$NH_4OAc/(NH_4)HCO_3$, pH 8) for 62 h at 37 °C. C-terminally desamidated bombesin was also digested in a control experiment. The three C-terminal amino acids of desamidated bombesin were released under the above conditions as determined by amino acid analysis whereas no amino acid was released from GRP under those conditions.

Sequence Analysis. Sequence analysis based on the Edman degradation of peptides was performed with a Beckman 890C spinning-cup sequencer modified according to Wittmann-Liebold.[28] A Quadrol single-cleavage program was used. Prior to the application of peptide, purified Polybrene was applied to the cup as a peptide carrier[48] and subjected to 8–10 sequencer cycles. More details about the sequence method employed here was described elsewhere.[39b] The identification and quantitation of the 3-phenyl-2-thiohydantoin derivatives of the cleaved amino acids (PTH amino acids) were accomplished by RP-HPLC with a Hewlett-Packard Liquid chromatograph, Model 1084 B. Details of the PTH amino acid determination will be described elsewhere (Spiess and Heil, in preparation). The sequencing method employed here allows for direct sequence analysis of 25–30 residues of 0.6–1.5 nmol of peptide, if one-third of each sequencer cycle is injected per HPLC run. The homogeneity of synthesized GRP was tested by subjecting GRP (14.4 nmol) to sequence analysis so that contaminating peptides with free N termini representing as little as 2% of the applied GRP could be detected.

Acknowledgment. This work was supported in part by an NINCDS grant (NS-14263) and in part by The Clayton Foundation for Research, California Division. Drs. Spiess, Brown, and Rivier are Clayton Foundation Investigators. W.M. is a recipient of a Swiss National Foundation Fellowship. Y.T. is a Scholar of the Medical Research Council of Canada. We thank Dr. M. Goodman, University of California, San Diego (USCD), for the CD spectrum and are indebted to W. Leupin and J. Feigon in Dr. Kearn's laboratory, UCSD, for providing us with the 300-MHz FT ^1H NMR spectrum. We also thank B. Karger and R. Eksteen of Northeastern University for developing the semipreparative C-18 RP-HPLC column (1 × 25 cm). R. Kaiser, R. Lee, F. Lux, J. Heil, R. McClintock, R. Allen, W. Bartholomeus, M. Newberry, L. Pacheco, and S. McCall are acknowledged for technical assistance.

(48) Tarr, G. E.; Beecher, J. F.: Bell, M.; McKean, D. J. *Anal. Biochem.* **1978**, *84*, 622–627.

Downloaded via SCRIPPS RESEARCH INST on December 19, 2022 at 15:52:40 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

Synthesis of Deoxyoligonucleotides on a Polymer Support[1]

M. D. Matteucci and M. H. Caruthers*

Contribution from the Department of Chemistry, University of Colorado, Boulder, Colorado 80309. *Received September 18, 1980*

Abstract: The development of a new method for synthesizing deoxyoligonucleotides is described. The synthesis begins by derivatizing high-performance liquid chromatography grade silica gel to contain 5′-*O*-(dimethoxytrityl)deoxynucleosides linked through the 3′-hydroxyl to a carboxylic acid functional group on the support. This matrix is then packed into a column which is attached to a pump and a series of valves. The chemical steps for the addition of one nucleotide to the support are as follows: (1) detritylation using $ZnBr_2$ in nitromethane (30 min); (2) condensation of a 5′-*O*-(dimethoxytrityl)deoxynucleoside (3′-methoxytetrazoyl)phosphine with the support-bound nucleoside (60 min); (3) blocking unreacted, support-bound nucleoside hydroxyl groups with diethoxytriazolylphosphine (5 min); (4) oxidation of phosphites to phosphates with I_2 (5 min). Completed deoxyoligonucleotides are isolated by sequential treatment with thiophenol and ammonium hydroxide, purification by reverse-phase chromatography, and treatment with 80% acetic acid. The method is extremely fast (less than 2.5 h are needed for each nucleotide addition cycle), yields in excess of 95% per condensation are obtained, and isolation of the final product is a simple one-step column purification. The syntheses of d(C-G-T-C-A-C-A-A-T-T) and d(A-C-G-C-T-C-A-C-A-A-T-T) were carried out as a test of this method. Yields of support-bound deoxyoligonucleotides were 64% and 55%; the isolated yield of deoxydecanucleotide was 30%. Both synthetic products were homogeneous and biologically active by every criteria so far tested.

Synthetic deoxyoligonucleotides of defined sequence have been used to solve important biochemical[2,3] and biophysical[4,5] problems. Moreover, recent advances in chemical methods have led to the synthesis of genes[6–10] and of deoxyoligonucleotides useful for manipulating natural DNA and RNA.[11] Despite these achievements, the synthesis and isolation of deoxyoligonucleotides remains a difficult and time consuming task. Ideally, chemical methods should be simple, rapid, versatile, and completely automatic. In this way the rapid synthesis of genes and gene control regions can be realized and many important biochemical studies which are presently not possible can be initiated.

Our approach to solving this problem has involved developing methods for synthesizing deoxyoligonucleotides on polymer supports. This concept is not new and has been investigated extensively.[12,13] Recently several promising approaches have been

(1) This is paper IV in a series on nucleotide chemistry. Paper III: M. H. Caruthers, S. L. Beaucage, J. W. Efcavitch, E. F. Fisher, M. D. Matteucci, and Y. Stabinsky, *Nucleic Acids Symp. Ser.*, No. 7, 215 (1980). This research was supported by the National Institutes of Health (Grants GM 21120 and GM 25680).

(2) H. G. Khorana, H. Büchi, H. Ghosh, N. Gupta, T. M. Jacob, H. Kössel, R. Morgan, S. A. Narang, E. Ohtsuka, and R. D. Wells, *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 39 (1966).

(3) M. H. Caruthers, *Acc. Chem. Res.*, **13**, 155 (1980).

(4) A. H.-J. Wang, G. J. Quiqley, F. J. Kolpak, J. L. Crawford, J. H. van Boom, G. vd Marel, and A. Rich, *Nature (London)*, **282**, 680 (1979).

(5) F. H. Martin and I. Tinoco, Jr., *Nucleic Acids Res.*, **8**, 2295 (1980).

(6) K. L. Agarwal, Büchi, M. H. Caruthers, N. Gupta, H. G. Khorana, K. Kleppe, A. Kumar, E. Ohtsuka, U. L. Rajbhandary, J. H. van de Sande, V. Sgaramella, H. Weber, and T. Yamada, *Nature (London)*, **227**, 27 (1970).

(7) H. G. Khorana, *Science*, **203**, 614 (1979).

(8) K. Itakura, T. Hirose, R. Crea, A. D. Riggs, H. L. Heyneker, F. Bolivar, and H. W. Boyer, *Science*, **198**, 1056 (1977).

(9) D. V. Goeddel, D. G. Kleid, F. Bolivar, H. L. Heyneker, D. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura, and A. Riggs, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 106 (1979).

(10) D. V. Goeddel, H. L. Heyneker, T. Hozumi, R. Arentzen, K. Itakura, D. G. Yansura, M. J. Ross, G. Miozzari, R. Crea, and P. Seeburg, *Nature (London)*, **281**, 544 (1979).

(11) R. J. Rothstein, L. F. Lau, C. D. Bahl, S. A. Narang, and R. Wu, *Methods Enzymol.*, **68**, 98 (1979).

(12) C. B. Reese, *Tetrahedron*, **34**, 3143 (1978).

(13) V. Amarnath and A. D. Broom, *Chem. Rev.*, **77**, 183 (1977).

0002-7863/81/1503-3185$01.25/0 © 1981 American Chemical Society

Shanghai Hongene Exhibit 1005

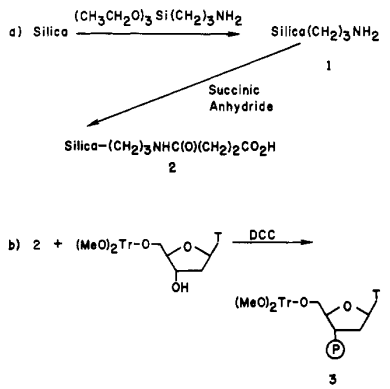

Figure 1. Steps in the synthesis of silica gel containing covalently joined deoxynucleosides.

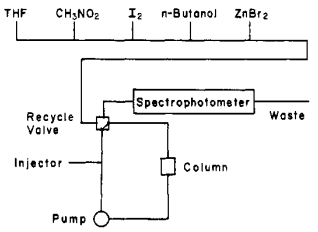

Figure 2. Schematic diagram of the apparatus used for polynucleotide synthesis. Five flasks containing two solvents (tetrahydrofuran and nitromethane) and three reactants are represented in the top part of the diagram. The complete compositions of these reaction solutions (I_2, *n*-butanol, and $ZnBr_2$) are included in the Experimental Section.

proposed.[14-17] These methods utilize various organic polymers and phosphate-activated di- and trinucleotides as condensing agents. We report in this paper a completely different method. The polymer is high-performance liquid chromatography (HPLC) grade silica gel, and activated nucleotide phosphites are used as condensing agents. A preliminary account of this approach in a form applicable only to deoxyoligopyrimidines has been reported.[18]

Outline of the Procedure

Support. Extensive research has been directed toward developing silica gels containing different functional groups for use in HPLC. These polymers have been designed for efficient mass transfer. We therefore anticipated and have since shown that reactants and reagents can rapidly be removed from these derivatized silica gels after various synthesis steps. Additionally, silica gel is a rigid, nonswellable matrix in common organic solvents. It can be packed into a column and reactants merely pumped through the column. These features make HPLC grade silica gel an attractive support for deoxyoligonucleotide synthesis. For our work, we use a silica gel that has been derivatized to contain a carboxylic acid group (200 μmol/g of silica). The scheme developed for synthesis of this support is outlined in Figure 1, reaction scheme a. The functionalized, insoluble support was prepared from a 20-μm particle size, macroporous (300 Å) silica gel. The initial step involved refluxing (3-aminopropyl)triethoxysilane with silica gel in dry toluene for 3 h. Succinic anhydride was next reacted with **1** in order to generate **2**. Excess silanol groups were eliminated by treatment with $(CH_3)_3SiCl$. For the synthesis of **3** (Figure 1, reaction scheme b), 5′-*O*-(dimethoxytrityl)thymidine was condensed with **2** by using dicyclohexylcarbodiimide (DCC) in anhydrous pyridine.[19] After 40 h, residual acid groups were converted to an inert amide by addition first of *p*-nitrophenol and then morpholine. The yield of **3** was 40 μmol/g of reactant. No attempt has been made to maximize the amount of nucleoside covalently joined to the support. This procedure is quite general and has been used for the synthesis of supports containing the 5′-*O*-dimethoxytrityl derivatives of *N*-benzoyldeoxycytidine, *N*-benzoyldeoxyadenosine and *N*-isobutyryldeoxyguanosine.[20]

Table I. Protocol for Machine-Assisted Polynucleotide Synthesis

reagent or solvent[a,b]	time, min	machine mode
satd $ZnBr_2/CH_3NO_2$	30	flush
$CH_3(CH_2)_2CH_2OH$/2,6-lutidine/THF	5	flush
THF	10	flush
activated nucleotide	60	recycle
$(CH_3CH_2O)_2P$ (triazole)	5	recycle
THF	2	flush
I_2 oxidation	5	flush
THF	5	flush
CH_3NO_2	3	flush

[a] THF = tetrahydrofuran. [b] A nitromethane solution saturated with $ZnBr_2$ is approximately 0.1 M in $ZnBr_2$.

Table II. Investigation of Detritylation and Depurination Conditions[a,b]

conditions[c]	detritylation time, min	depurination %/time
0.1 N toluenesulfonic acid/THF	5	50/20 min
5% BF_3/1% 2,6-di-*tert*-butylpyridine/acetonitrile	10	50/120 min
2.5% $AlCl_3$/1% 2,6-di-*tert*-butylpyridine/acetonitrile	10	50/8 h
2.0% $TiCl_4$/3% 2,6-di-*tert*-butylpyridine/acetonitrile	3	10/4 h
<0.1 M $ZnBr_2$ (saturated)/nitromethane	15	none/24 h

[a] Analytical reaction: $d[(MeO)_2TrbzA\text{-}T(P)] \xrightarrow{acid} d(bzA\text{-}T(P)) + (MeO)_2Tr\text{-}OH$. [b] All depurination results were obtained by analyzing reaction mixtures using reverse-phase HPLC. Estimates of the time required for 100% detritylation were by trityl assay (see Experimental Section). [c] 2,6-Di-*tert*-butylpyridine was used to specifically buffer protic acids [H. C. Brown and B. Kanner, *J. Am. Chem. Soc.*, 88, 986 (1966)] which potentially could be generated via hydrolysis of highly reactive Lewis acids (BF_3, $AlCl_3$, and $TiCl_4$) by trace amounts of water.

Synthesis Machine. A schematic of the apparatus is shown in Figure 2. Machine-assisted synthesis of deoxyoligonucleotides begins by loading the column with 0.20–0.30 g of **3** or one of the other silica gels containing a covalently joined deoxynucleoside. The protocol listed in Table I is then followed for the addition of one nucleotide. Solvents and reactants can be selected through a series of three-way valves and then either cycled once or recycled many times through the column. An injector port is used for the addition of activated nucleotides, and the efficiency of various wash cycles is monitored by a spectrophotometer. The major steps that form a part of this protocol are outlined in the following sections.

Removal of 5′-*O*-Dimethoxytrityl Ethers. *p*-Toluenesulfonic acid in acetonitrile rapidly removes the 5′-*O*-dimethoxytrityl group from either a deoxypyrimidine nucleoside such as **3** or a deoxyoligopyrimidine attached covalently to silica gel.[18] However, when purines were examined, this procedure was unsatisfactory. These results are outlined in Table II. When a dinucleotide attached to silica gel and containing *N*-benzoyldeoxyadenosine and thy-

(14) M. J. Gait, N. Singh, R. C. Sheppard, M. D. Edge, A. R. Greene, G. R. Heathcliff, T. C. Atkinson, C. R. Newton, and A. F. Markham, *Nucleic Acids Res.*, **8**, 1081 (1980).
(15) K. Miyoshi and K. Itakura, *Tetrahedron Lett.*, **38**, 3635 (1979).
(16) V. Potapov, V. Veiko, O. Koroleva, and Z. Shabarova, *Nucleic Acids Res.*, **6**, 2041 (1979).
(17) R. Crea and T. Horn *Nucleic Acids Res.*, **8**, 2331 (1980).
(18) M. D. Matteucci and M. H. Caruthers, *Tetrahedron Lett.*, **21**, 719 (1980).

(19) Abbreviations for nucleosides, nucleotides, oligonucleotides, and protected deoxyoligonucleotides are according to the IUPAC-IUB Commission on Biochemical Nomenclature Recommendations [*Biochemistry*, **9**, 4022 (1970)]. The symbol (P) represents the insoluble, derivatized silica gel.
(20) Unpublished results of S. Beauage and C. Becker.

Shanghai Hongene Exhibit 1005

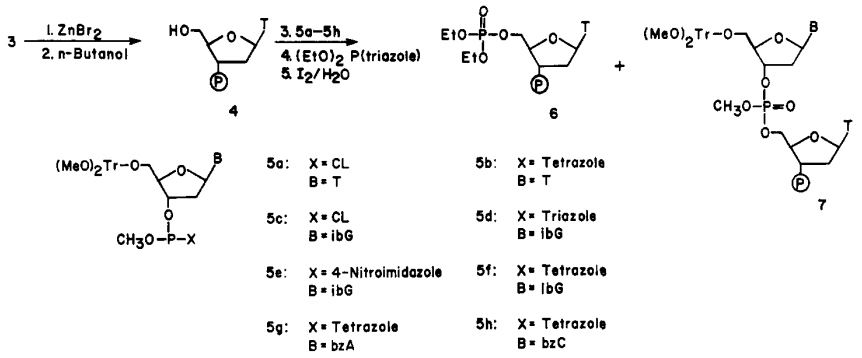

Figure 3. Steps in the synthesis of a dinucleotide. Purine bases (B) are abbreviated as bzA for *N*-benzoyladenine and ibG for *N*-isobutyrlguanine. Pyrimidine bases (B) are abbreviated as bzC for *N*-benzoylcytosine and T for thymine.

midine, d[$(MeO)_2$TrbzA-T(P)], was treated with *p*-toluenesulfonic acid, detritylation was complete in 5 min. However, in 20 min, 50% depurination had also occurred. Thus this procedure cannot be used for repetitive detritylation of purine containing deoxyoligonucleotides during their synthesis on a polymer support. Because of this depurination problem, various Lewis acids were investigated as potential detritylating reagents.[21] As can be seen from the results reported in Table II, a saturated solution containing $ZnBr_2$ in nitromethane was superior to the other Lewis acids tested. Detritylation was complete within 15 min and depurination was not detected even after 24 h. Detritylation was also most rapid when the 5′-deoxynucleoside was a purine (10–15 min) rather than a pyrimidine (30 min). On the basis of the results presented in Table II, $TiCl_4$ also appeared to be a potentially useful detritylating reagent. However, since $TiCl_4$ is very easily hydrolyzed, it was not examined further. The reaction of nucleosides with $ZnBr_2$ has been studied in some detail.[22,23] Deprotection appears to proceed via a bidentate chelation mechanism involving the 5′ and deoxyribose ring oxygens. Furthermore, in an anhydrous nitromethane solution containing $ZnBr_2$, deprotection generated a compound attached to the support which was not fully reactive toward the phosphite coupling reagent. Presumably this intermediate was a zincate ester. A mildly basic hydrolytic wash with *n*-butanol in tetrahydrofuran and 2,6-lutidine was sufficient to regenerate a free 5′-hydroxyl group. The detritylation reaction as outlined in Figure 3 therefore consisted of two steps. Intially, compound **3** or any other protected nucleoside attached to silica gel was allowed to react for 30 min with a saturated solution of $ZnBr_2$ in nitromethane. The next step (5 min) was a hydrolytic wash with *n*-butanol in tetrahydrofuran and 2,6-lutidine. This procedure was also used for removing a 5′-dimethoxytrityl group from a deoxyoligonucleotide attached to the silica gel.

Condensation of Activated Nucleotides to the Polymer Support. Letsinger and Lunsford have shown that thymidine deoxyoligonucleotides can be synthesized by using phosphite triester intermediates and that protecting groups commonly used in deoxyoligonucleotide synthesis are stable to phosphorodichloridites.[24] Because these reactions proceed rapidly in high yield (95% for synthesis of the dinucleotide), this method appeared very attractive for adaptation to polymer-supported deoxyoligonucleotide synthesis. We have modified this procedure by using methyl phosphorodichloridite in place of trichloroethyl phosphorodichloridite and by using an intermediate activated deoxynucleoside phosphite containing tetrazole in place of the deoxynucleoside phosphorochloridite. As outlined in Figure 3 for the synthesis of a dinucleotide, the formation of the internucleotide bond therefore involves a reaction between a deoxynucleoside attached to the silica gel and an appropriately protected deoxynucleoside phosphite.

Table III. Comparison of Phosphitylating Reagents[a]

deoxy-nucleotide[b]	reaction time, min	dinucleotide yield,[c] %
5a	60	90
5b	60	95
5a	5	30
5b	5	85
5c	60	80
5d	60	75
5e	60	75
5f	60	95

[a] The analytical reaction involved step 3 and step 5 of the reaction scheme outlined in Figure 3. [b] The deoxynucleotides tested are defined in the key presented in Figure 3. [c] The yield of dinucleotide was obtained by analyzing reaction mixtures using reverse-phase HPLC.

Selection of the methyl triester protecting group was based on several considerations. Our initial experiments with trichloroethyl dichlorophosphite gave satisfactory condensation yields which were comparable to those reported previously. Removal of this blocking group, however, created serious problems. Since a base-labile protecting group anchors the deoxyoligonucleotide to the support, the triester protecting group must be removed prior to release of the deoxyoligonucleotide from the support. Otherwise the basic conditions present during release from the support would cause considerable internucleotide bond cleavage and rearrangement of the deoxyribose skeleton.[25,26] Reductive cleavage procedures involving heterogeneous solutions[27] were not investigated, and those involving homogeneous solutions[28] gave unsatisfactory results. We therefore turned our attention to the methyl group which can be removed by using thiophenol.[29,30] Extensive investigations have not revealed any internucleotide bond cleavage with this reagent. A 5-min exposure of the methyl triester of d(T-T) to the thiophenoxide reagent resulted in complete deprotection to the diester. Further treatment of d(T-T) for 50 h resulted in no detectable degradation. This presumably is because a methyl group is much more reactive than a methylene group toward attack by thiophenoxide, and, once the methyl group has been removed, the phosphodiester is relatively stable toward nucleophilic attack.

Initial studies involving CH_3OPCl_2 suggested that the yields per deoxynucleotide additon were satisfactory (90%) but not

(21) We thank R. L. Letsinger for suggesting that BF_3 might be useful for detritylating nucleosides.
(22) M. D. Matteucci and M. H. Caruthers, *Tetrahedron Lett.*, **21**, 3243 (1980).
(23) V. Kohli, H. Blöcker and H. Köster, *Tetrahedron Lett.*, **21**, 2683 (1980).
(24) R. L. Letsinger and W. B. Lunsford, *J. Am. Chem. Soc.*, **98**, 3655 (1976).
(25) J. H. van Boom, P. M. J. Burgers, P. H. van Deursen, J. F. M. de Rooy, and C. B. Reese, *J. Chem. Soc., Chem. Commun.*, 167 (1976).
(26) A. Myles, W. Hutzenlaub, G. Reitz, and W. Pfleiderer, *Chem. Ber.*, **108**, 2857 (1975).
(27) F. Eckstein and I. Rizk, *Angew. Chem., Int. Ed. Engl.*, **6**, 695 (1967).
(28) R. L. Letsinger and J. L. Finnan, J. Am. *Chem. Soc.*, **97**, 7197 (1975).
(29) G. W. Daub and E. E. van Tamelen, *J. Am. Chem. Soc.*, **99**, 3526 (1977).
(30) Recently amines have also been shown to remove the methyl group without oligonucleotide degradation [D. J. H. Smith, K. K. Ogilvie, and M. F. Gillen, *Tetrahedron Lett.*, **21**, 861 (1980)].

Shanghai Hongene Exhibit 1005

sufficient for a repetitive, multistep synthesis.[18] Furthermore, the production of collidine hydrochloride during the coupling step led to blockage and flow restriction in our machine-assisted synthesis. Investigations of leaving groups other than chloride (triazole, tetrazole, and 4-nitroimidazole) were therefore initiated and these results are reported in Table III. As can be seen by comparing these results, the activated nucleotide containing tetrazole was superior since the yield exceeded 95% and the condensation rate was faster than the parent chloridite.

The activated deoxynucleotides were prepared via a two-step procedure involving the formation of the deoxynucleoside phosphorochloridite followed by conversion to the tetrazolide. An important feature of this procedure is that all key steps are completed under an inert gas atmosphere (nitrogen or argon). 5′-*O*-Dimethoxytrityl and base-protected deoxynucleosides in tetrahydrofuran were added dropwise to a well-stirred solution of CH_3OPCl_2 and 2,4,6-trimethylpyridine (collidine) in tetrahydrofuran at −78 °C. Collidine hydrochloride was removed by filtration. Initial results with **5a**, the phosphorochloridite, in the condensation reaction gave only about a 50% yield of d(T-T) and the synthesis of several side products. These side products were shown to result from trace amounts of CH_3OPCl_2 which were present in the reaction mixture. Since reactions on polymer supports usually require an excess (typically tenfold) of the incoming activated deoxynucleotide, trace amounts of unreacted CH_3OPCl_2 can become significant. Methyl dichlorophosphite would be kinetically more reactive and therefore effectively compete with the activated deoxynucleotide for the 5′-hydroxyl of the deoxynucleoside or deoxyoligonucleotide attached to the support. The unwanted side products persisted even when the molar ratio of deoxynucleoside to CH_3OPCl_2 was 1:0.75. However, these side products were completely eliminated and the condensation yield was improved to 85–90% by repeatedly concentrating reaction mixtures containing the activated nucleotide (the monochloridite) to a gum by using a solution of toluene and THF. This procedure presumably removes the volatile CH_3OPCl_2. By use of this repetitive evaporation step, the molar ratio of 5′-*O*-(dimethoxytrityl)deoxynucleoside (thymidine, *N*-benzoyldeoxycytidine, and *N*-benzoyldeoxyadenosine) to CH_3OPCl_2 could be lowered to 1:1.2. This ratio produces a minimum amount of 3′–3′ dimer and a maximum yield of the expected product. By use of polymer-support chemistry, the presence of 3′–3′ dimer is not a serious problem but simply reduces the yield of activated nucleotide. It is inert toward further reaction and, unlike conventional solution approaches, can be readily removed simply by washing the silica gel. For *N*-benzoyl- or *N*-isobutyryldeoxyguanosine, a molar ratio of deoxynucleoside to CH_3OPCl_2 of 1:0.9 was used because of difficulties encountered in removing methyl dichlorophosphite, even by repetitive evaporation with toluene and tetrahydrofuran. These deoxynucleoside phosphorochloridites were next converted to the tetrazolide by further reaction with 1 molar equiv of tetrazole in tetrahydrofuran. Collidine hydrochloride was removed by filtration, and the activated nucleotides were isolated by precipitation into pentane. After removal of pentane, these tetrazolides are stable for at least 3 months when stored as anhydrous glasses under an inert gas at −20 °C.

Condensations usually were completed by using approximately a tenfold excess of the activated mononucleotides. These activated nucleotides as anhydrous glasses were dissolved in tetrahydrofuran, applied to the column through the injector, and recycled through the column for 1 h at room temperature. Usually each reconstituted aliquot contained enough activated nucleotide for three or four condensations on 0.2–0.3 g of derivatized silica gel.

Capping and Oxidation Steps. On the basis of HPLC analysis of condensation reactions, approximately 1–5% of the deoxynucleoside or deoxyoligonucleotide bound to the support does not react with the activated deoxynucleotide. These unreactive compounds must be blocked or capped in order to prevent the formation of several deoxyoligonucleotides with heterogeneous sequences. This capping step can best be accomplished by using a large excess of a very reactive phosphite such as diethoxytriazoylphosphine which would react with deoxynucleosides and deoxyoligonucleotides to form a 5′-diethylphosphite, a relatively nonhydrophobic triester (step 4, Figure 3). More traditional reagents such as acetic anhydride and phenyl isocyanate were tried but reacted much more slowly with the unblocked 5′-hydroxyl group. Since purification involves reverse-phase HPLC, this capping reagent assures that all nonhydrophobic failure sequences can be readily separated from the synthetic deoxyoligonucleotide product containing a hydrophobic 5′-*O*-dimethoxytrityl group.

The final step involves oxidation of these phosphites to the corresponding phosphates with I_2 in water, 2,6-lutidine, and tetrahydrofuran (step 5, Figure 3). As has been reported previously[24] and confirmed by us, this oxidation step is sufficiently mild so that side products are not generated. We have attempted to postpone the oxidation until after all condensation steps, but the results have not been encouraging. Several uncharacterized side products were observed.

Removal of Protecting Groups. Upon completion of the synthesis, silica gel containing the deoxyoligonucleotide can be stored at −20 °C. The initial deprotection step involves removal of the methyl group from phosphotriesters by using triethylammonium thiophenoxide in dioxane. This step is followed by treatment with concentrated ammonium hydroxide at 20 °C for 3 h to hydrolyze the ester joining the deoxyoligonucleotide to the support. After centrifugation and recovery of the supernatant containing the deoxyoligonucleotide, the *N*-benzoyl groups from deoxycytosine, deoxyadenosine, and the *N*-isobutyrl group from deoxyguanosine are removed by warming at 50 °C for 12 h. The final purification steps are isolation of the 5′-*O*-dimethoxytrityl containing deoxyoligonucleotide by reverse-phase HPLC followed by removal of the 5′-dimethoxytrityl group by using 80% acetic acid.[31]

Results

The feasibility of this approach for synthesizing deoxyoligonucleotides was examined by preparing d(A-C-G-C-T-C-A-C-A-A-T-T) and d(C-G-T-C-A-C-A-A-T-T). These syntheses provide a rigorous test of our methodology because all four bases are present in each deoxyoligonucleotide. Moreover, *N*-benzoyldeoxyadenosine, the purine most susceptible to depurination, is present at multiple positions within the first half of the synthesized molecules. Therefore, *N*-benzoyldeoxyadenosine must undergo several detritylation cycles and, consequently, multiple exposures to potential depurination conditions before each synthesis is completed. Initially, d(T-C-A-C-A-A-T-T), a deoxyoctanucleotide common to both final products, was synthesized by using **5b,g,h** and the apparatus diagrammed in Figure 2. The procedure outlined in Table I was followed. Aliquots of silica gel containing this deoxyoctanucleotide were then separately extended to complete the synthesis of the deoxydecanucleotide and deoxydodecanucleotide. Since the dimethoxytrityl group was alternately joined to the support as part of each activated deoxynucleotide and then removed before the next deoxynucleotide addition, condensation reactions were monitored by measuring the amount of dimethoxytritanol released following each detritylation. A constant amount would be expected if condensations were quantitative. We observed in all cases that condensations did proceed with high yields of approximately 95%. The amount of dimethoxytritanol released after the final condensation steps indicated that the overall yields of deoxydecanucleotide and deoxydodecanucleotide were 64% and 55%, respectively. At the conclusion of each deoxyoligonucleotide synthesis, the triesters were converted to diesters by treatment with triethylammonium thiophenoxide. The expected products and all polymer-bound intermediates were then freed from the support and base-labile protecting groups by treatment with concentrated ammonium hydroxide and analyzed by reverse-phase HPLC. The elution profile of an aliquot of the reaction mixture obtained from the synthesis of d(C-G-T-C-A-C-A-A-T-T) is shown in Figure 4, panel A. Peak I which elutes in the void volume contains deoxyoligo-

(31) The preferred detritylating reagent after the removal of amino protecting groups is 80% acetic acid. Depurination is not observed with completely deprotected deoxyoligonucleotides, and, unlike $ZnBr_2$, 80% acetic acid is volatile and easily removed.

Shanghai Hongene Exhibit 1005

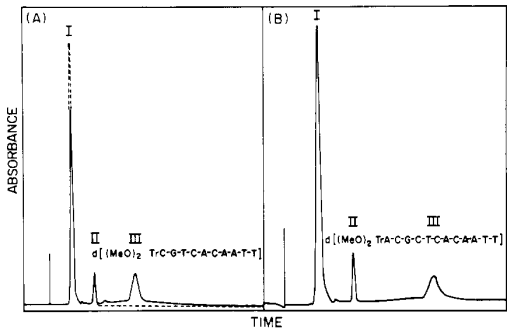

Figure 4. Analysis of reaction mixtures from the preparation of d(C-G-T-C-A-C-A-A-T-T) in panel A and d(A-C-G-C-T-C-A-C-A-A-T-T) in panel B: the solid line is the HPLC elution profile of reaction mixtures containing deoxyoligonucleotides with a 5′-dimethoxytrityl group; the dashed line is the HPLC elution profile of a reaction mixture containing deoxyoligonucleotides in a completely deprotected form. The eluting buffer was 0.1 M triethylammonium acetate in 26% acetonitrile.

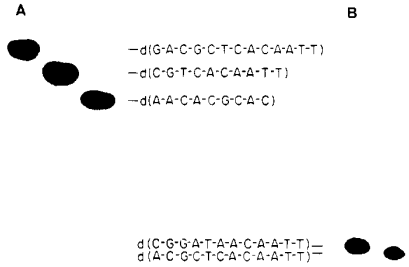

Figure 5. Analysis of ^{32}P-phosphorylated deoxyoligonucleotides by gel electrophoresis: gel A, the results with [5′-^{32}P]d(pC-G-T-C-A-C-A-A-T-T); gel B, the results with [5′-^{32}P]d(pA-C-G-C-T-C-A-C-A-A-T-T). The mobility of characterized markers of known sequence are also shown on the gels. Both gels were 20% acrylamide and 1% *N,N*-methylenebisacrylamide in 89 mM Tris borate (pH 8.3), 2.2 mM EDTA, and 7 M urea. (The positive electrode was at the bottom of the figure.)

nucleotides corresponding to failure sequences. Peak II was nonnucleotidic. Peak III was shown to be the expected product, d[(MeO)$_2$TrC-G-T-C-A-C-A-A-T-T], isolated in 30% overall yield based on the amount of thymidine attached to the silica gel.[32] As a first step in further characterization of the reaction mixture, a second aliquot was treated with 80% acetic acid and then analyzed by reverse-phase HPLC. The results are also presented in panel A of Figure 4. Peak III has disappeared, and the amount of UV-absorbing material recovered in the column void volume has increased considerably. Both observations are consistent with peak III containing a deoxyoligonucleotide with a hydrophobic dimethoxytrityl group. The deoxyoligonucleotide recovered from peak III was treated with 80% acetic acid and further characterized. One aliquot was shown to be completely degraded with snake venom phosphodiesterase. A second aliquot was analyzed by reverse-phase column chromatography using several acetonitrile concentrations in 0.1 M triethylammonium acetate as the eluting buffer. In all cases the detritylated materials eluted from the column as one peak, suggesting that the sample was homogeneous. A third aliquot was further characterized after phosphorylating the 5′-hydroxyl with [^{32}P]phosphate by using [γ-^{32}P]ATP and T4 kinase. On the basis of the UV absorbance and a calculated extinction coefficient, the phosphorylation was quantitative.[33] After removal of excess [γ-^{32}P]ATP by gel filtration, the phosphorylated material isolated from peak III was analyzed by gel electrophoresis, and these results are reported in Figure 5, panel

(32) We routinely recover only 40–60% of various deoxyoligonucleotides which are applied to C_{18} HPLC columns. Such low recoveries have been observed irrespective of which synthetic methodology is used.

(33) Because of hypochromic effects, the absolute amount of deoxydecanucleotide cannot be estimated exactly, and therefore the possibility exists that 5–10% of the sample was not phosphorylated.

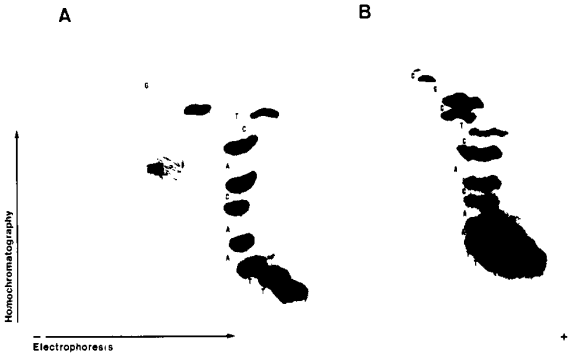

Figure 6. Two-dimension sequence analysis: panel A, [5′-^{32}P]d(pC-G-T-C-A-C-A-A-T-T); panel B, [5′-^{32}P]d(pA-C-G-C-T-C-A-C-A-A-T-T). Electrophoresis was along the longitudinal axis and homochromatography along the vertical axis. Nucleotide losses are recorded between the appropriate spots.

A. As can be seen by inspection of this gel, only one radioactive deoxyoligonucleotide was observed, indicating that the capping step was extremely efficient. Because separation on the reverse-phase column was dependent strictly on the presence of a dimethoxytrityl group and not the size of the deoxyoligonucleotide, incomplete capping would have led to several bands corresponding to deoxyoligonucleotides of shorter length. Furthermore, the mobility of the phosphorylated compound relative to characterized markers on the same gel indicated that the compound was a deoxydecanucleotide. The labeled deoxyoligonucleotide was then analyzed by the two-dimensional procedure,[34] and these results are reported in Figure 6, panel A. As can be seen by these results, the data were consistent with a decanucleotide having the structure [5′-^{32}P]d(pC-G-T-C-A-C-A-A-T-T). Moreover, the analysis indicated that the deoxydecanucleotide was essentially homogeneous since only trace amounts of labeled material migrate at positions other than those expected. This data also indicated that the capping procedure was quantitative and that the chemistry did not cause modifications of various nucleotides. Either circumstance would have led to additional spots as part of the two-dimensional analysis pattern.

A similar purification and analytical procedure was used to characterize d(A-C-G-C-T-C-A-C-A-A-T-T). Fractionation by reverse-phase HPLC of the ammonium hydroxide hydrolysate obtained from silica gel is shown in Figure 4, panel B. The isolated yield of deoxydodecanucleotide was not determined accurately. Peak III containing nucleotidic material and a hydrophobic dimethoxytrityl group was characterized following treatment with acetic acid and phosphorylation with [^{32}P]phosphate. Quantitative phosphorylation was observed. The analysis by gel electrophoresis is shown in Figure 5 (panel B), and the two dimensional analysis is shown in Figure 6, panel B. Once again, all these results are consistent with peak III being composed of an essentially homogeneous sample of the expected deoxyoligonucleotide, d(A-C-G-C-T-C-A-C-A-A-T-T).

Several new chemical reagents and procedures were developed in conjunction with this approach. These include the design of a silica gel matrix, the development of a new phosphitylating reagent, and the discovery of new procedures for removing trityl groups. The approach appears extremely promising as a method for synthesizing biologically active polynucleotides. Nucleotides can be added sequentially every 2 h to a growing deoxyoligonucleotide with yields exceeding 95% for each condensation. Work is in progress to interface this polymer-support procedure into a completely automatic, microprocessor-controlled machine.

Experimental Section

The solvents must be anhydrous and pure. Reagent grade tetrahydrofuran was distilled from sodium benzophenone ketal under inert gas

(34) F. Sanger, J. E. Donelson, A. R. Coulson, H. Kössel, and O. Fischer, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 1209 (1973).

Shanghai Hongene Exhibit 1005

as needed. Reagent grade toluene and reagent grade pentane were dried by distillation from CaH_2. 2,4,6-Trimethylpyridine, pyridine, and 2,6-lutidine were distilled first from *p*-toluenesulfonyl chloride and then CaH_2. Reagent grade nitromethane was distilled from CaH_2 and then stored over Linde 4A molecular sieves. 1,2,4-Triazole, 1*H*-tetrazole, 4-nitroimidazole, and diethyl phosphorochloridite were purchased from Aldrich Chemical Co. and used without further purification. 1,2,4-Triazole, 4-nitroimidazole, and 1*H*-tetrazole were dried in vacuo over Drierite before use.

The amount of 5′-*O*-(dimethoxytrityl)deoxynucleoside covalently joined to silica gel was determined quantitatively by measuring the dimethoxytrityl cation released after acid treatment of the silica gel. An aliquot of silica gel was accurately weighed (1–2 mg) and then treated with 5 mL of 0.1 M toluenesulfonic acid in acetonitrile.[35] After centrifugation, the absorbance was measured at 498 nm, and the amount of dimethoxytrityl cation was determined by using an extinction coefficient of 7×10^4. This assay was reproducible within ±5%.

Diethoxytriazolylphosphine was prepared by adding diethyl phosphorochloridite (1.0 mL, 5.8 mmol) dropwise to dry tetrahydrofuran (18 mL) containing 2,6-lutidine (2 mL) and dry triazole (0.50 g, .72 mmol) at 20 °C under nitrogen. After centrifugation to remove 2,6-lutidine hydrochloride, the supernatant containing diethoxytriazolylphosphine can be used directly as a capping reagent. This reagent can also be stored for at least 1 week at 20 °C under nitrogen. The activity of the diethoxytriazoylphosphine solution was assayed by measuring its ability to phosphitylate 5′-*O*-(dimethoxytrityl)thymidine in 1 min at 20 °C.

Thin-layer chromatrography (TLC) was routinely carried out on Merck analytical silica gel plates (No. 5775), and HPLC was completed on a Waters Associates apparatus equipped with a solvent programmer and a Waters Associates, C_{18}, reverse-phase, μ-Bondapak analytical column. The organic phase was acetonitrile, and the aqueous phase was 0.1 M triethylammonium acetate (pH 7).

Synthesis of the Support. HPLC grade silica gel (2 g, Vydac TP-20, Separation Group, 100 m^2/g surface area, 300-Å pore size, 20-μm particle size) was exposed to a 15% relative humidity atmosphere (saturated LiCl) for at least 24 h. The silica (2.0 g) was then treated with 3-(triethoxysilyl)propylamine (2.3 g, 0.01 M in toluene) for 12 h at 20 °C and 12 h at reflux under a Drierite drying tube.[36] This reaction was completed on a shaking apparatus because magnetic stir bars pulverize the silica gel and must be avoided. Compound **1** was isolated by centrifugation, washed successively (twice each) with toluene, methanol, and ether, and air-dried.

The carboxylic acid group was introduced by agitating **1** (2 g) and succinic anhydride (2.5 g, 0.025 M) in water. The pH was controlled (pH 2–6) by addition of 2 M NaOH. Completeness of the carboxylation reaction was qualitatively monitored by using a picrate sulfate test.[37] An aliquot of silica (approximately 2 mg) was treated with 0.5 mL of 0.1 M picrate sulfate in saturated sodium borate buffer (pH 10). Compound **1** reacted within 10 min and stained a bright yellow whereas compound **2** remained white. The succinic anhydride reaction was allowed to continue until the silica gel remained white during the picrate sulfate test. Usually the total reaction time was 1 h, and a second addition of succinic anhydride was required. After being washed successively (twice each) with water, 0.1 M trichloroacetic acid, water, methanol, and ether, compound **2** was air-dried, dried in vacuo, and then treated with trimethylsilyl chloride (1.25 mL, 0.01 M) in pyridine (7 mL) for 24 h at 25 °C. Compound **2** was then washed with methanol (four times) and ether. Analysis for extent of carboxylation involved a two-step procedure. An accurately weighed aliquot was treated with DCC and *p*-nitrophenol in pyridine. After several washings with tetrahydrofuran to remove unreacted *p*-nitrophenol, 10% piperidine in pyridine was added to the silica gel, and the amount of *p*-nitrophenol released was measured at 410 nm by using 1.57×10^4 as the extinction coefficient of *p*-nitrophenoxide. The incorporation of carboxylic acid was 200 μmol/g.

Deoxynucleosides were joined to **2** by using DCC. This is a general procedure, and therefore only one example will be presented. 5′-*O*-(Dimethoxytrityl)thymidine (1.1 g, 2.16 mmol), DCC (2 g, 0.01 mol), and **2** (4 g, 0.8 mmol, carboxylic acid) were agitated in dry pyridine (21 mL) for 2 days. *p*-Nitrophenol (1.4 g, 0.01 mol) was added, the mixture was agitated for an additional day, and then the reaction was quenched with morpholine (1 mL, 0.011 mol). After being washed with methanol and ether, the silica gel was analyzed for unreacted carboxylic acid by the procedure outlined previously. Usually a second treatment with DCC (2 g, 0.01 mol) and *p*-nitrophenol (1.4 g, 0.01 mol) in dry pyridine (20 mL) and finally morpholine (1 mL) was necessary to completely block the trace amount of free carboxylic acid (<10 μmol/g) that remains from the first blocking procedure.

Preparation of Activated Deoxynucleotides. Compounds **5b**, **5g**, and **5h** were synthesized by the same procedure. 5′-*O*-(Dimethoxytrityl)-thymidine, 5′-*O*-(dimethoxytrityl)-*N*-benzoyldeoxycytidine, and 5′-*O*-(dimethoxytrityl)-*N*-benzoyldeoxyadenosine were prepared by published procedures.[38] 5′-*O*-(Dimethoxytrityl)-*N*-isobutyrldeoxyguanosine was prepared according to a published procedure[39] except that dimethoxytrityl chloride was used instead of monomethoxytrityl chloride. These 5′-*O*-dimethoxytrityl deoxynucleosides were dried in vacuo over Drierite before use.

Methyl phosphorodichloridite was prepared according to a published procedure[40] and must be of the highest purity. After distillation through a column packed with glass helices, aliquots of methyl phosphorodichloridite were stored in vials under argon at −20 °C. Analysis by phosphorus-31 NMR indicated that the dichloridite was completely stable over several months when prepared in this manner. The phosphitylation procedure was completed in an apparatus designed so that all operations (additions, filtrations, concentrations in vacuo) could be completed under an inert gas atmosphere.

Compound **5b** was prepared by the following procedure. 5′-*O*-(Dimethoxytrityl)thymidine (1.6 g, 2.9 mmole in anhydrous tetrahydrofuran (5 mL) was added dropwise to a well-stirred solution at −78 °C of CH_3OPCl_2 (0.33 mL, 3.5 mmol) and collidine (1.86 mL, 14.1 mmol) in anhydrous tetrahydrofuran (5 mL). A white precipitate formed during the addition. The mixture was stirred for 15 min at −78 °C and then filtered through a sintered-glass funnel to remove collidine hydrochloride. The collidine hydrochloride was washed with dry tetrahydrofuran (1 mL). The filtrate was then diluted with dry toluene and concentrated to a gum. After dry argon had been bled into the apparatus, a solution (6 mL) containing toluene–tetrahydrofuran (2:1) was added, and the gum was allowed to dissolve completely in this solution. Solvent was removed by concentration in vacuo. This reconcentration cycle using a solution of toluene and tetrahydrofuran was repeated three times. After the final concentration, the gum was dissolved in dry tetrahydrofuran (3 mL) and cooled to −78 °C, and a solution of tetrazole (0.18 g, 2.6 mmol) in dry tetrahydrofuran (3 mL) was added dropwise. A white precipitate of collidine hydrochloride formed during the addition. The mixture was stirred an additional 10 min at −78 °C and then transferred by using positive argon pressure and a cannula to a centrifuge tube filled with argon. The supernatant recovered after centrifugation contained **5b** which can be used directly for synthesis of deoxyoligonucleotides. Alternatively, **5b** can be stored as a precipitate and reconstituted as needed. Typically aliquots (2 mL) of the supernatant (8 mL) were percipitated into 15 mL of pentane at 20 °C. Each aliquot therefore contained enough activated nucleotide for three or four condensations with 0.2–0.3 g of silica gel. The precipitation was carried out in 15-cm, screw-cap test tubes fitted with Teflon–silicon septums (Pierce Chemical Co.). After centrifugation, cannula decanting, and careful drying in vacuo, the test tubes containing the samples were recovered after reequilibrating the desiccator with argon. Each test tube was cooled in a dry ice/acetone bath under an argon atmosphere, sealed with Parafilm, and stored in a desiccator over Drierite at −20 °C. With these storage conditions, aliquots were stable for at least 3 months.

Compounds **5g** and **5h** were prepared by the same general procedure. However, for the preparation of **5f**, the stoichiometry of deoxynucleoside and CH_3OPCl_2 was changed. Typically 5′-*O*-(dimethoxytrityl)-*N*-isobutyrldeoxyguanosine (1.0 g, 1.56 mmol) and methyl phosphorodichloridite (0.13 mL, 1.4 mmol) were allowed to react in tetrahydrofuran (5 mL). Otherwise the preparation of **5f** was the same as for the other activated deoxynucleotides.

The amounts of **5b**, **5f**, **5g**, or **5h** per preparation were estimated qualitatively by the following procedure. One aliquot of the activated nucleotide in tetrahydrofuran was quenched with dry methanol and a second with water. A TLC comparison on silica gel plates (ethyl acetate; 10% methanol in $CHCl_3$) between the two quenches identified the bis(methyl nucleoside) phosphite. Estimated yields were 70% for **5b**, **5g**, and **5h** and 50% for **5f**. These estimates were based on the intensity of orange stain due to the dimethoxytrityl group when compounds containing this group were exposed to concentrated hydrochloric acid vapor.

Synthesis of Deoxyoligonucleotides. Syntheses were completed in a machine that is drawn schematically in Figure 2. The apparatus consists of a Milton Roy Minipump, three-way Altex slide valves, a recycle valve (a modified Altex valve), and an injector loop (a three-way Altex valve).

(35) M. H. Caruthers, Ph.D. Dissertation, Northwestern University, Evanston, IL, 1968.
(36) R. Majors and M. Hopper, *J. Chromatogr. Sci.*, **12**, 767 (1974).
(37) D. M. Benjamin, J. J. McCormack, and D. W. Gump, *Anal. Chem.*, **45**, 1531 (1973).
(38) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3821 (1963).
(39) H. Büchi and H. G. Khorana, *J. Mol. Biol.*, **72**, 251 (1972).
(40) D. R. Martin and P. J. Pizzolato, *J. Am. Chem. Soc.*, **72**, 4584 (1950).

Shanghai Hongene Exhibit 1005

All connections were with Teflon tubing and were designed to minimize the tubing volume in the recycle loop. The column was an 11-mm Ace glass column that had been shortened to approximately 1-mL capacity. Cellulose filters were used to support the silica bed. The filters were acetylated with a solution of acetic anhydride and pyridine (1:1 based on volume) for 4 h at 50 °C before use. the total volume contained within the recycle loop of this apparatus was approximately 2.5 mL. The tetrahydrofuran reservoir was protected from air with a nitrogen bubbler, and the $ZnBr_2$ solution was protected from moisture with a Drierite tube.

The various chemical operations that must be performed for the addition of one nucleotide to the silica are listed in Table I. Typically 0.25 g (10 μmol) of **3** (thymidine) was loaded into the column and the silica washed with nitromethane. The 5′-*O*-dimethoxytrityl group was removed by flushing the column (30 min) with nitromethane saturated with $ZnBr_2$ (approximately 0.1 M in $ZnBr_2$) at a pump speed of 1 mL/min. The completeness of deprotection was monitored visually or spectrophotometrically by observing the release of a bright orange dimethoxytrityl cation. By measuring the absorbance at 498 nm, the completeness of the previous condensation step was monitored. The step was followed successively by a wash with a solution of *n*-butanol–2,6-lutidine–tetrahydrofuran (4:1:5) for 5 min at a flow rate of 2 mL/min. The next step was a wash for 5 min (5 mL/min) with dry tetrahydrofuran. During the course of this washing step, the recycle valve and the injector port were also flushed with dry tetrahydrofuran, and the effectiveness of this wash was monitored at 254 nm by using a spectrophotometer. The condensation step was next completed by using activated nucleotide that had been reconstituted with dry tetrahydrofuran. The reconstituted solution was stored in a dry ice/acetone bath over argon, but condensation reactions were carried out at room temperature. When reconstituted, the activated nucleotide stored in this way was stable for several days. Approximately 10 equiv of activated nucleotide (100 μmol for 0.25 g of **4**) in 0.5–0.8 mL of tetrahydrofuran was injected into the apparatus and the machine switched to the recycle mode. The activated nucleotide was circulated through the silica gel for 1 h at a pump speed of 2 mL/min. Aliquots of activated nucleotide from the apparatus were then collected directly into dry methanol and water. Analysis as described previously indicated whether activated nucleotide was still present in the system. Usually this is the case. However, occasionally (approximately one time in ten) the dimethyl phosphite of the deoxynucleotide was not observed by this assay. When this occurred, the condensation step was repeated to prevent the possibility of incomplete reaction. The next step involves capping unreacted **4** or deoxyoligonucleotides containing a 5′-hydroxyl by adding diethoxytriazoylphosphine (1 mL of a 0.3 M solution in tetrahydrofuran) directly to the solution of activated nucleotide and continuing the recycle mode for 5 min at a pump speed of 2 mL/min. Residual activated nucleotide and the capping reagent were then flushed from the apparatus with dry tetrahydrofuran (2 min at 5 mL/min). This step was followed by the oxidation of phosphites with a solution of tetrahydrofuran–2,6-lutidine–water (2:1:1) containing 0.2 M I_2. The solution was flushed through the apparatus for 5 min (2 mL/min). Finally the cycle was completed by flushing the system first with dry tetrahydrofuran for 3 min (5 mL/min) and then with nitromethane for 2 min (5 mL/min).

Isolation of Deoxyoligonucleotides. The completely deprotected deoxyoligonucleotides were isolated by the following procedure. An aliquot (10 mg) of the silica gel containing the deoxydecanucleotide triester in protected form was first treated with thiophenol–triethylamine–dioxane (1:1:2 v/v). After 45 min of gentle shaking, the silica gel was recovered by centrifugation and washed with methanol (four times) and ethyl ether. After air drying, the deoxyoligonucleotide was removed from the support by a 3-h treatment with concentrated ammonium hydroxide at 20 °C followed by centrifugation. Base protecting groups were removed by warming the supernatant at 50 °C for 12 h in a sealed tube. The 5′-*O*-(dimethoxytrityl)deoxyoligonucleotide was isolated by concentrating the hydrolysate in vacuo, dissolving the residue in 0.1 M triethylammonium acetate (pH 7.0), and chromatographing this material on a C_{18} reverse-phase, HPLC column (Waters Associates). The eluting buffer was 0.1 M triethylammonium acetate containing 26% acetonitrile. The peak containing 5′-*O*-(dimethoxytrityl)deoxyoligonucleotide was concentrated in vacuo, and the residue was treated at 20 °C for 15 min with acetic acid–water (4:1 v/v) to remove the 5′-*O*-dimethoxytrityl group. The completely deprotected deoxyoligonucleotide was isolated by concentration of the acetic acid solution in vacuo, dissolving the residue in 25 mM triethylammonium bicarbonate (pH 7), and extraction of dimethoxytritanol with water-saturated ether.

Characterization of Deoxyoligonucleotides. The 5′-hydroxyl of each deoxyoligonucleotide was phosphorylated by using [γ-^{32}P]ATP and T4-kinase.[41] The amount of deoxyoligonucleotide used in a phosphorylation reaction was determined by measuring the absorbance and using a calculated extinction coefficient which assumed no hypochromicity for the deoxyoligonucleotide. Phosphorylated deoxyoligonucleotides were separated from excess ATP by desalting on a G-50-40 Sephadex column with 10 mM triethylammonium bicarbonate (pH 7) as eluant. Gel electrophoresis on polyacrylamide[42] and two-dimensional analysis[34] were completed by using standard procedures.

Synthesis of d(C-G-T-C-A-C-A-A-T-T). Compound **3** (0.25 g, 40 μmol/g) was loaded into the column, and the cycle was started by washing the silica gel with nitromethane and removing the 5′-dimethoxytrityl group with $ZnBr_2$. Elongation was performed as previously described by using an approximate tenfold excess of the incoming activated nucleoside phosphite (0.1 mM) at each condensation. Synthesis was continued to the completion of the deoxyoctanucleotide, d(T-C-A-C-A-A-T-T). At this point the silica was divided into two approximately equal portions. One portion was elongated to the deoxydecanucleotide in standard fashion. The overall yield was 64% based on the amount of dimethoxytrityl group bound to the support, and 30% was the yield isolated from a reverse-phase HPLC column.

Synthesis of d(A-C-G-C-T-C-A-C-A-A-T-T). The remaining portion of d(T-C-A-C-A-A-T-T) was elongated in the standard fashion in the machine to the deoxydodecanucleotide. The overall yield was 55% based on the dimethoxytrityl group bound to the support. The isolated yield was not accurately determined.

(41) V. Sgaramella and H. G. Khorana, *J. Mol. Biol.*, **72**, 427 (1972).
(42) T. Maniatis, A. Jeffrey, and H. van de Sande, *Biochemistry*, **14**, 3787 (1975).

Shanghai Hongene Exhibit 1005