleotide synthesis, are not suitable for the present purpose, because the alkali treatment used to eliminate the cyanoethyl group causes deprotection of 2'-OH groups and makes it impossible to elongate the chain by further condensations.

Synthesis of Ribooligonucleotides by the Use of Aromatic Phosphoramidate Groups as Protected Phosphate Groups

Several years ago we studied the decomposition of nucleotide phosphoramidates by means of nitrite and found that thymidine 5'-phosphoramidate is converted quantitatively to the corresponding phosphate[11] under neutral conditions at room temperature. We tested, therefore, aromatic phosphoramidates, which are known[12] to be stable in acidic and alkaline media.

Figure 2. Cloverleaf model of yeast alanine tRNA. Oligonucleotides in the boxes were synthesized.

$N,2'$-O-Dibenzoylcytidine 3'-phosphate (1) was converted smoothly to the corresponding 3'-phosphoranisidate (2) upon treatment with anisidine and DCC.[13] Compound 2 was then allowed to react with isoamyl nitrite in an aqueous acetic acid–pyridine buffer at pH 7.5 and room temperature. As expected, compound 1 was obtained in quantitative yield after 3–4[14] hr. By this experiment, it was proved that aromatic phosphoramidates could be used for the protection of the terminal phosphate and that other protecting groups, such as 2'-benzoyloxy or exocyclic N-benzoyl, remain intact. A separate experiment showed that a 5'-monomethoxytrityl (MMTr) group, which is labile to acid, is also stable to this nitrite treatment.

Using this aromatic phosphoramidate we attempted[15] first to synthesize a hexanucleotide, CpCpApCpCpA, having the sequence of yeast alanine tRNA[16] (Figure 2) at the 3' end. This was achieved by condensing two trinucleotide units, MMTrC^{Bz}(OBz)pC^{Bz}(OBz)pA^{Bz}(OBz)p (4) and HOC^{Bz}(OBz)pC^{Bz}(OBz)pA^{Bz}(OBz)$_2$ (5). Compound 4 was obtained by reaction of 3 with 5'-O-monomethoxytrityl-$N,2'$-O-dibenzoylcytidine 3'-phosphate (6) with DCC as condensing reagent. After the appropriate work-up, the protected dinucleotide was treated with excess isoamyl nitrite (RONO) to afford MMTrC^{Bz}(OBz)pC^{Bz}(OBz)p (7) which was purified by diethylaminoethyl (DEAE) cellulose column chromatography. The trinucleotide 4 was obtained by further condensation of a mononucleotide, A^{Bz}(OBz)pNHC$_6$H$_4$OCH$_3$ (8), with 7 by the use of DCC. The yield in the final step was around 30%. Thus, properly protected trinucleotide 4 became

(12) V. M. Clark, G. W. Kirby, and A. R. Todd, *J. Chem. Soc.*, 1470 (1957).

(13) Abbreviations: DCC, *N,N*-dicyclohexylcarbodiimide; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; C, cytidine; A, adenosine; U, uridine; G, guanosine; T, thymidine; pA means adenosine 5'-phosphate; Ap means adenosine 3'-phosphate; MMTr, monomethoxytrityl; Bz, benzoyl; An, anisyl; iBu, isobutyryl; Ac, acetyl. Thus, MMTrC^{Bz}(OBz)p-A^{Bz}(OBz)p-means 5'-O-monomethoxytrityl-N^4-benzoylcytidylyl-(3'→5')-N^6,2'-O-dibenzoyladenosine 3'-phosphate. In structural formula 1, and others in this Account, the vertical line represents the "backbone" of carbons 1', 2', 3', and 4' of the ribose moiety, and the upward-slanting line at bottom left represents the C4'–C5' bond; the 2'- and 3'-oxygens and groups attached thereto are represented by horizontal bars to the right. The 5'-oxygen and group attached thereto is shown at lower left. The heterocyclic base moiety is represented by the usual letter at the top (*e.g.*, C for cytosine in 1), and the superscript "Bz" represents benzoylation of the cytosine amino group. The ring oxygen atom of the ribose moiety is not shown.

(14) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, 91, 1537 (1969).

(15) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, 92, 3441 (1970).

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

(4) R. Lohrmann and H. G. Khorana, *J. Amer. Chem. Soc.*, 88, 829 (1966).

(5) T. M. Jacob and H. G. Khorana, *J. Amer. Chem. Soc.*, 87, 368 (1965).

(6) R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H. G. Khorana, *J. Amer. Chem. Soc.*, 88, 819 (1966).
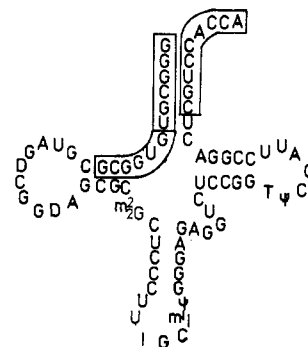
(7) H. G. Khorana, H. Büchi, H. Ghosh, N. Gupta, T. M. Jacob, W. Kössel, R. Morgan, S. A. Narang, E. Ohtsuka, and R. D. Wells, *Cold Spring Harbor Symp. Quant. Biol.*, 31, 39 (1966).

(8) V. E. Wünsch, *Angew. Chem.*, 83, 773 (1971).

(9) H. Kössel, M. W. Moon, and H. G. Khorana, *J. Amer. Chem. Soc.*, 89, 21483(1967).

(10) G. M. Tener, *J. Amer. Chem. Soc.*, 83, 159 (1961).

(11) M. Ikehara, S. Uesugi, and T. Fukui, *Chem. Pharm. Bull.*, 15, 440 (1967).

available in reasonable yield and in relatively large quantity (1 mmolar scale). However, the yields in the condensation steps were not satisfactory partly because of cleavage of internucleotidic linkages during the reaction.
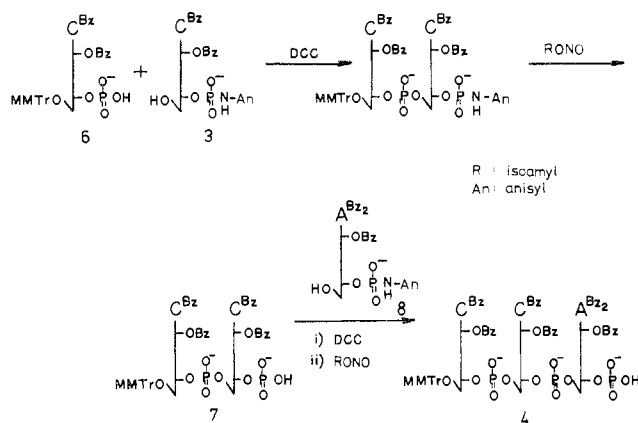

A trinucleotide, CpGpUp, corresponding to the seventh to ninth nucleotides of yeast alanine tRNA 3′ end, was synthesized also by essentially the same procedure.[17] The total yield was superior to that obtained by the cyclic phosphate method described below. The phosphoramidate method was also successfully applied to the synthesis of a deoxytrinucleotide and of three deoxydinucleotides containing N^6-benzoyl-2′-deoxyadenylic acid residues; the latter are known to be easily depurinated in acidic media.[18]

Synthesis of Ribotrinucleotides by the Use of Cyclic Phosphate as Protecting Group

Söll and Khorana[19] developed a method, using 2′,3′-cyclic phosphate protection of the 3′-end phosphate during the condensation reaction, to obtain dinucleotides. In order to obtain longer oligonucleotides, the terminal 2′,3′-cyclic phosphate must be cleaved by an appropriate RNase to regenerate the 3′-phosphate and the 2′-OH should be reprotected. This procedure is, therefore, applicable only when Up is at the 3′ terminus, because any protecting group on the heterocyclic NH_2 inhibits enzymatic hydrolysis.

A trinucleotide, CpGpUp, corresponding to the seventh to ninth nucleotides of yeast alanine tRNA 3′ end, was synthesized[20] as follows. MMTrG^{Ac}-(OAc)p (9) and U-2′- (or -3′-)p were condensed by the use of DCC to yield a dinucleotide 2′,3′-cyclic phosphate which was then treated with acid to expose the 5′-OH; a second nucleotide, MMTrC^{Bz}(OBz)p (11), was then condensed to afford a trinucleotide. In the course of work on this reaction, we found that a 2′- or 3′-phosphorylurea, which arose together with compound 10, could be quantitatively converted to the 2′,3′-cyclic phosphate by treatment with acetic acid–pyridine solution. Treatment of the product with pancreatic RNase gave the

3′-phosphate and reacylation of the 2′-OH using acetic anhydride in tetraethylammonium acetate gave properly protected MMTrC^{Bz}(OBz)pG^{Ac}(OAc)-pU(OAc)p (12). By means of the same procedure,
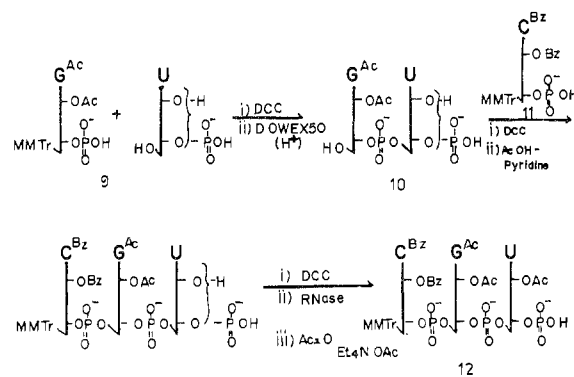
GpUpA was synthesized[21] by condensation of 2′,5′,N^2-triacetylguanosine 3′-phosphate with uridine 3′-phosphate followed by RNase cleavage and acetylation to yield AcG^{Ac}(OAc)pU(OAc)p, which was further condensed with 2,3′,N^6-tribenzoyladenosine. In this procedure it was possible to avoid time-consuming column chromatography and yet obtain a pure final product.

Condensation of Protected Oligonucleotide Blocks

Heretofore the condensation of ribooligonucleotides longer than three nucleotide units had not been done. To effect such a condensation without the assistance of enzymes would be of interest, especially because two trinucleotides described above (4 and 12) have defined sequences corresponding to units 69–71 and 72–74, respectively, of yeast alanine tRNA from the 3′ end.

Trinucleotide 5 having the sequence of units 75–77 of the tRNA was prepared according to Khorana's trinucleoside diphosphate synthesis[6] except that MMTrC^{Bz}(OBz)p was used for the 5′-terminal nucleotide. The condensation of the two trinucleotides 4 and 5, suitably provided with protecting groups, was attempted first by using DDC,[22] but it was not possible to isolate any condensation products. As it was postulated[23] that cyclic trimetaphosphates might be intermediates in the activation of phosphomonoesters, the reactive intermediate in the condensation seems to be too bulky for access to the 5′-OH end group of 5. Aromatic sulfonyl chlorides had been previously[4] found to be suitable activators of phosphomonoesters. Employing triisopropylbenzenesulfonyl chloride (TPS) as the condensing agent in this reaction enabled a hexanucleotide, MMTr-C^{Bz}(OBz)pC^{Bz}(OBz)A^{Bz}(OBz)pC^{Bz}(OBz)pC^{Bz}(OBz)-pA^{Bz}(OBz)$_2$ (13), to be isolated by column chromatography successively on Sephadex LH-20 and TEAE-cellulose though in 15% yield only.[22] Analysis of the deprotected hexanucleotide by digesting with RNase M[24] showed satisfactory agreement with the expected nucleotide ratio. Thus it was

(17) E. Ohtsuka, M. Ubasawa, S. Morioka, and M. Ikehara, *J. Amer. Chem. Soc.*, 95, 4725 (1973).

(18) E. Ohtsuka, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, 92, 5507 (1970).

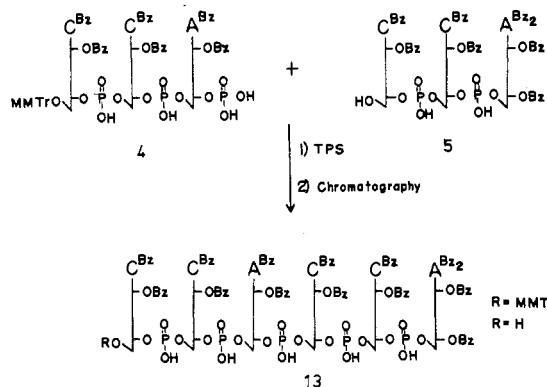(19) D. Söll and H. G. Khorana, *J. Amer. Chem. Soc.*, 87, 350 (1965).

(20) E. Ohtsuka, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, 92, 3445 (1970).

(21) E. Ohtsuka, H. Tagawa, and M. Ikehara, *Chem. Pharm. Bull.*, 19, 139 (1971).

(22) E. Ohtsuka, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, 93, 2296 (1971).

(23) G. Weimann and H. G. Khorana, *J. Amer. Chem. Soc.*, 84, 4329 (1962).

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

first proved that the block condensation method is useful for synthesis of longer ribooligonucleotides.

For further elongation of the hexanucleotide to no-nananucleotides with sequence authentic to alanine tRNA 3′ end, two routes were chosen: (i) Condensation of the hexa- with a trinucleotide and (ii) successive condensation of the hexa- with a mono- and then a dinucleotide.
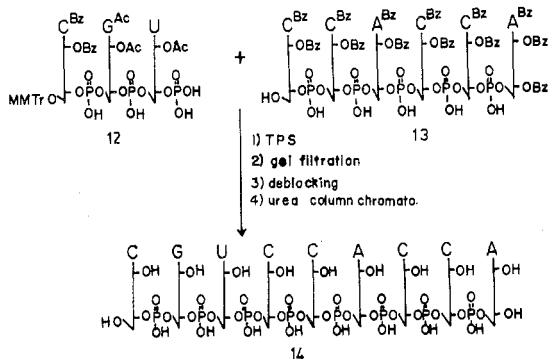
The hexanucleotide 13 (R = H) was condensed with trinucleotide 12 by the use of TPS. Purification of the nonanucleotide 14 was achieved after total deprotection using DEAE-cellulose in the presence of .7 M urea[25] at 50°. Essentially pure CpGpUpCpCp-ApCpCpAp (14) was obtained in a yield of 8%. The successive condensation of mono- and dinucleotides also afforded yields of 8 and 5% in two successive condensation steps. Characterization of these nonan-ucleotides by digestion with pancreatic RNase, RNase T_1, and spleen phosphodiesterase showed satisfactory results. The nonanucleotide 14, thus obtained, is the longest ribonucleotide ever synthesized chemically.
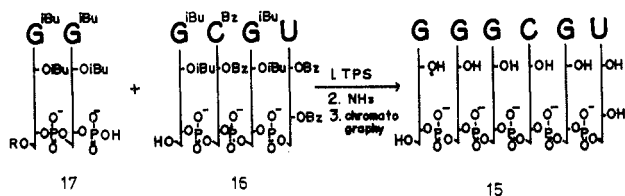
A hexanucleotide, GpGpGpCpGpU (15), having the sequence of yeast alanine tRNA 5′ end was synthesized[16] by condensing a tetranucleotide, G^{iBu}-(OiBu)pC^{Bz}(OBz)pG^{iBu}(OiBu)pU(OBz)$_2$ (16), with a dinucleotide, G^{iBu}(OiBu)pGiBu(OiBu)p (17), which was obtained by the polymerization of protected Gp. In this case also TPS was used as the condensing re-

(25) R. V. Tomlinson and G. M. Tener, *Biochemistry*, 2, 697 (1963).

agent and the isolated yield was 15%. Nonanucleotide (14) and this hexanucleotide (15), thus obtained, form the amino acid accepting arm of yeast alanine tRNA (Figure 2, in the upper two boxes). Reconstitution reactions of these chemically synthesized fragments with fragments obtained by RNase digestion of tRNA are now in progress.

Another feature of the synthesis by the phosphoramidate–nitrite method was attempted in the synthesis of yeast tyrosine tRNA[26] 5′ end. The previous finding that a limited digestion of this tRNA by RNase T_1 gave three portions involving one-fourth molecule of 5′ end led us to the synthesis of a dodeca-nucleotide having this sequence, because reconstitution of the synthetic dodecamer with other two parts of the molecule may restore the amino acid acceptor activity of the tRNA. For this purpose we condensed MMTrC^{Bz}(OBz)pG^{iBu}(OiBu)pG^{iBu}-(OiBu)p and HOU(OBz)pA^{Bz}(OBz)pm^{2}G(OBz)-pC^{Bz}(OBz)pC^{Bz}(OBz)$_2$ by the use of TPS. The resulting octanucleotide was purified successively by TEAE-cellulose, Sephadex LH-20, and Bio-Gel 2 column chromatography.[27] Further elongation of the octamer to the dodecanucleotide is being attempted.

In all of these block condensation reactions it was observed that (i) the yield was relatively low and (ii) chain scission of the block oligonucleotide occurred. In order to avoid chain scission during the reaction, protection of the phosphodiester linkage[28] may be necessary. Further investigation to find a more satisfactory activating agent for phosphomonoesters should be continued.[29]

Polymer Support Synthesis of Ribooligonucleotides

The polymer support synthesis[30] of polypeptides has been developed extensively. In the polynucleotide field, however, it has limited usefulness, because of relatively low yields in condensation steps and difficulties in the recovery of the product oligonucleotides after detachment from the polymer. Before 1971, investigations on the synthesis of polynucleotides were restricted to the deoxy series.[31] After we had completed several experiments, Yip and Tsou[32] reported a ribotrinucleotide synthesis using an isotactic polystyrene support.

We investigated polymer support synthesis[33] with aromatic phosphoramidate anchoring and detachment with isoamyl nitrite. Fully protected ribotrinucleotides, which could be used as the intermediate in the liquid-phase synthesis described above, were successfully isolated, demonstrating for the first time the feasibility of the method.

The terminal mononucleotide (17) was attached to an aminophenoxymethylenepolystyrene[34] (2% cross-

(26) T. Madison, G. A. Everett, and H. Kung, *Science*, 153, 531 (1966).

(27) A. Honda, unpublished experiments.

(28) R. L. Letsinger and K. K. Ogilvie, *J. Amer. Chem. Soc.*, 91, 3350 (1969).

(29) Recently the synthesis of GpUpm^{1}GpGpCp having the sequence of nucleotides 7–11 of the yeast alanine tRNA (Figure 2, in the third box) was accomplished by the stepwise condensation of 3′-nucleotides (M. Ohashi, unpublished experiments).

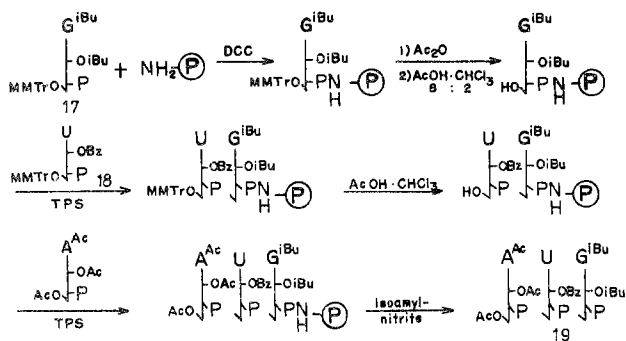(30) R. B. Merrifield, *Science*, 150, 178 (1965).

(31) References in L. R. Melby and D. R. Strobach, *J. Amer. Chem. Soc.*, 89, 450 (1967).

(32) K. F. Yip and K. C. Tsou, *J. Amer. Chem. Soc.*, 93, 3272 (1971).

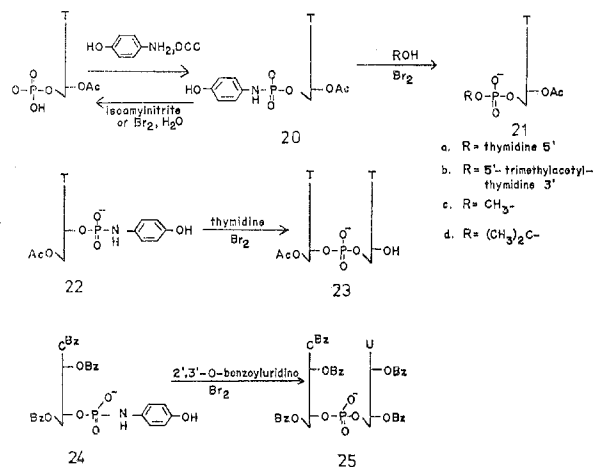(33) E. Ohtsuka, S. Morioka, and M. Ikehara, *J. Amer. Chem. Soc.*, 94, 3229 (1972).

Ribotrinucleotide Synthesis on the Polymer

linkages) by phosphoramidate bonds. After treatment with anhydrous acetic acid in chloroform to remove the methoxytrityl group, the second nucleotide (18) was condensed by the use of TPS. Repetition of these treatments and final column chromatography provided AcA^{Ac}(OAc)pU(OBz)pG^{iBu}(OiBu)p (19), which could be used as such for further condensation in liquid-phase synthesis or could be deprotected to afford ApUpGp. Thus, this phosphoramidate resin method may be useful for large-scale production of block oligonucleotides for use in the liquid-phase synthesis described above.

## Phosphate Protecting Groups Removable by Oxidation

Our previous investigation[11] of the decomposition of phosphoramidate by nitrite failed to produce phosphodiesters. However, if we use the amino moiety of a phosphoramidate as a leaving group, which after oxidation abstracts a pair of electrons from the phosphate, the resulting metaphosphate intermediate[35] must react either with an alcoholic or a phosphate hydroxyl group. Todd and coworkers[36] demonstrated the activation of hydroquinone monophosphate by oxidation with bromine. When an amidate of p-aminophenol (20) was synthesized from thymidine 5'-phosphate and oxidized with bromine in an excess of alcohol, thymidine 5'-phosphate alkyl esters (21) were produced in yields of 51–91%.[37] Reac-

a. R = thymidine 5'
b. R = 5'-trimethylacetyl-thymidine 3'
c. R = CH$_3$-
d. R = (CH$_3$)$_2$C-

tion of 5'-O-acetylthymidine 3'-phosphoro-p-oxanilidate (22) with thymidine gave TpT (23) in a yield around 40%. Although only in 24% yield, BzC^{Bz}(OBz)pU(OBz)$_2$ (25) was obtained from BzC^{Bz}(OBz)pNHC$_6$H$_4$OH-p (24) and U(OBz)$_2$. With improvement of the oxidation conditions and the leaving group, this reaction may qualify for practical use.

We found recently that p-aminophenoxy esters of nucleotides are also susceptible toward oxidative cleavage.[38] We synthesized, therefore, p-tritylaminophenol (26) and coupled it with 3'-O-acetylthymidine 5'-phosphate (27) using DCC. The resulting tritylaminophenoxy ester of 5'-pT(OAc) (28) was deacylated with alkali and a second nucleotide pT(OAc) (27) was condensed. This process was repeated twice more; the final product, TrNHC$_6$H$_4$pTpTpTpT, was treated with iodine at pH 7.5 to give (pT)$_4$ (29). The use of a tritylaminophenoxy group at the terminal phosphate enabled the extraction of condensation products in each step. Thus time-consuming column chromatography could be omitted except for the final stage. p-Trityl anilidate has been used by Yamazaki, et al.,[39] in deoxydinucleotide synthesis.

a : R Ac
b : R H
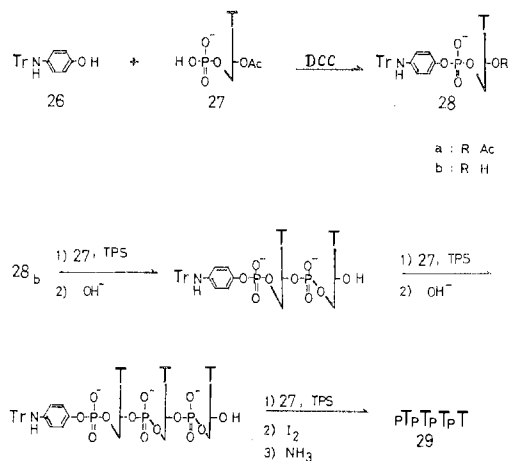
Concluding Remarks

I have described the chemical synthesis of *ribooli*gonucleotides to the level of nona- to dodecanucleotides. Such synthetic oligonucleotides are of interest on the one hand because they may be reconstituted with fragments obtained by the enzymatic digestion of natural tRNA to reconstitute biologically active nucleic acid. Such experiments, which have been performed in regard to proteins (*e.g.*, RNase S[40]), may provide valuable information on enzyme-nucleotide recognition. On the other hand, the total synthesis of a tRNA molecule by joining such oligoribonucleotides might be important in its own right. Furthermore, the chemical synthesis of unnatural, tRNA-like polynucleotides may open avenues to a more penetrating understanding of protein biosynthesis.

(34) G. M. Blackburn, M. J. Brown, and M. R. Harris, *J. Chem. Soc.*, 2438 (1967).

(35) P. Jencks and M. Gilchrist, *J. Amer. Chem. Soc.*, 86, 1410 (1964).

(36) V. M. Clark, D. W. Hutchinson, G. W. Kirby, and A. Todd, *J. Chem. Soc.*, 715 (1961).

(37) E. Ohtsuka, S. Morioka, and M. Ikehara, *Tetrahedron Lett.*, 2553 (1972).

(38) E. Ohtsuka, S. Morioka, and M. Ikehara, *J. Amer. Chem. Soc.*, 95, 8437 (1973).

(39) K. L. Agarwal, A. Yamazaki, and H. G. Khorana, *J. Amer. Chem. Soc.*, 93, 2754 (1971).

(40) K. Hofmann, J. P. Visser, and F. M. Finn, *J. Amer. Chem. Soc.*, 91, 4883 (1969).