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Synthesis of Deoxyoligonucleotides on an Isotactic Polymer Support

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ABSTRACT

An insoluble and noncross-linked polymer of isotactic polystyrene having a p-methoxytrityl group as part of its structure was chosen as a solid polymer support for deoxyribonucleotide synthesis. The p-methoxytrityl functional group was introduced into the isotactic polymer by benzoylation and Grignard reaction. Judging by the NMR spectra, the isotacticity of the polymer remains unaffected after the modifications. Deoxynucleosides were linked to the polymer via 5' ether group. Condensation of the polymer-nucleoside derivative with 3' protected nucleotides in the presence of mesitylene sulfonyl chloride resulted in 36-63% coupling to the bound nucleoside. Subsequent condensation gave the trinucleoside diphosphate in 25-57% conversion, based on polymer-bound dinucleoside phosphate. The limitation of the polymer support still lies in the low yield of the coupling step, but the value of an isotactic polymer backbone as a "dendritic" center for chain lengthening remains attractive.

INTRODUCTION

In order to extend our enzyme study [1, 2] on mammary tissue and tumor endonuclease, there is a need for some specific sequence deoxyoligonucleotides (milligram). For such a preparative purpose the synthesis of oligonucleotides on a solid polymer support system appears attractive to us. Soluble atactic polystyrene and cross-linked polystyrene had been used as the polymer support. The advantages and disadvantages of these two polymer systems can be summarized as follows: For the soluble polystyrene system that was investigated by Khorana [3] and in other laboratories [4], the yield of the internucleotide condensation steps are usually good, but the attachment of the first nucleoside unit to the polymer is low. The polymeric materials that are used in the experiments cannot be recovered completely; the loss is sometimes around 10-15%. The insoluble cross-linked polystyrene that was used by Letsinger [5] and others [6-9] has the advantage of high yield for the initial reaction between the polymer and nucleoside. However, most of the subsequent internucleotide condensation yields dropped off, probably because of the problem of reagent diffusion. The isotactic polystyrene that we use in our laboratory [10] has the advantages of both systems. Because of its isotacticity, isotactic polystyrene is not soluble in ordinary organic solvents. Therefore, it can be separated from the reaction mixture easily after each reaction. Since it is not a cross-linked polymer, it should have neither the problem of reagent diffusion nor the tendency to trap reagents or products inside the polymer pores. While a number of methods for covalently linking the first nucleoside or nucleotide to the polymer support could be considered, in our work we used essentially a modified procedure of Hayatsu and Khorana [3] whereby the introduction of the methoxytrityl group can be achieved by a simple organic reaction. Furthermore, the reaction of the subsequent formation of the methoxytrityl derivatives can be easily controlled by varying the reaction conditions, thus providing various types of reactive polymer.

DISCUSSION AND RESULTS

The isotactic polystyrene prepared by the method of Tsou et al. [11] was modified by benzoylation in carbon disulfide with aluminum chloride as catalyst (Fig. 1). The IR spectra of the final products from different runs are almost the same, but characteristic differences can be found if the degree of benzoylation is different. Compared with the isotactic polystyrene, new peaks appear at 1650, 1400, 1295, 1270, 840, and 790 cm^{-1}. The absorbance of these new peaks is different for different degrees of benzoylation. The most characteristic absorption for the

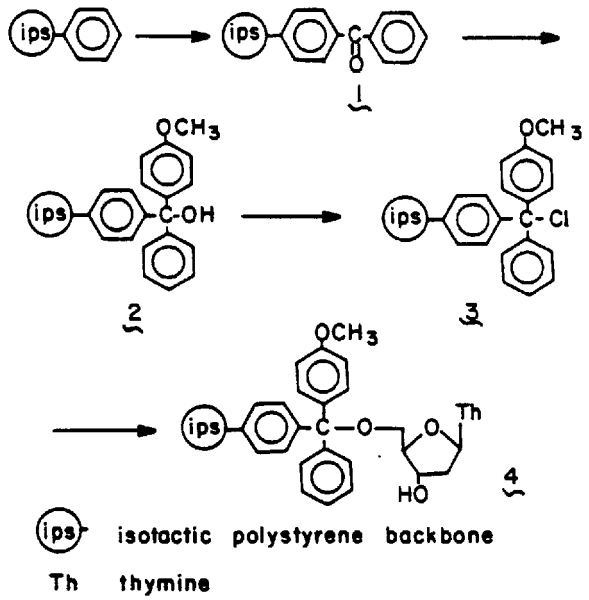

FIG. 1. Modification of the isotactic polystyrene.

derivative is the carbonyl absorption at 1650 cm^{-1}. The extinction coefficient of this absorption peak is derived from the monomer benzophenone based on their similar structure. The extinction coefficient (1.6×10^2 $mole^{-1}$ 200 mg KBr) then served as the standard for the quantitative determination of the carbonyl absorption in the polymer. The results of this reaction are listed in Table 1. The degree of benzoylation varied from 10 to 55% and could be controlled by changing the reagent-polymer ratio and the reaction time. We found that only about 50% of the phenyl groups are available for reaction, which probably could be anticipated from the three repeating helical units of the backbone structure of the isotactic polymer. In other words, the phenyl group that is sandwiched between two benzoyl groups should be sterically hindered from reaction.

The benzoylated isotactic polystyrene was then treated with the Grignard reagent, p-magnesium bromoanisole, by two different methods: 1) The finely powdered polymer was poured into the Grignard reagent directly and more absolute ether was added; and 2) the finely powdered polymer was first mixed with dry benzene, then the Grignard reagent was added.

The percentage of Grignard reaction was estimated from the decrease

TABLE 1. Benzoylation of Isotactic Polystyrene

Experiment No.	1	2	3	4	5	6
Polystyrene (mmole)	19.2	50.0	50.0	50.0	25.0	300.0
Benzoyl chloride (mmole)	8.5	25.0	50.0	75.0	34.0	500.0
Aluminum chloride (mmole)	7.5	22.5	45.0	67.5	22.5	300.0
Carbon disulfide (ml)	120	130	130	130	80	800
Reaction time (hr)	4	6	6	6	12	12
Yield percentage[a]	15.6	9.7	16.6	28.9	55.2	49.0

[a]Percentage of benzoylated styrene unit was estimated by IR spectroscopy.

in the intensity of the IR carbonyl absorption band between the starting benzoylated polymer and the product. Alternately, the amount of methoxy group on the polymer could be measured by the Zeisel method [12, 13]. A good correlation of these two methods can be seen in Table 2.

The conversion of the benzophenone group to the monomethoxytrityl alcohol under different conditions ranged from 13.7 to 63.2%. The conversion was low when ether was used as solvent. High loading polymer could be obtained when benzene was used as the solvent. In order to check the isotacticity of the polymer after the modification by Friedel-Craft and Grignard reactions, the NMR spectra of the reaction products were taken (Fig. 2). There are no significant differences between the signal of the main chain protons of the parent isotactic polystyrene and its derivatives [14, 15]. The configuration of the polymer was thus proven to be unchanged after the modification.

The reaction of acetyl chloride with the hydroxyl group of the polymeric methoxytrityl alcohol yields the corresponding methoxytrityl chloride. After the reaction, the excess reagent was removed by two methods: 1) The crude product was washed with dry n-hexane and then recovered by filtration, or 2) the reagent was evaporated under reduced pressure and repeatedly lyophylized from dry benzene. The second treatment was favored because there was less chance for the product to be exposed to atmospheric moisture which would change the chloride group back to the hydroxyl group. The yield of this reaction, following the second treatment, was almost quantitative. The amount of chloride was detected by following the chloride content

TABLE 2. Grignard Reaction of Benzophenone Derivatives of Isotactic Polystyrene

Experiment No.	1	2	3	4	5	6	7
Percent of benzoyl group per styrene unit	55.2	28.9	49.0	55.2	16.6	55.2	28.9
Total carbonyl group (mmole)	3.4	4.3	124	2.3	7.9	3.3	1.6
Magnesium p-bromo anisole (mmole)	10	4	150	4	12	12	2
Reaction time (hr)	44	12	12	12-2	10	15	24
Reaction temperature	RT	RT	Reflux	RT-reflux	Reflux	Reflux	Reflux
Solvent for polymer	Ether	Ether	C_6H_6	C_6H_6	C_6H_6	C_6H_6	C_6H_6
Reacted carbonyl[a] group mmole/g polymer (%)	0.33 (9.8)	0.21 (10.0)	0.43 (13.7)	1.12 (32.9)	0.47 (34.5)	1.48 (43.5)	1.36 (63.2)
Calculated mmole of OCH_3/g of polymer	0.22	0.17	0.33	0.91	0.43	1.23	1.25
Expt mmole OCH_3/g[b]	0.199	0.154	-	0.980	0.463	1.240	1.540

[a]Calculated values are based on the IR spectroscopy results.
[b]Zeisel method determination of methoxyl group.

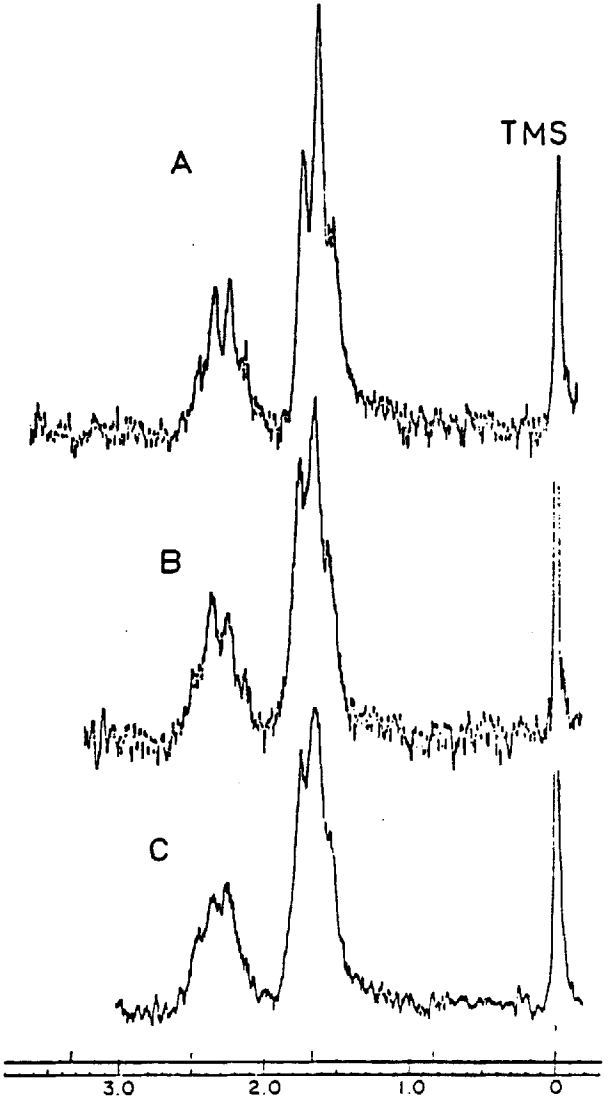

FIG. 2. NMR spectra of main chain protons in O-dichlorobenzene at 185°: (A) isotactic polystyrene, (B) benzophenone derivative 1, and (C) methoxytrityl alcohol derivative of isotactic polystyrene 2.

in the water-methanol solution by the Mohr method [16]. However, the chloride content was found to decrease markedly after storing over P_2O_5 for some time. Thus it was desirable to use the active chloride-containing polymer for reaction with the appropriate nucleoside as soon as possible.

After the reaction of the nucleoside with the active polymer, the polymer nucleoside linkage was found to be stable in neutral or basic solution as expected.

The nucleoside was released by treatment with acid. Trifluoroacetic acid (10%) in chloroform was found to be the most effective reagent for release of the nucleosidic material from the polymer. Thymidine was found to be released completely into the solution in 10 min at 0° C with acid. No degradation of nucleoside was found under these conditions. This reagent was also effective for the same purpose with oligonucleotide-polymer. Dilute ammonium hydroxide was used to extract the nucleoside into aqueous solution after the acid treatment. The nucleoside solution was then adjusted to pH 6-7 and the amount of nucleoside was estimated by UV absorption. The results are listed in Table 3.

As shown in Table 3, it was found that the method of handling of the chlorinated polymer plays an important role in this reaction. A prolonged exposure of the chlorinated polymer to atmospheric moisture during the purification process or storage gives a very low yield. High yield, however, is obtained if lyophylization is used to purify the chlorinated polymer. The reaction time is also a very important factor. The optimum time was found to be 48 hr at 80° C. When thymidine was used as the nucleoside, a large excess was not necessary, as shown in Experiments 9 and 5, which give about the same yield, though the ratio of thymidine to chloride increases from 2 to 5.

For internucleotide bond synthesis, the polymers carrying the first nucleoside unit were treated with 3'-O-acetylthymidine-5'-phosphate (pT3'OAc) or 3'-O-acetyl-5-iododeoxyuridine-5'-phosphate (pIUdR3'-OAc) in the presence of mesitylene sulfonyl chloride (MSC) and dry pyridine (Fig. 3). 3'-O-Acetylthymidine-5'-phosphate was prepared from thymidine-5'-phosphate according to the conventional method developed by Khorana and co-workers [17]. 5-Iododeoxyuridine-5'-phosphate was prepared by iodination of deoxyuridylic acid [18] or phosphorylation of 5-iododeoxyuridine [19] followed by column chromatography. Acetylation of the nucleotide gave 3'-O-acetyl-5-iododeoxy-uridine-5'-phosphate. The extent of reaction was determined by paper chromatographic analysis after the nucleotides were released from the polymer with TFA treatment.

The next requirement for lengthening of the oligonucleotide chain was the selective removal of the 3'-O-acetyl group. After numerous trials the best deacetylation conditions are those of Hayatsu and Khorana [3]. At room temperature, complete deacetylation was obtained in

TABLE 3. Conversion of Methoxytrityl Chloride-Polymer to Nucleoside Derivatives

Expt. No.	Loading[a]	Cl mmole/g[b] polymer	Nucl	Nucleoside / Cl	Reaction conditions	Cl → Nucl (%)	Nucl mmole/g polymer
1[c]	5.7	0.477	T	7.5	8 hr-RT 1 hr-80°	1.0	0.005
2[c]	5.7	0.477	T	16.0	12 hr-80°	1.6	0.008
3[c]	18.3	1.49	T	2	8 hr-RT 1 hr-80°	1.0	0.015
4[c]	18.1	0.97	T	8	12 hr-80°	4.1	0.040
5	6.7	0.40	T	5	48 hr-80°	85.0	0.340
6	6.7	0.36	T	5	52 hr-80°	82.0	0.295
7	6.7	0.37	T	5	12 hr-80°	20.0	0.074
8	6.7	0.39	T	5	24 hr-80°	53.1	0.206
9	6.7	0.40	T	2	48 hr-80°	83.0	0.306
10	6.7	0.36	dA	8	36 hr-RT	47.2	0.170
11	6.7	0.33	IUdR	7	25 hr-80°	28.0	0.093
12	3.3	0.17	IUdR	2	48 hr-80°	24.0	0.041

[a]Percentage of phenyl ring carrying the methoxytrityl group in the polymer.

[b]Chloride content determined by the Mohr method after the methoxytrityl group was converted to methoxytrityl chloride.

[c]Polymer chlorinated by Method 1.

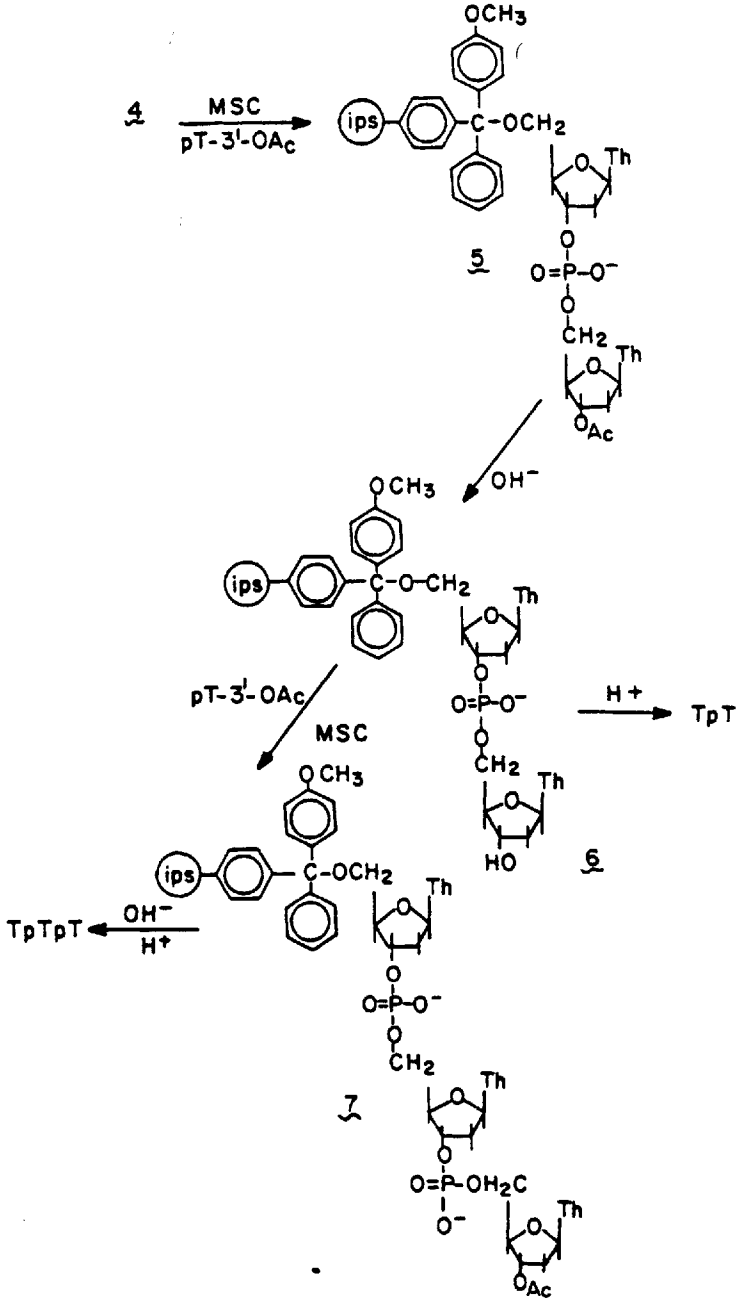

FIG. 3. Synthesis of deoxyoligonucleotides on isotactic polystyrene.

10 min. The polymer-bound dinucleotide, now having a free 3'-OH, was then subjected to repeated condensation with pT3'-OAc. The product of condensation was then treated again with base followed by acid to release the oligonucleotide, which was analyzed by paper chromatography (Table 4). A DEAE-Sephadex A-25 column was also found useful to separate and purify large quantities of nucleotides released from the polymer.

The dinucleotides and trinucleotides thus prepared were identified by their characteristic chromatographic properties as shown in Table 5. The nucleotides were completely degradable by spleen [20] and snake venom phosphodiesterase [21] which catalyzed the hydrolysis of 3',5'-phosphodiester links only as shown in Table 6. There was also evidence that the chain lengthening step was a successful feature of this new polymer support in that there appears to be less drop in yield as the chain length is increased.

EXPERIMENTAL SECTION

UV absorption spectra were measured on a Beckman Model DB-G recording spectrophotometer. NMR spectra were measured with a Vari spectrometer HA-100 or A-60. Chemical shifts are given in parts per million on a δ scale; coupling constants are expressed in cycles/second TMS was used as an internal standard. Thin-layer chromatography was carried out by the ascending method with Eastman Chromagram Sheets 6060 (silica gel with fluorescent indicator) and Sheets 6065 (cellulose with fluorescent indicator). Paper chromatography was carried out by the descending technique on Whatman 3MM paper. For product analyses absorbances of the blank were cut from the paper near the product and treated in the same manner as the product. Solvent systems are: S_1, 2-propanol:ammonium hydroxide:water (7:1:2); S_2, chloroform:2-propanol (2:8); S_3, ethanol:ammonium acetate 1 M (7:3). Isotactic polystyrene, mol wt = 5.02×10^6, was ground to a fine powder with a Waring blender before use. Pyridine was dried by distillation over CaH_2 and was stored over Linde Molecular Sieves, Type 4A. The elemental analysis were performed by Galbraith Laboratories, Knoxville, Tennessee.

Enzyme Assay

A solution containing about 1 μmole of substrate and 1-2 unit spleen phosphodiesterase (Worthington) in 0.1 ml of 0.01 M sodium pyrophosphate buffer (pH = 6.5) and 0.2 ml of 0.05 M ammonium acetate (pH = 6.5) was incubated at 37°C. After 5 hr the mixture was chromatographed and the UV absorbing bands were eluted and measured

TABLE 4. Oligonucleotide Synthesis on Isotactic Polystyrene Support

	Nucleotide components	Nucl[b]/polymer bound comp.	MSC[b]/ nucl	Reaction[c] time (hr)	Product	%[d]
ips-MeOTr-T[a]	pT3'OAc	1.5	2	4	TpT	52
ips-MeOTr-T	pT3'OAc	3.0	2	8	TpT	55
ips-MeOTr-T	pT3'OAc	4.0	1	6	TpT	43
ips-MeOTr-T	pT3'OAc	4.0	4	20	TpT	50
ips-MeOTr-T	pIUdR3'OAc	7.0	2	8	TpIUdR	63
ips-MeOTr-dA	pT3'OAc	3.0	2	8	dApT	58
ips-MeOTr-IUdR	pT3'OAc	6.0	5	8	IUdRpT	36
ips-MeOTr-TpT	pT3'PAc	6.0	5	8	TpTpT	57
ips-MeOTr-TpT	pT3'OAc	3.0	2	8	TpTpT	40
ips-MeOTr-IUdRpT	pT3'OAc	6.0	5	8	IUdRpTpT	25

[a]The symbol ips denotes the isotactic polystyrene backbone and MeOTr the methoxytrityl group. Other abbreviations are: T = thymidine, pT3'OAc = 3'-O-acetythymidylic acid, dA = deoxyadenosine, IUdR = 5-iodo-2'-deoxyuridine, pIUdR3'OAC = 3'-O-acetyl-5-iodo-2'-deoxyuridylic acid, MSC = mesitylene sulfonyl chloride. The oligomers are abbreviated in the conventional way.

[b]Molar ratio.

[c]All reactions were run in dry pyridine suspension.

[d]Yield calculated based on the amount of polymer-bound components.

TABLE 5. Chromatographic Properties of Nucleoside Derivatives and Deoxyoligonucleotides

	TLC$_1$[a]				Paper[c]	
	S_1	S_2	S_3	TLC$_2$,[b] S_3	S_1	S_3
T	0.63	0.53	0.66	0.95	0.65	0.83
pT	0.18	0	0.32	-	0.12	0.44
Tp	-	-	-	-	0.30	0.50
pT3'OAc	0.27	0	0.40	-	-	-
TpTpT	0.49	0.09	0.61	-	0.16	0.68
TpT	0.50	0.14	0.66	-	0.40	0.77
IUdR	0.69	0.50	0.68	-	0.56	0.60
pUdR	-	-	-	0.25	-	-
pIUdR	0.06	0	-	0.28	-	-
pIUdR3'OAc	-	-	-	0.51	-	-
TpIUdR	0.43	0	-	0.60	0.45	-
IUdRpT	-	-	-	-	0.33	0.47
IUdRpTpT	-	-	-	-	-	0.32
dA	0.59	0.32	0.70	-	0.61	0.60
dApT	-	-	-	-	0.35	0.42

[a]TLC$_1$ was performed on Kodak Chromagram 6060 (solvent systems, see Experimental Section).
[b]TLC$_2$ was performed on Kodak Chromagram 6065.
[c]Paper chromatography was carried out on Whatman 3MM paper.

spectrophotometrically. A solution containing about the same amount of substrate and 40 units of Russel's viper venom phosphodiesterase (Calbiochem) in 0.2 ml of 0.1 M tris-HCl buffer (pH = 9.4) was incubated at 37°C for 5 hr.

Benzophenone Derivative of Polystyrene 1

White powder of isotactic polystyrene [11] (31.0 g, 300 mmole styrene unit) was mixed with dry carbon disulfide (500 ml) and aluminum chloride

TABLE 6. Enzymatic Hydrolysis of Deoxyoligonucleotide by Phosphodiesterase[a]

Substrate	Enzyme	Products	Products ratio
TpT	Spleen	Tp/T	1.05
TpT	Snake venom	pT/T	0.99
TpIUdR	Spleen	Tp/IUdR	1.02
dApT	Snake venom	pT/dA	1.10
TpTpT	Spleen	Tp/T	2.15
TpTpT	Snake venom	pT/T	2.05
IUdRpT	Snake venom	pT/IUdR	1.05
IUdRpTpT	Snake venom	pT/IUdR	2.18

[a]See Experimental Section.

(40.0 g, 300 mmole). Heat was applied to bring the mixture into a gellike suspension. Benzoyl chloride (60 ml, 500 mmole) dissolved in carbon disulfide (300 ml) was then added to the suspension. Hydrogen chloride was liberated after the mixture was stirred and heated. After refluxing for 12 hr the reaction mixture was cooled to room temperature and poured into ice water (800 ml) and chloroform (200 ml). The mixture was stirred for 3 hr and the aqueous layer was discarded. The polymer was recovered from the mixture after washing with methanol (1500 ml), filtering, and again washing well with chloroform-methanol and hot water. The product was finally dried at 60° under vacuum to give 45.0 g of white solid 1.

Monomethoxytrityl Alcohol Derivative of Isotactic Polystyrene 2

(A) Magnesium turnings (0.25 g, 0.01 g-atom), p-bromoanisole (2.00 ml, 16 mmole), and absolute ether (20 ml) were used to prepare the Grignard reagent. The reagent was carried out in a nitrogen bag filled with prepurified nitrogen. The magnesium turnings were pressed with a glass rod to start the reaction. After the spontaneous boiling, heat was applied to keep the reaction mixture refluxing. No metal remained in the yellowish liquid after 3 hr. The fine powder of benzophenone derivative of isotactic polystyrene 1 (1.00 g, 3.40 mmole carbonyl group) was added to the Grignard reagent and stirred for 44 hr. The solid, which was

slightly yellow in color, turned to pink when ether (50 ml) was added. The color then turned to white on addition of 10% sulfuric acid (50 ml). After washing again thoroughly with fresh ether twice (50 ml) and two portions of methanol (50 ml), the solid was dried at 50° in vacuo and gave 1.05 g of slightly colored solid 2.

(B) Magnesium turnings (3.7 g, 0.15 g-atom), p-bromoanisole (28.0 g, 150 mmole), and absolute ether (40 ml) were used to prepare the Grignard reagent in a nitrogen bag following essentially the same procedure as in (A). The fine powder of 1 (30 g, 124 mmole carbonyl grou was first suspended in benzene (200 ml) and then added to the Grignard reagent. After the reaction started, the mixture was refluxed overnight The reaction mixture was then cooled to room temperature and the nitrogen bag removed. Sulfuric acid (10%, 100 ml) was added to the reaction product and stirred for 5 hr at room temperature. The precipitate was separated by filtration; washed thoroughly by large amount of water, ether, and methanol; and dried to give 33.1 g of slightly yellow solid 2.

Monomethoxytrityl Chloride Derivative of Isotactic Polystyrene 3

(A) A mixture of the monomethoxytrityl alcohol derivative of isotact polystyrene 2 (4.35 g, 1.90 mmole alcohol) and acetyl chloride (40 ml) was refluxed for 6 hr. Dry n-hexane (100 ml) was then added to the bright red gellike reaction mixture, which was then filtered in a dry nitrogen bag. The solid was washed with dry n-hexane until no chloride ion was detected in the washing solution (silver nitrate test). The brown polymer 3 was dried in a vacuum at room temperature and weighed 4.30 g.

(B) The monomethoxytritylphenyl alcohol derivative of isotactic poly styrene 2 (11.0 g, containing 3.6 mmole alcohol group) was suspended i dry benzene (90 ml). Acetyl chloride (40 ml) was added dropwise into t suspension with stirring. Heat was supplied to the red mixture for 2 hr after which the mixture was allowed to stand at room temperature overnight. Acetic acid, benzene, and excess acetyl chloride were removed b distillation. Four portions of dry benzene (50 ml) were added to the residue and distillation continued until no chloride ion detected in the distillate. The residue, after lyophylization, gave 11.5 g of polymer 3.

Thymidine Derivative of iPS 4

The mixture of 3 (11.0 g, 4.4 mmole chloride), thymidine (5.1 g, 22.0 mmole), and dry pyridine (150 ml) was stirred and heated at 80° for 48 hr. The mixture was then poured into 200 ml of ice-ethanol mixture

(1:1 vol). The solid that separated was recovered by filtration and washed thoroughly by methanol until no thymidine was detected from the filtrate. 11.5 g of dry polymer 4 (0.34 mmole T/g) was obtained after drying. Deoxyadenosine and 5-iodo-2'-deoxyuridine derivatives of the polymer were prepared using a procedure similar to that of thymidine.

The results are summarized in Table 3.

Removal of Thymidine from the Polymer

Trifluoroacetic acid (10%) in chloroform (1 ml) was added to the polymer 4 (5.0 mg) and the orange suspension was kept at 0° for 10 min. The suspension was then extracted with ammonium hydroxide (0.5 N, 5 ml) to remove the product. 2 ml of the extract were then adjusted to 7 by 0.5 N HCl and diluted to 5 ml. The optical density units were determined at 267 mμ to be 16.50, indicating 0.34 mmole thymidine per gram of polymer 4. (E_{267} = 9600 for thymidine.)

Preparation of Iododeoxyuridine-5'-monophosphate

Iodination of Deoxyuridylic Acid

Deoxyuridine-5'-monophosphate disodium salt (0.99 g, 2.8 mmoles) in 10 ml of water was allowed to pass through a 1.5 × 15 cm Dowex 50 H^+ form column which was then washed by water until no UV absorption at 260 mμ was detected from the eluate. 5'-Deoxyuridylic acid was recovered from the eluate by freeze drying and was then mixed with 0.5 N nitric acid (6 ml), p-dioxane (24 ml), and iodine (1.5 g, 6 mmole), and the mixture was refluxed for 1 hr at 100°. After the reaction, the solvent and most of the iodine were evaporated off by rotary evaporator. The residue was dissolved in ethanol (5 ml) which was then added to absolute ether (30 ml) dropwise. The hygroscopic white precipitate was separated by filtration, dissolved in water, neutralized by 0.5 N ammonium hydroxide, and then lyophylized to give 0.64 g white product. The ether layer was concentrated and the residue was dissolved in water. The excess free iodine was removed by extraction with carbon tetrachloride. After being neutralized by 0.5 N ammonium hydroxide, the aqueous layer gave a 0.25-g second crop of product after freeze drying. Total yield, 68%, UV (H_2O, pH = 2) max 278 mμ, min 256 mμ, (H_2O, pH = 12) max 288 mμ, min 250 mμ; NMR (D_2O) = 8.30 (s, 1, H_6) 6.29 (t, 1, J = 7.0, H_1) 4.59 (m, 1, H_3), 4.16 (m, 1, H_4), 3.98 (m, 2, H_5), 2.40 (q, 2, H_2).

Analysis: Calculated for $C_9H_{18}N_4O_8PI$: C, 23.09; H, 3.88; N, 11.97; P, 6.62; I, 26.90. Found: C, 23.12; H, 3.80; N, 11.46; P, 6.53; I, 26.93.

By Phosphorus Oxychloride

The suspension of 5-iododeoxyuridine (95 mg, 0.27 mmole) in trimethylphosphate (4 ml) was frozen in a Dry Ice-acetone bath before phosphorous oxychloride (0.6 ml) was added. The final suspension was kept at 0° overnight. Excess phosphorous oxychloride was driven off by evaporation under reduced pressure. Ice water (2 ml) was added to the colorless syrup and half of the water was then removed by evaporation under reduced pressure again. The solution was adjusted to pH = 6.6 with ammonium hydroxide (0.5 N, 6.5 ml). The solution was diluted to 155 ml and charged onto a 1.5 × 25 cm bicarbonate form Sephadex A-25 column which was then eluted with a linear gradient solution of 0.01 (2 liter) to 0.35 M (2 liter) ammonium bicarbonate solution. Flow rate was 64 ml/hr, and 16 ml fraction were collected. The fractions 85-100, exhibiting the expected UV spectrum were combined and freeze dried to give 37 mg (30%) of product with the identical chromatographic and spectroscopic properties of the product from Procedure A.

Acetylation of 5-Iododeoxyuridine Phosphate

5-Iododeoxyuridine phosphate diammonium salt (0.47 g, 1 mmole) was dissolved in 5 ml water and charged onto a 1.1 × 9 cm Dowex-50 resin (H^+ form). Ten 5-ml portions of water were used to elute the column. The eluates were combined with pyridine (10 ml) and the solvent was removed by vacuum distillation. More dry pyridine was added and the distillation was repeated until all the moisture was driven off. The syrup was dissolved in dry pyridine (4 ml) and acetic anhydride (2 ml) and allowed to stand at room temperature overnight. After the reaction, pyridinium acetate was removed by rotary evaporation. Water (5 ml) was then added, and the aqueous solution was freeze dried to give 5-iododeoxyuridine-3'-acetyl phosphate pyridinium salt as a glassy hygroscopic solid; NMR (d_5-pyridine) = 2.00 (S, 3, $COCH_3$).

Preparation of Thymidylyl-(3', 5')-thymidine Polyme Derivative 5

Isotactic polystyrene supported thymidine 4 (1 g, 0.34 mmole thymidine per gram of polymer) was mixed with pyridinium thymidine-3 acetyl phosphate (0.51 mmole), mesitylene sulfonyl chloride (216 mg, 1 mmole), and dry pyridine (20 ml). The mixture was stoppered and stirred at 30° for 4 hr, and ice-water (30 ml) was added to stop the reaction. The solid was recovered by filtration and washed thoroughly with water and methanol to give 1.0 g polymer 5 after drying.

Removal of 3'-O-Acetyl Group from Polymer 5, 6

Polymer 5 (1.0 g) was mixed with pyridine (20 ml), dimethyl sulfoxide (16 ml), and sodium methoxide (1 M in methanol, 8 ml). The suspension was stirred at room temperature for 15 min after which the polymer was recovered by mixing the suspension with water (50 ml), filtering, and washing with methanol and water to yield 0.98 g of product 6 after drying.

Removal of Thymidylyl-(3',5')-thymidine from Polymer 6

(A) Polymer 6 (5.0 mg) was mixed with trifluoroacetic acid (10% in chloroform, 1 ml) at 0° for 10 min. Ammonium hydroxide (0.5 N, 5 ml) was added to extract the product. The aqueous solution was separated and the pH was adjusted to 7 by dilute hydrochloric acid. The solution was then concentrated to 0.3 ml and chromatographed on Whatman 3MM paper in the solvent system S_3 for one day. The band with the R_F value = 0.68 was eluted with water to give 16.3 O.D. units at 267 mμ of the thymidylyl-(3',5')-thymidine which equaled 176 μmole of TpT per gram of polymer 6 (52%) using E_{267} = 18,500 for TpT. The yield was calculated based on the amount of thymidine in the starting polymer 4. Unreacted thymidine (R_F = 0.68) of 7.8 O.D. units was also recovered along with the dinucleotide.

(B) Polymer 6 (0.9 g) was mixed with chloroform (5 ml) and cooled to 0° in an ice bath before trifluoroacetic acid (10% in chloroform, 15 ml) was added. The suspension was stirred at 0° for 15 min and methanol (20 ml) was added. The solid polymer was separated by filtration and washed with methanol (20 ml). The filtrate and the methanol were combined and extracted with ammonium hydroxide (0.5 N, three 25-ml portions). The aqueous solutions were combined and adjusted to pH = 7, diluted to 1500 ml, and then charged onto a 1.5 × 20 cm Sephadex A-25 bicarbonate form column which was eluted with a linear gradient solution of 0.01 M (2 liters) to 0.35 M (2 liters) ammonium bicarbonate. The flow rate was set at 80 ml/hr and 20 ml fractions were collected. The fractions 30 to 40 were combined and freeze dried to give 84 mg (50%) of TpT.

Preparation of Thymidylyl-(3',5')-thymidylyl-(3',5')-thymidine-polymer Derivative 7

A mixture of thymidylyl-(3',5')-thymidine-polymer derivative 6 (100 mg, 18 μmole TpT), pyridinium thymidine-3'-acetate-5'-phosphate

(180 μmole), mesitylene sulfonyl chloride (115 mg, 0.54 mmole), and dry pyridine (2 ml) was sealed and stirred at 30° for 8 hr. Ice water (5 ml) was added to the mixture and stirred overnight. The solid that separated was recovered by filtration and washed several times with water and methanol to give polymer 7 (98 mg).

Deacetylation of Polymer 7

Polymer 7 (98 mg) was mixed with pyridine (6 ml), dimethyl sulfoxide (8 ml), and sodium methoxide (1 M solution in methanol 2 ml) at room temperature for 15 min. Water (30 ml) was added and the solid was separated by filtration and washed several times with water and methanol to give 97 mg of deacetylated product.

Removal of Thymidylyl-(3',5')-thymidylyl-(3',5')-thymidine from Polymer

Polymer derivative (90 mg) was mixed with trifluoroacetic acid (10% in chloroform, 4 ml) at 0° for 10 min. Ammonium hydroxide (0.5 N, 10 ml) was added to extract the product. The aqueous solution was separated and water (two 10-ml portions) was used to wash the chloroform solution. The aqueous solutions were combined and neutralyzed with 0.5 N acid and the solution was diluted to 500 ml and charged onto a Sephadex A-25 bicarbonate form column. A linear gradient of ammonium bicarbonate solution was used to elute the column. Fractions 64 to 74 were combined to give 250 O.D. units of TpTpT. Using E_{267} = 25,400 for TpTpT, 97 μmole of TpTpT was found per gram of polymer or 56.8% yield based on the amount of TpT on the starting polymer. Chromatographic properties and enzyme assay of TpTpT were shown in Tables 5 and 6, respectively.

The procedure for the preparation of other oligonucleotides was similar to that of TpT and TpTpT. The results were summarized in Table 3.

CONCLUSION

The present work shows that it is possible to create deoxyoligonucleotides by sequential addition of known nucleotides on an isotactic polystyrene. The yield of the condensation reaction to form the internucleotide linkage was found to be among the highest in the insoluble polymer systems. The yield of the successive condensation step remains as high as or better than the first condensation step.

Also, the polymeric material was found to be completely recovered easily after each reaction. A major problem remaining unsolved, however, is the lack of quantitative yield of the internucleotide condensation reaction. The condensation agents generally used today are carbodiimide and aromatic sulfonyl chloride. The yield of the internucleotide bond obtained is satisfactory for general purposes in organic synthesis, but it is not sufficient for the ideal polymer support synthesis. So far, no activating agent in the nucleotide field gives a yield approaching 100% in the conventional or polymer support method. The success of the development of the automated polynucleotide synthesis depends on the improvement of such activating agents.

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