Use of the Lipophilic *tert*-Butyldiphenylsilyl Protecting Group in Synthesis and Rapid Separation of Polynucleotides†

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ABSTRACT: Butyldiphenylchlorosilanes react with deoxy- and N-protected deoxymononucleotides to give, in each case, the 3′-*O*-butyldiphenylsilyl ether in high yield. A comparative study of *n*-, *sec*-, and *tert*-butyldiphenylchlorosilanes showed, as expected, a wide variation in the rate of formation and in the stability of the 3′-*O*-silyl ethers, the *tert*-butylphenylsilyl group being the one of choice in polynucleotide synthesis. The silyl group can be readily removed on treatment with fluoride ion in buffered pyridine at room temperature. This property markedly increases flexibility of the current methodology for polynucleotide synthesis. Secondly, because of its high lipophilicity, the silyl group allows rapid solvent extraction of synthetic intermediates, and thirdly, it greatly facilitates the isolation of the required product in synthetic reactions because of the selective and strong retention of the condensation product during reverse-phase high-pressure liquid chromatography. Several examples of synthetic procedures, including a stepwise synthesis of an undecanucleotide, are given which demonstrate overall simplification of polynucleotide synthesis.

Efficiency in organochemical synthesis of polynucleotides depends to a large extent on the design of suitable protecting groups. In the general methodology developed in this laboratory (Khorana et al., 1972), one recurring step is the specific deprotection of the terminal 3′-hydroxyl group after every condensation in order that further elongation of the growing chain may be carried out. The acetyl group has served reasonably well for this purpose, but even the very mildly alkaline conditions adequate for its removal have proved not to be completely safe, especially because of the necessity to repeat the treatment in a multistep synthesis. Further, more recent developments in the overall strategy of polynucleotide synthesis (Agarwal et al., 1972, 1976) have made it increasingly desirable that an alternative group, which should meet new requirements, be designed for the protection of 3′-hydroxyl group in mono- and oligonucleotide chains. For example, an improved and rapid synthesis of the dinucleotide blocks carrying 5′-phosphate groups utilizes the very lipophilic and alkali-sensitive TPSE[1] group for the protection of the 5′-phosphate group (Agarwal et al., 1976). But, straightforward use of this group for the synthesis of tri- and tetranucleotide blocks is not possible in conjunction with the acetyl group because both groups are base sensitive. For the synthesis of the tri- and tetranucleotide blocks the 2-(*p*-tritylphenyl)thioethyl (TPTE) group has been used (Agarwal et al., 1976), but the latter group requires oxidation with *N*-chlorosuccinimide to the TPSE analogue before its removal can be effected. Clearly, the scope of the above method for the synthesis of oligonucleotide blocks would be much enhanced if a group, which is alkali stable but is removable under suitably mild conditions, could be used to protect the hydroxyl group at the 3′-terminus. Indeed, the ideal situation would be one in which the groups protecting either the 5′- or 3′-termini can be removed, selectively and without affecting the N-protecting groups. This would allow chain elongation to proceed in either direction and permit maximal use of oligonucleotides prepared as synthetic intermediates.

A second important consideration is the recent introduction of the use of high-pressure liquid chromatography in polynucleotide synthesis (Fritz et al., 1978a, b). This rapid separation method, which replaces the earlier anion exchange chromatography, shortens the time required in oligonucleotide synthesis by about 50%. From the previous study of the influence of the different protecting groups present in mono- and oligonucleotides on the retention by the reverse-phase liquid chromatography columns, it is clear that the effectiveness of the technique could be greatly enhanced by further manipulation of the protecting groups (Fritz et al., 1978b). Thus, in conjunction with the protecting groups currently used, if there were to be present at the 3′-hydroxyl end of every incoming oligonucleotide block an extremely hydrophobic group, then the required condensation product would be set apart from the remainder of the reaction mixture because of its greatly increased hydrophobicity and this would result in its uniquely long retention on reverse phase columns.

In the present paper, we describe a study and the use of the *tert*-butyldiphenylsilyl group for the protection of the 3′-hydroxyl group of mono- and oligonucleotides. The group has the highly desirable feature of being readily removable by fluoride ion under mild and neutral conditions. Furthermore, being very hydrophobic it has the desired attribute of causing great retardation of the oligonucleotides which carry it during elution from a reverse-phase column by high-pressure liquid chromatography. Advantages accruing from the use of this group in polynucleotide synthesis are illustrated by the synthesis of several oligonucleotides. In particular, the marked advantages in separation by reverse-phase liquid chromatography are documented by the synthesis of an undecanucleotide, d(G-G-A-A-G-C-G-G-G-G-C). The latter polynucleotide,

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[1] Abbreviations used in this paper are as described in the preceding paper (Fritz et al., 1978a).

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whose synthesis has also been previously carried out (R. Belagaje, E. L. Brown, M. J. Gait, K. E. Norris, & H. G. Khorana, manuscript in preparation), forms the bridge between the promoter and structural gene of the tyrosine suppressor tRNA.

Results and Discussion

A number of novel groups have been proposed for the protection of the 3′-hydroxyl function of nucleosides and nucleotides (for recent reviews see Amarnath & Broom, 1977; Kössel & Seliger, 1975), but none has evidently proven to be of general use in polynucleotide synthesis. Recently, a number of *tert*-butyldimethylsilyl derivatives of carbohydrates as well as of certain nucleosides (thymidine and uridine) have been prepared (Ogilvie et al., 1976; Ogilvie, 1973). Furthermore, the *tert*-butyldimethylsilyl ethers were shown to be cleavable by fluoride ion (Ogilvie et al., 1976; Ogilvie, 1973). In addition, some *tert*-butyldiphenylsilyl derivatives of carbohydrates have been reported and their marked stability toward hydrolysis in acid and base has been observed (Hanessian & Lavallee, 1975). Previously, 3′-*tert*-butyldiphenylsilylthymidine has been prepared by first suitably protecting and subsequently deprotecting the 5′-hydroxyl group (Ogilvie, 1973). In the present work, the first aim was to study direct silylation of the 3′-hydroxyl groups in the readily available deoxyribonucleoside 5′-phosphates, as well as their N-protected derivatives, which have served as the starting materials in all of the synthetic work reported from this laboratory. Since the ionized phosphomonoester groups in the nucleotides are much more reactive than the 3′-hydroxyl groups, silyl–phosphate linkages would be expected to be formed. However, the latter would be expected to be selectively hydrolyzed in aqueous pyridine. Furthermore, although their lipophilicities would not significantly differ, the preparation of *n*-, *sec*-, and *tert*-butyldiphenylsilyl derivatives of the mononucleotides was undertaken. In this way, three series of derivatives with a wide range of stabilities would be available for a systematic evaluation of their use in oligonucleotide synthesis.

In the case of *n*-$BuPh_2SiCl$, the use of 5 mol equiv gave the 3′-*O*-silyl derivatives of all the mononucleotides in practically quantitative yield in pyridine at room temperature within 2 h. In order to complete the analogous reaction with *s*-$BuPh_2SiCl$ in under 24 h at room temperature, it was necessary to use a larger excess (10 mol equiv) of the chlorosilane. The addition of 5 equiv of imidazole (with 5 mol equiv of chlorosilane) reduced the time for the completion of reaction to 2 h. With *t*-$BuPh_2SiCl$, only silyl–phosphate formation was observed when the reaction was attempted in pyridine alone. However, the addition of 5 equiv of imidazole was effective in bringing about complete 3′-*O*-silylation in 15 h. Imidazole, especially in dimethylformamide, is a well-known silylation catalyst (Corey & Venkateswarlu, 1972; Ogilvie et al., 1976; Ogilvie, 1973; Hanessian & Lavallee, 1975).

As expected, the differences observed above in the reactivities of the *n*-, *s*-, and *t*-$BuPh_2SiCl$ were also reflected in the stabilities of the corresponding silyl ethers. This was first evident with the silyl–phosphate intermediates (Figure 1). In the case of the *n*-butylsilyl derivatives, the silyl–phosphate intermediate was too unstable to be detected on thin-layer chromatography (TLC). In the reaction between thymidine 5′-phosphate and the *sec*-butylchlorosilane, a fast-travelling material corresponding, presumably, to the silyl–phosphate was observed and it persisted for several minutes after the reaction mixture was quenched with water. The *tert*-butylsilyl phosphate derivative of thymidine 5′-phosphate was much more stable (5 h) in aqueous pyridine at room temperature and,

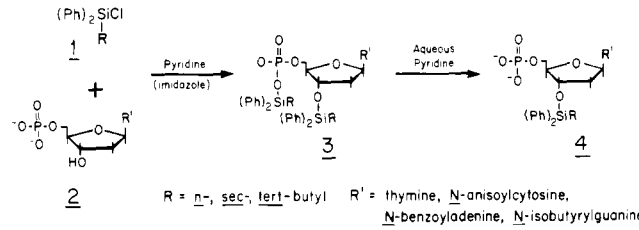

FIGURE 1: General method for the preparation of 3′-*O*-(*n*-, *sec*-, or *tert*)-butyldiphenylsilyl mononucleotides by the reaction of the chlorosilanes (**1**) with mononucleotides (**2**). The intermediate silyl-phosphate derivative (**3**) hydrolyzes to give the required products **4**.

consequently, all the three possible silyl derivatives (the monosilyl phosphate derivative, the disilyl derivative, namely silyl–phosphate and 3′-*O*-ether, and the required mono-3′-*O*-silyl derivative) could all be isolated.

The 3′-*O*-silyl ethers exhibited, as expected, a wide range of stabilities. The 3′-*O*-*n*-butyldiphenylsilyl derivatives of nucleotides suffered extensive hydrolysis in aqueous pyridine in 24 h[2] and their isolation and storage necessitated the preparation of the disodium salt (see Materials and Methods). Both the 3′-*O*-*sec*- and *tert*-butylsilyl ethers of nucleotides were stable to aqueous pyridine at room temperature. The difference in stability in the two series was, however, evident during treatment with 9 N ammonia, conditions which are routinely used for removal of the N-protecting groups in polynucleotide synthesis. While complete cleavage of the 3′-*O*-*sec*-butyl derivative was observed in 4 h at 25 °C, the *tert*-butyl analogue underwent less than 10% cleavage in 76 h. Because of this stability to basic conditions and because of the fact that no difficulty was experienced in fluoride ion catalyzed removal of the *tert*-butyldiphenylsilyl group, the latter group was selected for the synthetic applications herein described. It should, however, be emphasized that the groups now studied, and related ones carrying alternative substituents on the silyl group that could be prepared, offer enormous flexibility and choice in the strategy for polynucleotide synthesis.

Removal of the 3′-*O*-silyl group in the presence of tetra-*N*-butylammonium fluoride with anhydrous pyridine as the solvent occurred without difficulty at room temperature, conditions which are completely safe for the usual protected oligonucleotides. Thus, although the minimal conditions necessary for the removal of the different isomeric butylsilyl ethers have not been determined, the removal of all the butylsilyl ethers of mononucleotides was complete in less than 20 min on treatment with 3.5 mol equiv of fluoride ion at room temperature. Although there appeared to be a drop[3] in the rate of cleavage in going from the mononucleotides to the oligonucleotides, the use of 5–10 mol equiv of fluoride ion always gave complete removal of the most stable *tert*-silyl group in 4–8 h.

Synthesis of the Protected Di- and Trinucleotide Blocks. The syntheses shown in Figure 2 illustrate the advantages and flexibility that the *t*-$BuPh_2Si$ group offers in the preparation

[2] The stability in pyridine is minimally adequate to be of use in oligonucleotide synthesis in certain situations. For example, the use of the 3′-*O*-formyl group, which is very inconvenient to prepare and which is removed by treatment with aqueous pyridine, has been proposed in oligonucleotide synthesis (Seliger et al., 1975).

[3] Substantial differences in rates of removal of the protecting groups in differently substituted nucleosides and nucleotides have frequently been observed in previous studies. For example, the presence of the 5′-monoester phosphate group in nucleotides may influence markedly, relative to the corresponding nucleosides, the stability of a protecting group on the 3′-hydroxyl group.

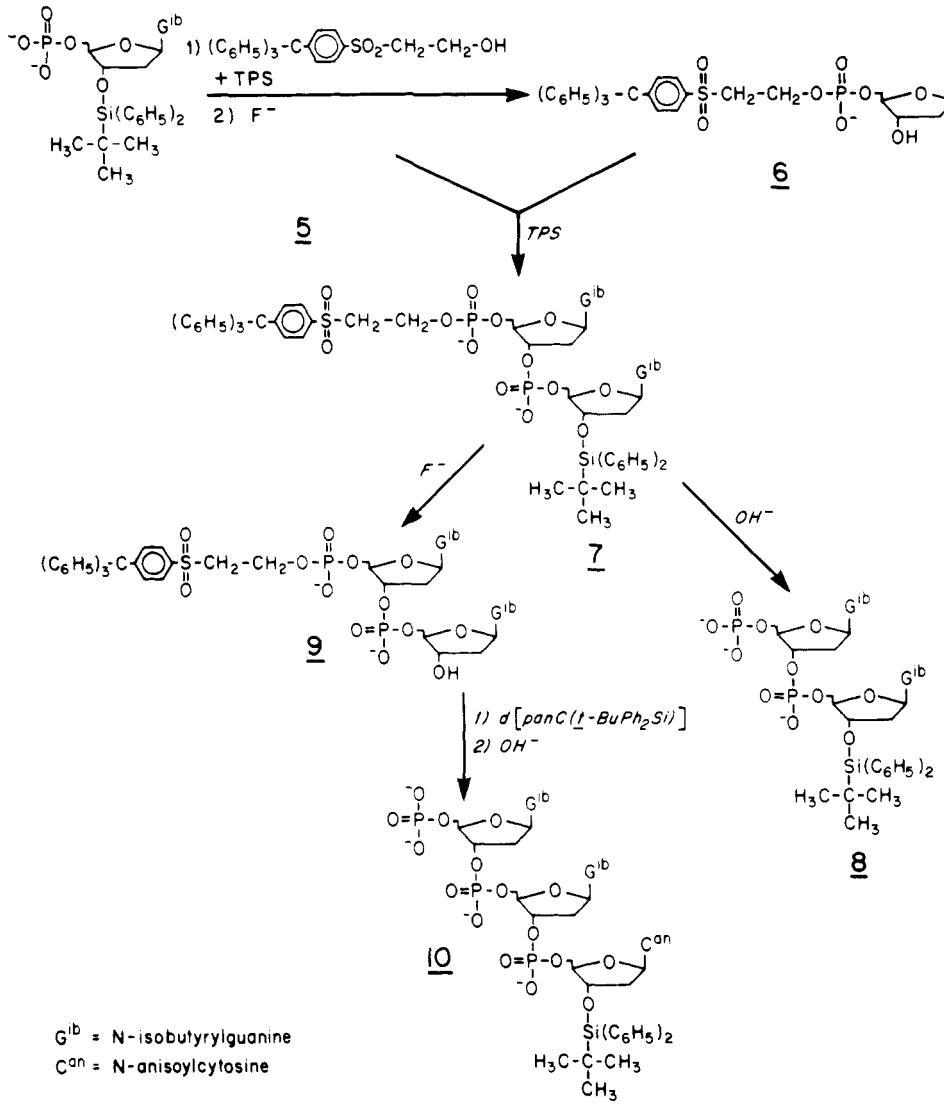

FIGURE 2: The use of 3′-*O*-*tert*-butyldiphenylsilyl-*N*-isobutyrylguanosine 5′-phosphate (**5**) in the synthesis of suitably blocked di- and trinucleotides (**8**-**10**). The 3′-O- and N-protected mononucleotide (**5**) is converted to d(TPSE-pibG) (**6**). Condensation between **5** and **6** affords the fully protected dinucleotide, **7**; the latter on mild base treatment gives useful dinucleotide blocks of the type **8**. Alternatively, treatment of **7** with fluoride ion gives the dinucleotide **9**, which on condensation with protected mononucleotides, e.g., d[panC(*t*-BuPh$_2$Si)], gives the trinucleotide block, **10**. The use of the latter in the final step of the undecanucleotide synthesis is shown in Figures 5 and 8.

of oligonucleotide blocks. The protected dinucleotide, d[pibG-ibG(*t*-BuPh$_2$Si)] (**8**), and the protected trinucleotide, d[pibG-ibG-anC(*t*-BuPh$_2$Si)] (**10**), whose syntheses are shown, were required for the synthesis of the undecanucleotide described below. The conversion of the 3′-*O*-silyl derivative of dpibG (**5**) to **6** was performed by first preparing the TPSE derivative as worked out previously (Agarwal et al., 1976) and the silyl group was then removed by treatment with tetra-*n*-butylammonium fluoride. Condensation between **5** and **6** gave the fully protected dinucleotide **7**. The main feature of the protecting groups used at the two termini in **7** is that either one of them may be removed with complete selectivity. Thus, mild alkaline treatment affords **8**, which is especially suitable as an intermediate in the undecanucleotide synthesis described below. Alternatively, treatment of **7** with fluoride ion gives **9** having the 3′-hydroxyl end group free; the latter can be elongated to the trinucleotide **10** as shown in Figure 2. The protected trinucleotide **10** was also required for the undecanucleotide synthesis described below.

The differences in the hydrophobicities of the components used in the synthesis of the trinucleotide (**10**) were such that the latter could be readily purified effectively by solvent extraction. The final preparation was analyzed by high-pressure liquid chromatography (HPLC) as shown in Figure 3. Clearly, the product was remarkably pure as obtained directly by the extraction method.

The preparation of a second protected trinucleotide block d[TPSE-pbzA-bzA-anC(*s*-BuPh$_2$Si)], as described under Materials and Methods, proceeded equally well. The dinucleotide, d[(TPSE)pbzA-bzA], was reacted with the mononucleotide, d[panC(*s*-BuPh$_2$Si)], and the required protected trinucleotide was isolated using a simple extraction procedure.

The Silyl Group in Separation and Identification of the Required Products by HPLC. In addition to the marked advantages of the silyl group described above in chemical methodology, a highly useful feature of the hydrophobic silyl group is that, following every condensation step, there is a marked increase in the hydrophobicity of the required product, relative to the starting material, by virtue of the silyl group in the incoming oligonucleotide block. Consequently, the required products are readily separable because of their uniquely strong

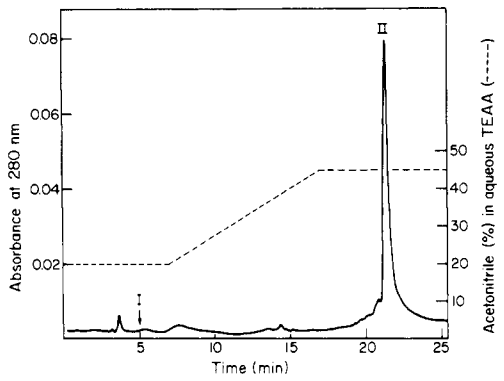

FIGURE 3: Analysis of high-pressure liquid chromatography of the preparation of the protected trinucleotide, d[pibG-ibG-anC(*t*-BuPh$_2$Si)] (**10**). The latter was prepared as described in Figure 2 and in the text, only solvent extraction being used for purification. Peak I, with a retention time of 5 min at 20% acetonitrile concentration, corresponds to d(pibG-ibG). Elution of **10**, the trinucleotide, required 45% acetonitrile concentration.

retention on the reverse-phase columns. One example of the facilitation of separation of the synthetic product is shown in Figure 4. Condensation of d[(MeOTr)bzA-bzA-T] (R. Belagaje, E. L. Brown, H.-J. Fritz, R. G. Lees, and H. G. Khorana, manuscript in preparation) with dpT(*t*-BuPh$_2$Si) gave a mixture which, after fractionation with diethyl ether, was applied to an HPLC column. The separation obtained is shown in panel A (Figure 4). Thus, the desired tetranucleotide (peak III) was well separated from the unreacted starting materials (peaks I and II). In panel C is shown the influence of the *tert*-butyldiphenylsilyl group on retention of the tetranucleotide d(A-A-T-T). The fully unprotected tetranucleotide eluted with 12% acetonitrile while the silyl derivative required 35% acetonitrile concentration.

The main synthetic application of the silyl group in conjunction with the HPLC technique has been in the synthesis of the undecanucleotide d(G-G-A-A-G-C-G-G-G-G-C). The steps used are shown in Figure 5, the preparation of the protected hexanucleotide (**11**) having been described previously (Fritz et al., 1978a) (analytical HPLC in Figure 6A). Condensation with the protected dinucleotide **8**, whose preparation has been discussed above (Figure 2), gave the fully protected octanucleotide **12**. The latter was well separated from the other components of the reaction mixture and appeared last as shown in the analytical HPLC run (Figure 6B) as well as in the preparative run (Figure 6C). Panel D (Figure 6) shows another analytical run of the isolated protected octanucleotide. The latter was not purified further at this stage: the contamination very likely was from the dinucleotide and not the hexanucleotide, which was widely separated. Instead, the octanucleotide was desilylated to **13** (Figure 5) and then subjected to preparative HPLC again (Figure 7, panel A). Upon desilylation, the dinucleotide should undergo a marked shift to shorter retention times relative to the octanucleotide since only the latter (**13**) has the lipophilic 5′-methoxytrityl group. The octanucleotide (**13**) thus obtained was pure as seen in Figure 7, panel B. Furthermore, after removal of the 3′-*O*-*tert*-butyldiphenylsilyl groups, the hexa- and octanucleotides showed very similar retention times, the latter eluting first as seen in Figure 7C (cf. Fritz et al., 1978a). This result thus confirmed that the large separation of the octanucleotide **12** from the hexanucleotide **11** was due entirely to the 3′-*O*-silyl group. This was again evident in the next and the last step in the synthesis, namely the condensation of **13** with the protected trinucleotide (**10**, Figure 2) to give the protected undecanucleotide **14** (Figure 5). Preparative HPLC of the reaction mixture gave the pattern shown in Figure 8 (panel A), peak IV containing the required product. An analytical HPLC of the pooled peak gave the pattern in panel B. Desilylation followed by preparative HPLC gave a product which from its HPLC behavior (panel C, Figure 8) was pure. A co-injection of the hexanucleotide **11**, desilylated octanucleotide **13** and undecanucleotide **15** gave the elution pattern shown in panel D of Figure 8. This again illustrated the relatively minor differences in retention times of the three oligonucleotides after desilylation. Interestingly, the 3′-*O*-silylocta- and undecanucleotides showed wider separation (Figure 9). Finally, complete deprotection of the undecanucleotide by the standard method gave a product which was analyzed by the two-dimensional fingerprinting procedure (Sanger et al., 1974). As shown by the pattern obtained (Figure 10), the undecanucleotide was completely pure.

Materials and Methods

TPS (triisopropylbenzenesulfonyl chloride) was obtained from Aldrich Chemical Co. and was recrystallized from diethyl ether/petroleum ether before use. Diphenyldichlorosilane was

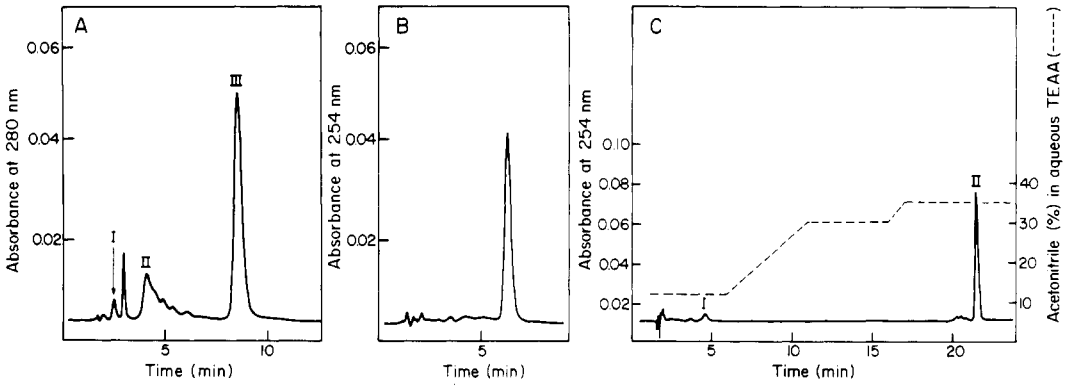

FIGURE 4: High-pressure liquid chromatography (HPLC) in the synthesis and characterization of the tetranucleotide d(A-A-T-T). (A) HPLC of the total reaction mixture obtained from the condensation of d[(MeOTr)bzA-bzA-T] with dpT(*t*-BuPh$_2$Si). HPLC was performed at a constant acetonitrile concentration of 43%. The methoxytrityl-protected trinucleotide appeared in peak I, while dpt(*t*-BuPh$_2$Si) was in peak II. The required product d[(MeOTr)bzA-bzA-T-T(*t*-BuPh$_2$Si)] was in peak III. (B) Characterization of the purity of the protected tetranucleotide (peak III in A) after purification by solvent extraction as described in text. HPLC was performed at a constant acetonitrile concentration of 45%. (C) Influence of the *tert*-butyldiphenylsilyl group on retention of the tetranucleotide, d(A-A-T-T). The fully unprotected tetranucleotide, d(A-A-T-T), and d[A-A-T-T(*t*-BuPh$_2$Si)], prepared as described in the text, were subjected to HPLC. The unprotected tetranucleotide eluted with 12% acetonitrile concentration while the silyl derivative required 35% acetonitrile concentration.

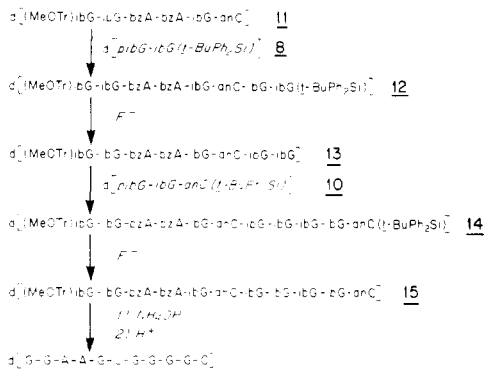

FIGURE 5: Steps in the synthesis of the protected undecanucleotide (**15**) from the protected hexanucleotide (**11**).

obtained from Petrarch Systems (Levittown, Pa.), *n, sec-*, and *tert*-butyllithium were obtained from Alfa Inorganics (Beverly, Mass.), and tetra-*n*-butylammonium hydroxide (25% methanolic solution) was obtained from Eastman. Pyridine was distilled in the presence of chlorosulfonic acid (1 g/L), redistilled from sodium hydroxide, and stored over predried molecular sieves (Linde 4A).

An anhydrous pyridine solution of the reactants for a condensation reaction was prepared by repeated evaporation (four times) of dry pyridine added after each evaporation. Dry pyridine and the specified amount of TPS were then added (in a drybox) and the volume reduced in vacuo to that specified. Workup included cooling the reaction mixture to −25 °C and adding a 2 M solution (2 mol equiv/mol equiv of TPS) of diisopropylethylamine in pyridine and water, such that the final concentration of the latter was about 25%.

Thin-layer chromatography was carried out on strips (2.2 × 10 cm) cut from EM5775 silica gel F-254 plastic sheets eluted with 25–50% methanol in ethyl acetate, as appropriate.

Nuclear magnetic resonance spectra were determined on a Varian T-60 spectrophotometer with tetramethylsilane as an internal standard. Infrared spectra were recorded on a Beckman 4210 spectrophotometer.

High-pressure liquid chromatography (HPLC) was performed using a Waters Associates system as described in detail elsewhere (Fritz et al., 1978a).

Unless otherwise indicated, products were pure by TLC and/or HPLC and by appropriate spectroscopic measurements.

Buffered Tetra-n-butylammonium Fluoride. The procedure previously developed for the cleavage of hindered silyl ethers involves the use of tetrahydrofuran (Corey & Venkateswarlu, 1972; Ogilvie et al., 1976; Hanessian & Lavallee, 1975), a solvent which is unsatisfactory for oligonucleotides. Pyridine is now found to be an excellent solvent for the preparation of the fluoride reagent. An anhydrous solution of tetra-*n*-butylammonium fluoride in pyridine was conveniently prepared as follows: To 15 mL (12.6 mmol) of a 25% methanolic solution of tetra-*n*-butylammonium hydroxide was added 25.2 mmol (10.9 mL) of a 2.3 M solution of hydrofluoric acid in pyridine. The solution was evaporated to an oil, 5 mL of dry pyridine was then added, and evaporation and pyridine addition were repeated two more times. The residue was then diluted to 50 mL with dry pyridine giving a 0.25 M solution of tetra-*n*-butylammonium fluoride and 0.25 M pyridinium fluoride.

Experimental Procedure

General Procedure for the Preparation of Butyldiphenylchlorosilanes

To an anhydrous solution of 100 mL (0.5 mol) of diphenyldichlorosilane and 400 mL of diethyl ether maintained at 0 °C under a nitrogen atmosphere was added dropwise 0.55 mol of a 1.5–2.3 M solution of the butyllithium in cyclohexane, hexane, or pentane (as commercially obtained) over 2 h. Stirring was continued for an additional 2 h at room temperature. The LiCl precipitate was then allowed to settle overnight and the supernatant was transferred without exposure to air via a double-tipped needle using nitrogen pressure to a dry flask; solvents were removed by distillation at atmospheric pressure. The residue was then transferred to a smaller flask and distilled under vacuum. In this way *n*-butyldiphenylchlorosilane was obtained in 76% yield over the range of 120–126 °C (0.05 mm) (oil bath 184 °C) [NMR (CCl_4) δ 0.93 and 1.38 (m and m, 9), 7.27 and 7.50 (m and m, 10); IR (neat) 2960, 2930, 2875, 1431, 1190, 1117, 736, and 698 cm^{-1}]; *sec*-butyldiphenylchlorosilane was obtained in 82% yield over the range 125–130 °C (0.03–0.05 mm) (oil bath 184 °C) [NMR (CCl_4) δ 1.07 and 1.33 (m and m, 9), 7.28 and 7.55 (m and m, 10); IR (neat)

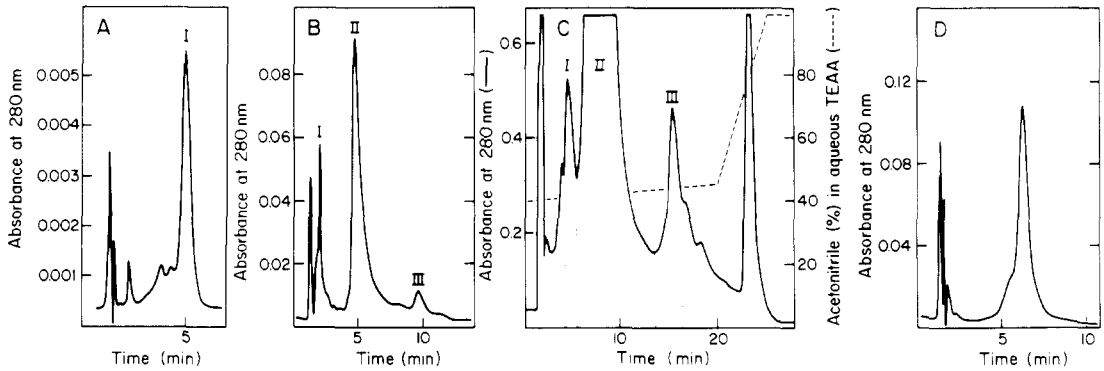

FIGURE 6: Analysis and purification of the octanucleotide (**12**, Figure 5) prepared from the hexanucleotide (**11**, Figure 5). (A) Examination of the hexanucleotide (**11**) preparation used in the synthesis of the octanucleotide. HPLC was at 38% acetonitrile concentration. The first peaks eluting immediately from the column are nonnucleotidic. Peak I eluting at about 5 min is the hexanucleotide **11**. (B) HPLC (at 45% acetonitrile concentration) of the total reaction mixture from the condensation of the hexanucleotide **11** (peak I of A) with the dinucleotide d[pibG-ibG(*t*-$BuPh_2Si$)] (**8**, Figure 2.) The unreacted hexanucleotide is in peak I, the dinucleotide is in peak II, while the required octanucleotide (**12**, Figure 5) is in peak III. (C) Preparative separation by HPLC of the reaction mixture shown in panel B. The fractions were pooled as shown by vertical dashed lines. The required product, peak III, eluted at 40–45% acetonitrile concentration. (D) Analysis of the protected octanucleotide (**12**) isolated from C by HPLC. The concentration of acetonitrile used was 46%.

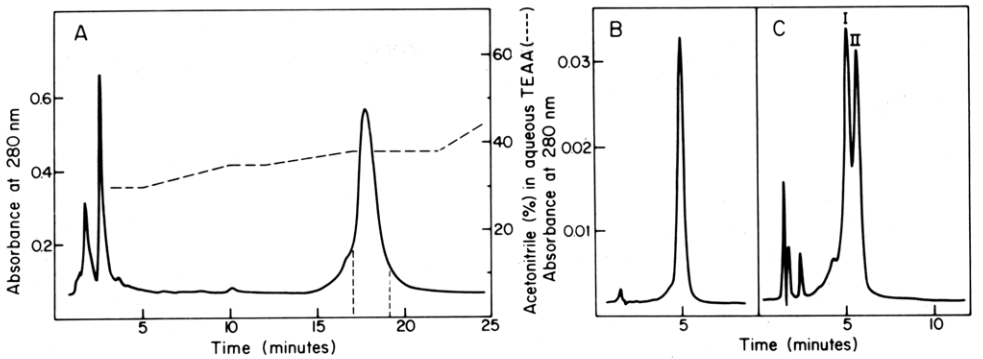

FIGURE 7: (A) Preparative purification by HPLC of the octanucleotide (**13**) after removal of the 3′-*O*-silyl group. The product was isolated by pooling the fractions between vertical dashed lines. The increments in acetonitrile concentration used at different time intervals during HPLC are shown. (B) Analysis of the octanucleotide (**13**) as isolated in panel A. HPLC was at 38% acetonitrile concentration. (C) Chracterization by co-injection of the octanucleotide **13** (peak I) and the hexanucleotide **11** (peak II) by HPLC. Acetonitrile concentration was 38%.

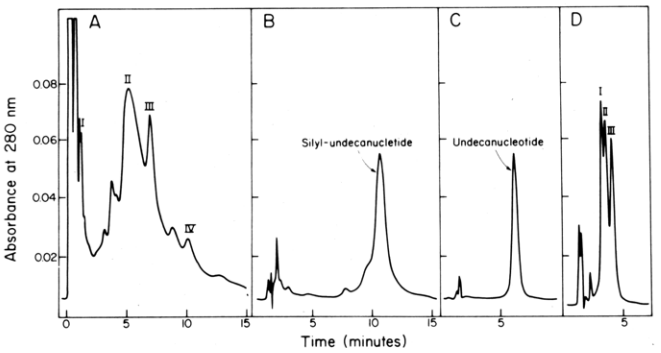

FIGURE 8: (A) HPLC, at 44% acetonitrile concentration, of the total reaction mixture obtained after condensation of the octanucleotide **13**, with the trinucleotide d[pibG-ibG-anC(*t*-BuPh$_2$Si)] (**10**) (Figure 2). The octanucleotide is in peak I, the trinucleotide in peak II, the pyrophosphate of the latter in peak III, while the required undecanucleotide (**14**, Figure 5) is in peak IV. (B) HPLC, at 44% acetonitrile concentration, of the undecanucleotide (**14**) isolated by preparative HPLC (panel A). (C) HPLC (37% acetonitrile concentration) of the undecanucleotide **15** obtained after removal of the 3′-*O*-silyl group and purification by preparative HPLC. (D) HPLC of a co-injected mixture of the undecanucleotide **15** (peak I), of the octanucleotide **13** (peak II), and of the hexanucleotide **11** (peak III). Acetonitrile concentration was 39%.

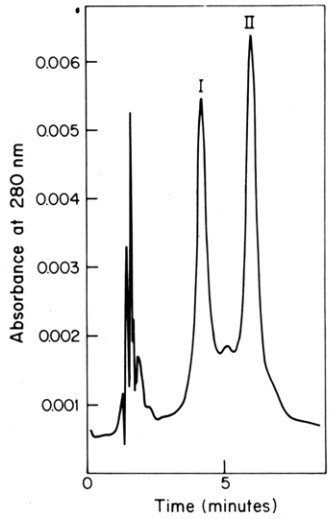

FIGURE 9: HPLC (46% acetonitrile) of a co-injected mixture of the undecanucleotide **14** and the octanucleotide **12**. The former was in peak I, while the octanucleotide was in peak II.

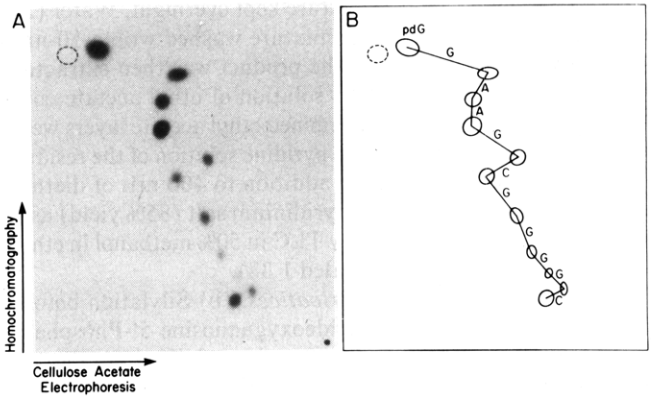

FIGURE 10: (A) A two-dimensional fingerprint of a partial snake venom phosphodiesterase digest of the undecanucleotide, d(G-G-A-A-G-C-G-G-G-G-C), after it was phosphorylated using [γ-^{32}P]ATP and polynucleotide kinase. (B) An artist's conception of the two-dimensional fingerprint shown in panel A. The dashed circle in panel A indicates the position of the dye marker, xylene cyanol.

2975, 2942, 2815, 1119, 1003, 855, 745, and 705 cm^{-1}], while *tert*-butyldiphenylchlorosilane was obtained in 77% yield over the range 120–125 °C (0.06 mm) (oil bath 184 °C) [NMR (CCl_4) δ 1.15 (s, 9), 7.28 and 7.70 (m and m, 10); IR (neat) 2965, 2935, 2865, 1431, 1365, 1112, 821, 742, and 700 cm^{-1}].

General Procedure for the Preparation of 3′-O-Butyldiphenylsilyl Deoxymononucleotides

n-Butyldiphenylsilyl Derivatives. 3′-*O*-Butyldiphenylsilyl-*N*-isobutyryldeoxyguanosine 5′-phosphate {d[pibG(*n*-BuPh$_2$Si)]}. To a dry solution of pyridinium *N*-isobutyryldeoxyguanosine 5′-phosphate (1.0 mmol) in pyridine (15 mL) was added 1.5 mL of *n*-butyldiphenylcholorosilane and the sealed mixture was stirred at room temperature. After 0.5 h the mixture was poured into 20 mL of 5% aqueous sodium bicarbonate solution. The excess of the silane was removed by extraction with diethyl ether (20 mL). Extraction with a 20-mL portion of ethyl acetate followed by ethyl acetate containing increasing amounts of 1-butanol (a 20-mL portion of 10% 1-butanol, a 30-mL portion of 33% 1-butanol, and a 30-mL portion of 50% 1-butanol in ethyl acetate) was required. The combined ethyl acetate extracts were evaporated and a concentrated pyridine solution of the residue precipitated by dropwise addition to 400 mL of diethyl ether. This gave 780 mg of the dipyridinium salt (96% yield) as a white powder, homogeneous by TLC in 35% methanol in ethyl acetate (A_{260}/A_{280} = 1.52, calcd 1.45; A_{280}/A_{300} = 2.29, calcd 2.50).

sec-Butyldiphenylsilyl Derivatives. (a) Using Imidazole: 3′-*O*-*sec*-Butyldiphenylsilyl-*N*-anisoyldeoxycytidine 5′-

phosphate {d[panC(*s*-BuPh$_2$Si)]}. To a dry solution of pyridinium *N*-anisoyldeoxycytidine 5′-phosphate (4.2 mmol) and 2.0 g (30 mmol) of imidazole in pyridine (75 mL) was added 6.5 mL (25 mmol) of *sec*-butyldiphenylchlorosilane. Although the reaction was judged by TLC (35% methanol in ethyl acetate) to be complete after 2 h, it was stirred overnight at room temperature. Water (75 mL) was then added and the mixture was washed with a 75-mL portion of petroleum ether. The product was extracted with three 75-mL portions of ethyl acetate, the combined ethyl acetate layers were evaporated, and a concentrated pyridine solution of the residue was precipitated by dropwise addition to 1.2 L of diethyl ether. This gave 3.2 g of the dipyridinium salt (95% yield) as a white powder, homogeneous by TLC in 35% methanol in ethyl acetate (A_{260}/A_{280} = 0.69, calcd 0.07; A_{280}/A_{300} = 0.88, calcd 0.80).

(b) Without Imidazole: 3′-*O*-*sec*-Butyldiphenylsilylthymidine 5′-Phosphate {d[pT(*s*-BuPh$_2$Si)]}. To a suspension of disodium thymidine 5′-phosphate (1 mmol) dried by evaporation from pyridine in dry pyridine (15 mL) was added 2.1 mL (10 mmol) of *sec*-butyldiphenylchlorosilane. After stirring at room temperature for 4 h, water (25 mL) was added and the mixture was washed with a 50-mL portion of petroleum ether. The product was then extracted with two 50-mL portions of a solution of ethyl acetate containing 25% 1-butanol. The combined ethyl acetate layers were evaporated and a concentrated pyridine solution of the residue was precipitated by dropwise addition to 400 mL of diethyl ether. This gave 580 mg of the dipyridinium salt (88% yield) as a white powder, homogeneous by TLC in 50% methanol in ethyl acetate (A_{260}/A_{280} = 1.33, calcd 1.38).

tert-Butyldiphenylsilyl Derivatives. (a) General Procedure Starting from Thymidine 5′-Phosphate or N-Protected Deoxynucleotides: 3′-*O*-*tert*-Butyldiphenylsilylthymidine 5′-Phosphate {d[pT(*t*-BuPh$_2$Si)]}. To a suspension of disodium thymidine 5-phosphate (1 mmol) dried by evaporation from pyridine and 400 mg (5.9 mmol) of imidazole in dry pyridine (15 mL) was added 1.3 mL (5 mmol) of *tert*-butyldiphenylchlorosilane. After stirring for 5 h at room temperature, water (5 mL) was added and the mixture kept overnight. Water (25 mL) was then added and the mixture washed wtih a 50-mL portion of petroleum ether. The product was then extracted with two 50-mL portions of a solution of ethyl acetate containing 25% 1-butanol. The combined ethyl acetate layers were evaporated and a concentrated pyridine solution of the residue was precipitated by dropwise addition to 400 mL of diethyl ether.This gave 560 mg of dipyridinium salt (85% yield) as a white powder, homogeneous by TLC in 50% methanol in ethyl acetate (A_{260}/A_{280} = 1.65, calcd 1.38).

tert-Butyldiphenylsilyl Derivatives. (b) Silylation before N-Protection: 3′-*O*-Isobutyryldeoxyguanosine 5′-Phosphate {d[pibG(*t*-BuPh$_2$Si)]}. To a suspension of disodium deoxyguanosine 5′-phosphate (10 mmol) dried by evaporation from pyridine and 6.8 g (0.1 mmol) of imidazole in dry pyridine (200 mL) was added 15 mL (60 mmol) of *tert*-butyldiphenylchlorosilane. After stirring for 18 h at room temperature, water (150 mL) was added and the mixture was washed with a 100-mL portion of petroleum ether. The product was then extracted with a 100-mL portion of ethyl acetate containing 30% 1-butanol and a 100-mL portion of ethyl acetate containing 50% 1-butanol. The combined ethyl acetate layers were evaporated and a concentrated pyridine solution of the residue precipitated by dropwise addition to 1 L of diethyl ether. This gave a quantitative yield of the product as a white powder, homogeneous by TLC in 50% methanol in ethyl acetate. It was then dissolved in dry pyridine (200 mL) and the solution treated with isobutyric anhydride (60 mL). After stirring for 5 days at room temperature, water (150 mL) was added and the mixture washed with three 100-mL portions of diethyl ether. The product was then extracted with a 100-mL portion of ethyl acetate followed by a 100-mL portion of ethyl acetate containing 30% 1-butanol. The combined ethyl acetate layers were evaporated and a concentrated pyridine solution of the residue precipitated by dropwise addition to 1 L of diethyl ether. This gave 5.14 g of the dipyridinium salt (64% yield) as a white powder, homogeneous by TLC in 35% methanol in ethyl acetate (A_{260}/A_{280} = 1.46, calcd 1.45; A_{280}/A_{300} = 2.44, calcd 2.50).

3′-*O*-*tert*-Butyldiphenylsilyl-*N*-benzoyldeoxyadenosine 5′-Phosphate {d[pbzA(*t*-BuPh$_2$Si)]}. To a suspension of deoxyadenosine 5′-phosphate (1 mmol) dried by evaporation from pyridine and 400 mg (5.9 mmol) of imidazole in dry pyridine (15 mL) was added 1.5 mL (5.8 mmol) of *tert*-butyldiphenylchlorosilane. After stirring for 17 h at room temperature, water (15 mL) was added and the mixture was washed with a 50-mL portion of petroleum ether. The product was then extracted with three 50-mL portions of ethyl acetate. The combined ethyl acetate layers were evaporated and dried by evaporation from dry pyridine and the residue was dissolved in dry pyridine (20 mL). The solution was then treated with 21 mL of benzoyltrifluoroacetate (Emmons et al., 1953). After stirring for 3 h at room temperature the solution was cooled (0 °C) and a 25-mL portion of aqueous pyridine (50%) was added. After being kept overnight the mixture was washed with a 50-mL portion of petroleum ether and the product extracted into three 50-mL portions of ethyl acetate. The combined ethyl acetate layers were evaporated, dried by evaporation from dry pyridine, and the residue dissolved in dry pyridine (20 mL). The solution was then treated with acetic anhydride (6 mL). After stirring for 2 h at room temperature, the reaction was cooled (0 °C) and water was added (25 mL). After being kept overnight, the mixture was washed with a 50-mL portion of petroleum ether and the product was extracted into two 50-mL portions of ethyl acetate. The combined ethyl acetate layers were evaporated and a concentrated pyridine solution of the residue precipitated by dropwise addition to diethyl ether. This gave 440 mg of the pyridinium salt (58% yield) as a white product homogeneous on TLC in 35% methanol in ethyl acetate (A_{260}/A_{280} = 0.65, calcd 0.59; A_{280}/A_{300} = 3.00, calcd 2.62).

3′-*O*-*tert*-Butyldiphenylsilyl-*N*-anisoyldeoxycytidine 5′-Phosphate {d[panC(*t*-BuPh$_2$Si)]}. To a suspension of deoxycytidine 5′-phosphate (1 mmol), dried by evaporation from pyridine, and 400 mg (5.9 mmol) of imidazole in dry pyridine (15 mL) was added 1.5 mL (5.8 mmol) of *tert*-butyldiphenylchlorosilane. After stirring for 17 h at room temperature, water (25 mL) was added and the mixture washed with a 50-mL portion of petroleum ether. The product was then extracted with two 50-mL portions of ethyl acetate. The combined ethyl acetate layers were evaporated and dried by evaporation from dry pyridine and the residue was dissolved in 20 mL of dry pyridine. This solution was then treated with 0.8 mL (6 mmol) of anisoyl chloride. After being stirred for 1 h at room temperature, the solution was cooled (0 °C) and 50% aqueous pyridine (15 mL) was added. The mixture was washed with a 50-mL portion of petroleum ether and three 25-mL portions of a 3:1 (v/v) mixture of diethyl ether and petroleum ether. The product was then extracted into three 50-mL portions of ethyl acetate. The combined ethyl acetate layers were evaporated and dried by evaporation from dry pyridine and the residue was dissolved in dry pyridine (20 mL). This solution was treated with acetic anhydride (4 mL). After

stirring for 5 h at room temperature, the reaction was cooled (0 °C) and a 15-mL portion of aqueous pyridine (50%) was added. After being kept overnight the mixture was washed with two 50-mL portions of diethyl ether. The product was then extracted into two 50-mL portions of ethyl acetate. The combined ethyl acetate layers were evaporated and the product was precipitated by dropwise addition of a concentrated pyridine solution to 350 mL of diethyl ether. This gave 505 mg of the dipyridinium salt (66% yield), as a white powder homogeneous on TLC in 35% methanol in ethyl acetate (A_{260}/A_{280} = 0.69, calcd 0.70; A_{280}/A_{300} = 0.86, calcd 0.80).

3′-*O*-*tert*-Butyldiphenysilyl-*N*-isobutyryldeoxyguanosine 5′-[2-(*p*-Tritylphenyl)sulfonylethyl] Phosphate {d[(TPSE)-pibG(*t*-BuBh$_2$Si)]} (**6**). An anhydrous pyridine solution (20 mL) of 3′-*O*-*tert*-butyldiphenylsilyldeoxyguanosine 5′-phosphate (0.92 mmol), 2-(*p*-tritylphenyl)sulfonylethanol (1.3 mmol), and *N,N*′-dicyclohexylcarbodiimide (9.2 mmol) was allowed to react for 22 h at room temperature. The mixture was then filtered and a 10-mL portion of water was added to the filtrate. The product was then extracted into two 20-mL portions of ethyl acetate. The combined ethyl acetate layers were evaporated and a concentrated pyridine solution of the residue precipitated by dropwise addition to 400 mL of 1:1 mixture (v/v) of diethyl ether and petroleum ether. This gave an 83% yield of a white powder homogeneous on TLC in 25% methanol in ethyl acetate.

The Dinucleotide d[(TPSE)pibG-ibG(*t*-BuPh$_2$Si)] (**1**). *N*-Isobutyryldeoxyguanosine 5′-[2-(*p*-tritylphenyl)sulfonylethyl] phosphate was prepared from d[(TPSE)pibG(*t*-BuPh$_2$Si)] (**6**) by the standard procedure in quantitative yield. To its anhydrous pyridine solution (3 mmol in 35 mL) were added 3′-*O*-*tert*-butyldiphenylsilyl-*N*-isobutyryldeoxyguanosine 5′-phosphate (4.5 mmol) and TPS (12 mmol) and the mixture was allowed to react for 17 h at room temperature. The reaction mixture was quenched and allowed to stand overnight. The mixture was then evaporated to a gum, dissolved in 100 mL of 0.2 M TEAB, and washed with two 50-mL portions of diethyl ether. The product was then extracted into three 50-mL portions of ethyl acetate containing a 20% 1-butanol. The combined ethyl acetate layers were evaporated and a concentrated pyridine solution of the residue precipitated by dropwise addition to 1 L of diethyl ether to give 3.2 g of the product. A 1.9-g sample was further purified on a 3 × 34 cm column of EM7734 silica gel 60, eluted with 650 mL of 50% and 500 mL of 60% methanol in ethyl acetate. Fractions comprising 400 to 1000 mL of the eluate were combined and evaporated to give 1.1 g (41%) of a tan powder homogeneous on TLC in 50% methanol in ethyl acetate (A_{260}/A_{280} = 1.43, calcd 1.45; A_{280}/A_{300} = 1.90, calcd 2.50).

The Dinucleotide d[(TPSE)pbzA-bzA(*n*-BuPh$_2$Si)]. d[pbzA(*n*-BuPh$_2$Si)] was prepared from d(pbzA) in 90% yield by the standard method. An anhydrous pyridine solution (20 mL) of *N*-benzoyldeoxyadenosine 5′-[2-(*p*-tritylphenyl)sulfonylethyl] phosphate (3 mmol), 3′-*n*-butyldiphenylsilyl-*N*-benzoyldeoxyadenosine 5′-phosphate (2 mmol), and TPS (7 mmol) was allowed to react for 5.5 h at room temperature; the reaction mixture was then quenched and allowed to stand overnight. The mixture was evaporated to a gum, dissolved in 100 mL of 0.2 M TEAB, and washed with a 100-mL portion of diethyl ether. The product was extracted with a 100-mL portion of ethyl acetate containing 10% 1-butanol and a 100-mL portion of ethyl acetate containing 20% 1-butanol. The combined ethyl acetate layers were evaporated and the product was precipitated by the dropwise addition of a concentrated pyridine solution of the residue to 1 L of diethyl ether to give 3.4 g. Preparative HPLC gave 831 mg (28% yield).

The Dinucleotide d[(TPSE)pbzA(*t*-BuPh$_2$Si)]. An anhydrous pyridine solution (25 mL) of *N*-benzoyldeoxyadenosine 5′-[2-(*p*-tritylphenyl)sulfonylethyl] phosphate (3 mmol), 3′-*O*-*tert*-butyldiphenylsilyl-*N*-benzoyldeoxyadenosine 5′-phosphate (4.5 mmol), and TPS (12 mmol) was allowed to react for 5.5 h; the reaction mixture was quenched and allowed to stand overnight. The mixture was then evaporated to a gum, dissolved in 100 mL of 0.2 M TEAB, and washed with two 50-mL portions of diethyl ether. The product was extracted with a 50-mL portion of ethyl acetate containing 10, 15, 20, and 25% 1-butanol. The combined ethyl acetate layers were evaporated and a concentrated pyridine solution of the residue was added dropwise to 1 L of diethyl ether to precipitate the product (6.4 g). Preparative HPLC of a 2.4-g sample gave 694 mg (30% yield) of the pure product.

The Dinucleotide d[(MeOTr)T-T(*n*-BuPh$_2$Si)]. An anhydrous pyridine solution (1 mL) of 5′-*O*-*p*-methoxytritylthymidine (0.1 mmol), 3′-*O*-*n*-butyldiphenylsilylthymidine 5′-phosphate (0.15 mmol), and TPS (0.3 mmol) was allowed to react for 5.5 h, the mixture was quenched, and a 50-mL portion of 10% aqueous sodium bicarbonate was added. This was extracted with two 25-mL portions of ethyl acetate and the extract was evaporated to a gum. The gum was dissolved in 15% methanol in ethyl acetate and the solution applied to a 7-g column of EM 7734 silica gel 60 (1 × 20 cm). Elution was carried out with 90 mL of ethyl acetate containing 15% methanol followed by 50 mL of ethyl acetate containing 25% methanol. The fractions comprising 70 to 120 mL of the eluate were evaporated and the residue, as a concentrated pyridine solution, was precipitated by dropwise addition to 40 mL of diethyl ether to give 50 mg (50% yield) of the product as the pyridinium salt. This was homogeneous by TLC in 25% methanol in ethyl acetate.

The Dinucleotide d[(TPSE)pibG-ibG]. An anhydrous solution of d[(TPSE)pibG-ibG(*t*-BuPh$_2$Si)] (**7**) (0.23 mmol), tetra-*n*-butylammonium fluoride (1 mmol), and pyridinium fluoride (1 mmol) in dry pyridine (4 mL) was allowed to react for 17 h. The mixture was then passed over a 1-mL column of pyridinium Dowex-50 resin; the eluate was diluted with water (20 mL) and washed with 2-mL portions of diethyl ether. The product was then extracted into one 50-mL and two 25-mL portions of ethyl acetate containing 50% 1-butanol. The combined ethyl acetate layers were evaporated and the residue as a concentrated pyridine solution was precipitated by dropwise addition to 300 mL of diethyl ether. This gave 300 mg (94%) of the product which was homogeneous by TLC in 50% methanol in ethyl acetate and HPLC (A_{260}/A_{280} = 1.63, calcd 1.45; A_{280}/A_{300} = 2.96, calcd 2.50).

The Dinucleotide d[pibG-ibG(*t*-BuPh$_2$Si)]. To 200 mg (0.133 mmol) of d[(TPSE)pibG-ibG(*t*-BuPh$_2$Si)] (**7**) dissolved in 7.5 mL of methanol–pyridine–water (30:65:5) at 0 °C was added 2.5 mL of a 0.5 N NaOH solution in the same solvent mixture. TLC (50% methanol in ethyl acetate) of a sample removed after 1 min showed complete removal of the TPSE group. After 3 min, the mixture was neutralized with pyridinium Dowex-50 resin and the solution was filtered and evaporated. The residue was dissolved in a small amount of dry pyridine and the product was precipitated from 40 mL of diethyl ether to give 130 mg (84%) yield) of a powder which was homogeneous by HPLC (A_{260}/A_{280} = 1.52, calcd 1.45; A_{280}/A_{300} = 2.37, calcd 2.50).

The Dinucleotide d[(TPSE)pbzA-bzA]. To an anhydrous pyridine solution (3 mL) of d[(TPSE)pbzA-bzA(*n*-BuPh$_2$Si)] (0.18 mmol) was added 0.64 mmol of tetra-*n*-butylammonium fluoride in 3 mL of dry pyridine. After 20 min the reaction was passed through a 1-mL column of pyridinium Dowex-50 and

the effluent was evaporated to a gum which was dissolved in 10 mL of 0.1 M TEAB. This was washed with 10 mL of diethyl ether and the product was extracted with three 10-mL portions of ethyl acetate containing 30% 1-butanol. The combined ethyl acetate layers were evaporated and the product was precipitated by the dropwise addition of a concentrated pyridine solution to an excess of diethyl ether; yield 231 mg (89%).

The Trinucleotide d[(TPSE)pbzA-bzA-anC(*s*-BuPh$_2$Si)]. An anhydrous pyridine solution (12 mL) of d[(TPSE)pbzA-bzA] (0.45 mmol), 3′-*O*-*sec*-butyldiphenylsilyl-*N*-anisoyldeoxycytidine 5′-phosphate (2.25 mmol), and TPS (5.4 mmol) was allowed to react for 5.5 h. The mixture was then quenched and allowed to stand overnight. Water (20 mL) was then added and the mixture washed with a 75-mL portion of diethyl ether and the excess of the mononucleotide was removed by extraction with two 100-mL portions of ethyl acetate and two 50-mL portions of ethyl acetate containing 10% 1-butanol. The product was then extracted with four 50-mL portions of ethyl acetate containing 20% 1-butanol. The combined product fractions were evaporated and the residue, as a concentrated pyridine solution, was added dropwise to 400 mL of diethyl ether to give 700 mg (70% yield) of the product which was homogeneous by HPLC (A_{260}/A_{280} = 0.72, calcd 0.63; A_{280}/A_{300} = 1.56, calcd 1.50).

The Trinucleotide d[(TPSE)pibG-ibG-anC(*t*-BuPh$_2$Si)]. An anhydrous pyridine solution (3 mL) of 0.1 mmol of [(TPSE)pibG-ibG] (0.1 mmol), 3′-*O*-*tert*-butyldiphenylsilyl-*N*-anisoyldeoxycytidine 5′-phosphate (0.5 mmol), and TPS (1 mmol) was allowed to react for 16.5 h; the reaction was then quenched and the mixture allowed to stand for 1.5 h. Water (20 mL) was then added and the mixture was washed with a 30-mL portion of diethyl ether. The excess of the mononucleotide was removed by extraction with a 25-mL portion of ethyl acetate followed by a 25-mL portion of ethyl acetate containing 10% 1-butanol. The product was then extracted into a 25-mL portion of ethyl acetate containing 25% 1-butanol, which was evaporated. A concentrated pyridine solution of the residue was added dropwise to 40 mL of diethyl ether to give 150 mg (70% yield) of a product homogeneous by TLC in 50% methanol in ethyl acetate and HPLC (A_{260}/A_{280} = 1.17, calcd 1.15; A_{280}/A_{300} = 1.42, calcd 1.26).

The Tetranucleotide d[(MeOTr)bzA-bzA-T-T(*t*-BuPh$_2$Si)]. An anhydrous pyridine solution (2 mL) of d[(MeOTr)bzA-bzA-T] (0.1 mmol), 3′-*O*-*tert*-butyldiphenylsilylthymidine 5′-phosphate (0.4 mmol), and TPS (1 mmol) was allowed to react for 17 h at room temperature. The reaction mixture, after quenching, was allowed to stand for 5 h. Water (25 mL) was then added and the mixture was washed with a 50-mL portion of diethyl ether and part of the excess mononucleotide was removed by extraction with a 50-mL portion of ethyl acetate containing 10% 1-butanol. Extraction with two 50-mL portions of ethyl acetate containing 50% 1-butanol brought all nucleotidic material into the organic phase. Extraction of these ethyl acetate layers with three 5-mL portions of water isolated the product, leaving only mononucleotide in the ethyl acetate layers. The aqueous extracts were then diluted with 100 mL of pyridine, the solution was evaporated, and a concentrated pyridine solution of the residue was added dropwise to 40 mL of diethyl ether to give 210 mg (95% yield) of the product, which was homogeneous by HPLC (A_{260}/A_{280} = 0.84, calcd 0.80; A_{280}/A_{300} = 3.10, calcd 3.43).

The Trinucleotide d[pibG-ibG-anC(*t*-BuPh$_2$Si)]. To a solution of 75 mg (0.035 mmol) of d[(TPSE)pibG-ibG-anC(*t*-BuPh$_2$Si)] in 1.7 mL of methanol–pyridine–water (30:65:5) at 0 °C was added 0.3 mL of 1 N NaOH in the same solvent. After 3 min the reaction mixture was neutralized with pyridinium Dowex-50 resin and the solution filtered. Water (5 mL) was then added to the filtrate and the mixture washed, successively, with a 10-mL portion of diethyl ether, a 5-mL portion of ethyl acetate and a 5-mL portion of ethyl acetate containing 20% 1-butanol. The aqueous layer was then evaporated and the product was precipitated by the dropwise addition of a concentrated pyridine solution to 10 mL of diethyl ether to give 50 mg (80% yield) of a powder which was homogeneous by HPLC (A_{260}/A_{280} = 1.17, calcd 1.15; A_{280}/A_{00} = 1.17, calcd 1.26).

The Octanucleotide d[(MeOTr)ibG-ibG-bzA-bzA-ibG-anC-ibG-ibG(*t*-BuPh$_2$Si)]. An anhydrous solution (1.4 mL) of d[(MeOTr)ibG-ibG-bzA-ibG-anC] (14 μmol), d[pibG-ibG(*t*-BuPh$_2$Si)] (**8**) (123 μmol), and TPS (440 μmol) was allowed to react for 6 h. The reaction was quenched and the solution allowed to stand overnight. The mixture was then diluted with 10 mL of 0.2 M TEAB and washed with a 10-mL portion of diethyl ether, a 5-mL portion of ethyl acetate, and a 5-mL portion of ethyl acetate containing 20% 1-butanol. The aqueous layer was evaporated and a concentrated pyridine solution of the residue precipitated by dropwise addition to 40 mL of diethyl ether to give 210 mg. This mixture was separated in three runs on a semipreparative HPLC reverse-phase column using 43% acetonitrile plus 0.1 M TEAA and one run as a 20-min gradient from 40 to 45% acetonitrile plus 0.1 M TEAA. Appropriate fractions were combined and evaporated and the product was precipitated from pyridine–ether by the standard method. In this way, 11 mg of the hexanucleotide (**11**) (28%) and 94 mg of the dinucleotide (**8**) (58%) were recovered. The yield of product, octanucleotide (**12**), was 12 mg (21%) (A_{280}/A_{280} = 1.15, calcd 1.05; A_{280}/A_{300} = 1.66, calcd 1.62).

The condensation was repeated using the recovered hexanucleotide **11** (11 mg, 2 μmol) and the dinucleotide **8** (94 mg, 72 μmol) with 60 mg (200 μmol) of TPS in 0.8 mL of anhydrous pyridine for 18 h. Treatment analogous to that described above gave 120 mg of a mixture which was separated in two runs as a gradient (40 to 45%, as above). Evaporation and precipitation gave 35 mg (37%) of recovered dinucleotide (**8**) and 40 OD_{260} units (12%) of the product octanucleotide **12**.

The Octanucleotide d[(MeOTr)ibG-ibG-bzA-bzA-ibG-anC-ibG-ibG] (**13**). To an anhydrous pyridine solution (0.2 mL) of 300 OD_{260} units of d[(MeOTr)ibG-ibG-bzA-bzA-ibG-anC-ibG-ibG(*t*-BuPh$_2$Si)] (**12**) was added 0.2 mL of 0.25 M tetra-*n*-butylammonium fluoride in pyridine. After 5 h, desilylation had occurred to about 80% as shown by HPLC. After an additional 4 h the mixture was passed over a 1-mL column of pyridinium Dowex-50 resin, the solution was evaporated, and the product was precipitated as usual to give 18 mg. This was further purified in one run on the semipreparative reverse-phase column as a gradient from 30 to 38%. This gave 120 OD_{260} units (40%) of the pure octanucleotide (A_{260}/A_{280} = 1.10, calcd 1.05; A_{280}/A_{300} = 1.94, calcd 1.62).

The Undecanucleotide d[(MeOTr)ibG-ibG-bzA-bzA-ibG-anC-ibG-ibG-ibG-ibG-anC(*t*-BuPh$_2$Si)] (**14**). An anhydrous pyridine solution (0.3 mL) of 120 OD_{260} units (1 μmol) of **13**, 36 mg (20 μmol) of d[pibG-ibG-anC(*t*-BuPh$_2$Si)] (**10**), and TPS (70 μmol) was allowed to react for 17 h, quenched, and allowed to stand for 1 h. Water (10 mL) was then added and the mixture washed with a 10-mL portion of diethyl ether, a 10-mL portion of ethyl acetate, and two 5-mL portions of ethyl acetate containing 20% 1-butanol. Evaporation and precipitation of the aqueous layers gave 47 mg. This was separated in one run on the semipreparative column as a 30-min gradient from 35 to 40%, giving 18.5 OD_{260} of the

undecanucleotide (A_{260}/A_{280} = 1.09, calcd 1.68).

The Undecanucleotide d[(MeOTr)ibG-ibG-bzA-bzA-ibG-anC-ibG-ibG-ibG-ibG-anC] (**15**). An anhydrous pyridine solution (110 μL) of **14** (6 OD_{260} units) and tetra-*n*-butylammonium fluoride (5 μmol) was allowed to stand 19 h. (A sample removed after 2 h showed approximately ⅔ desilylation by HPLC.) The mixture was then evaporated and the residue was dissolved in 100 μL of 0.1 M TEAA and purified in three runs on the analytical HPLC column to give 3 OD_{260} units of **15** (50%) (a_{260}/A_{280} = 1.12, calcd 1.07; A_{280}/A_{300} = 1.47, calcd 1.68).

The Deprotected Undecanucleotide d(G-G-A-A-G-C-G-G-G-G-C). The product recovered from the HPLC column was completely deprotected by the standard method. The resultant undecanucleotide, without any purification, was phosphorylated by polynucleotide kinase using [γ-^{32}P]ATP and subjected to the two-dimensional fingerprinting procedure. The pattern obtained is shown in Figure 10.

General Comments

Efforts continue to be made to devise new, as well as more rapid, methods for the chemical synthesis of short polynucleotides. The work now reported is significant, first, in regard to the general methodology for polynucleotide synthesis and, second, for introducing a new concept in facilitating rapid separation of the required product after every condensation step.

Chemically, even the preparation of protected mononucleotides has benefited from the use of the silyl groups. Since amino groups do not form stable silyl derivatives, silylation of mononucleotides can be carried out before protection of the amino groups. Thus, disodium deoxyguanosine 5′-phosphate was first silylated with *t*-$BuPh_2SiCl$; the 3′-*O*-*tert*-butyldiphenylsilyl derivative was isolated by extraction and treated with isobutyric anhydride to protect the amino group. Similarly, preparation of TPSE-protected mononucleotdies has also been improved in that following protection of the 3′-hydroxyl group by silylation only a limited (about 1 equiv) amount of TPSE is required to derivatize the 5′-phosphate group, the concomitant formation of homooligomers of the mononucleotides being precluded.

The synthesis outlined in Figure 2 demonstrates the increase in scope and flexibility in oligonucleotide synthesis made possible by the use of the 3′-*O*-*tert*-butyldiphenylsilyl group in conjunction with the TPSE group. Thus, for the first time in a direct manner, it is possible to go from **7** to either the protected dinucleotide **8**, which is suitable for chain elongation reactions, or to go to the corresponding trinucleotide **10**, via **9**. The use of the classical 3′-*O*-acetyl group in conjunction with the established 5′-protecting groups, TPSE or cyanoethyl, entails additional steps which make the synthetic procedure much less efficient. Furthermore, the very convenient and selective removal of the 3′-*O*-silyl group by fluoride ions promises to open new doors to polynucleotide synthesis and, indeed, it appears highly desirable to reevaluate the protecting group methodology. For example, the use of a base-labile 5′-protecting group in place of the monomethoxytrityl is now feasible and would have the important advantage of avoiding the acidic treatment required for removal of the trityl group. Finally, it should be emphasized that although the present work has mainly used the *tert*-butyldiphenylsilyl group, other silyl ethers should no doubt be of use in special cases and in specific combinations of protecting groups, e.g., the *sec*-butyldiphenylsilyl group can be removed with ammonia.

The second general application of the present work is in the separation of the required product from the other components of the reaction mixtures. Only the condenstation product contains highly lipophilic groups at both the 5′- and 3′-termini. The influence of the 3′-*O*-silyl group outweighs the effects of the other constituents and the N-protecting groups in the oligonucleotide chains. Thus, without exception, the condensation products carrying 3′-*O*-silyl groups emerged last from the HPLC columns (Figures 4, 6, 7, and 8). Thus, identification of the required product should present no difficulty. Further, it should be noted that in contrast with the classical anion exchange chromatography, where separations are largely determined by charge, present separations are based on the lipophilic "handles".

Finally, another important possibility raised by the present work is that the method of choice for the synthesis of short chains of deoxyribopolynucleotides may be stepwise elongation using 3′-*O*-silyl mononucleotides alone. The merits of this approach, in contrast with the one using preformed oligonucleotide blocks, have been discussed from time to time. The facile preparation of 3′-*O*-silyl mononucleotides and the extremely rapid separations made possible by HPLC make this classical approach (Khorana, 1968a, b) more attractive. Indeed, it would seem that this approach, involving rapid purification of the product at every step, would favorably compete with the concept of solid-phase synthesis unless the latter accomplished exactly 100% yields at every step.

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References

Agarwal, K. L., Yamazaki, A., Cashion, P. J., & Khorana, H. G. (1972) *Angew. Chem. Int. Ed. Engl. 11,* 451.

Agarwal, K. L., Berlin, Y. A., Fritz, H.-J., Gait, M. J., Kleid, D. G., Lees, R. G., Norris, K. E., Ramammoorthy, B., & Khorana, H. G. (1976) *J. Am. Chem. Soc. 98,* 1065.

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

Corey, E. J., & Venkateswarlu, A. (1972) *J. Am. Chem. Soc. 94,* 6190.

Emmons, W. D., McCallum, K. S., & Ferris, A. F. (1953) *J. Am. Chem. Soc. 75,* 6947.

Fritz, H.-J., Belagaje, R., Brown, E. L., Fritz, R. H., Jones, R. A., Lees, R. G., & Khorana, H. G. (1978a) *Biochemistry 17* (preceding paper in this issue).

Fritz, H.-J., Belagaje, R., Brown, E. L., Fritz, R. H., Jones, R. A., Lees, R. G., & Khorana, H. G. (1978b) *Adv. Chem. Ser.* (in press).

Hanessian, S., & Lavallee, P. (1975) *Can. J. Chem. 53,* 2975.

Khorana, H. G. (1968a) Proceedings of the Seventh International Congress of Biochemistry, Tokyo, Aug 19–25, p 17.

Khorana, H. G. (1968b) *Biochem. J. 109,* 709.

Khorana, H.G., Agarwal, K. L., Büchi, H., Caruthers, M. H., Gupta, N., Kleppe, K., Ohtsuka, E., RajBhandary, U. L., van de Sande, J. H., Sgaramella, V., Terao, T., Weber, H., & Yamada, T. (1972) *J. Mol. Biol. 72,* 209, and succeeding papers.

Kössel, H., and Seliger, H. (1975) *Fortschr. Chem. Org. Naturst. 32.*

Ogilvie, K. K. (1973) *Can. J. Chem. 51,* 3799.
Ogilvie, K. K., Beaucage, S. L., Entwistle, D. W., Thompson, E. A., Guilliam, M. A., & Westmore, J. B. (1976) *J. Carbohydr., Nucleosides, Nucleotides 3,* 197.
Sanger, F., Donelson, J. E., Coulson, A. R., Kössel, H., & Fischer, D. (1974) *J. Mol. Biol. 90,* 315.
Seliger, H., Schütz, H., Saur, E., & Phillips, M. (1975) *J. Carbohydr., Nucleosides, Nucleotides 2,* 79.

Conformation of Dinucleoside Monophosphates Modified with Benzo[*a*]pyrene-7,8-dihydrodiol 9,10-Oxide as Measured by Circular Dichroism†

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ABSTRACT: The conformational properties of GpU modified with the reactive derivative of benzo[*a*]pyrene, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, have been investigated utilizing circular dichroism spectroscopy. Binding of this carcinogen to the N^2 of G residues in GpU resulted in the formation of four compounds (I to IV) representing two pairs of diastereoisomers. The molar ellipticity values of the modified dimers were approximately twofold higher than those of the modified guanosine monomers. These values were decreased appreciably when the spectra of the dimers were obtained at 80 °C or in methanol rather than at 25 °C in water, suggesting that under the latter conditions there is a stacking interaction between the carcinogen and the neighboring uridine residue. Based on these results, a conformation is proposed for modified GpU. It includes insertion of the benzo[*a*]pyrene moiety, by rotation of the modified guanine residue about its glycoside bond, coplanar to the neighboring uridine and perpendicular to the phosphodiester backbone.

Benzo[*a*]pyrene, one of the most abundant environmental carcinogens (Committee on Biologic Effects of Atmospheric Pollutants, 1972), interacts with the genetic material of cells (Miller & Miller, 1974; Heidelberger, 1975). Recent studies indicate that the major reactive metabolite of benzo[*a*]pyrene with respect to covalent binding to nucleic acids, both in vitro and in vivo, is (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE I)[1] (Sims et al., 1974; Daudel et al., 1975; King et al., 1976; Weinstein et al., 1976; Koreeda et al., 1976). The in vitro modification of DNA or poly[G] with BPDE yields derivatives which are the same as those found in cellular RNA and DNA obtained from bovine and human bronchial explants incubated with [^{3}H]BP (Jeffrey et al., 1976, 1977). More recently, BPDE adducts have also been detected in mouse skin RNA following topical application of [^{3}H]BP (Moore et al., 1977). It has been established that the major nucleic acid derivative results from the formation of a covalent bond between the 10 position of BPDE and the N^2 position of guanine (Weinstein et al., 1976; Jeffrey et al., 1976; Moore et al., 1977). Complete stereochemistry of the major guanine adduct present in bovine and human bronchial RNA and DNA has also been established (Nakanishi et al., 1977; Jeffrey et al., 1977; Yang et al., 1977).

The biochemistry of BPDE is complicated by the fact that it can exist as four isomers. In BPDE isomer I the 7-OH and 9,10-oxide groups are on opposite sides of the ring system and in isomer II they are on the same side. Both isomers I and II consist of enantiomeric pairs, designated 7α and 7β. All four of these have been prepared (Harvey & Cho, 1977; Yagi et al., 1977) and found to react with guanine and to a lesser extent with adenine and cytosine residues in nucleic acids (Weinstein et al., 1976; Jennette et al., 1977). It appears that both isomers I and II are formed in vivo, although the relative abundance of their corresponding in vivo nucleic acid adducts varies among cell cultures, tissues, and species (Weinstein et al., 1976; Jeffrey et al., 1977; Shinohara & Cerutti, 1977; Moore et al., 1977; Baird and Diamond, 1977). In addition, although trans addition to the 10 position appears to be the predominant reaction during nucleic acid modification, there is also evidence for cis addition products (Jeffrey et al., 1977; Moore et al., 1977).

The covalent attachment of this bulky carcinogen to nucleic acids presents steric and conformational problems which are associated with structural and functional changes in the modified nucleic acids and may be relevant to the biology of carcinogens (Pulkrabek et al., 1977; Gamper et al., 1977; Leffler et al., 1977). We have found that BPDE modification of calf thymus DNA produces small localized regions of destabilization of the native structure (Pulkrabek et al., 1977) and inhibits chain elongation when the modified DNA is used as a template for transcription by *E. coli* RNA polymerase (Leffler et al., 1977). To find out in greater detail how attachment of the bulky hydrocarbon molecule to the N^2 position of the guanosine residue alters the tertiary structure of nucleic acids, the present study on the conformation of BPDE modified

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[1] Abbreviations used: BP, benzo[*a*]pyrene; BPDE I, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BPDE II, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; G*, guanosines containing a covalently bound hydrocarbon residue as a result of modification by BPDE; HPLC, high pressure liquid chromatography.

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