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Synthesis of oligodeoxyribonucleotides on silica gel support

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ABSTRACT

A rapid solid phase method of oligonucleotide synthesis based on monomeric protected nucleosides has been developed.

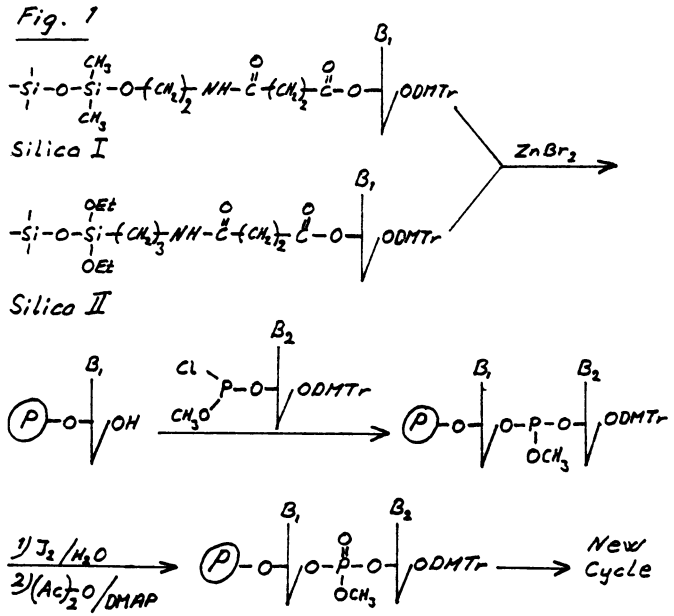
INTRODUCTION

The solid phase synthesis of oligonucleotides on silica gel described by Caruthers et al.[1] using monomeric baseprotected 5'-O-dimethoxytrityl-nucleoside-3'-O-methylphosphochloridites has been further studied. A new method for the functionalization of silica, deprotection of the 5'-O-dimethoxytrityl group with zinc bromide/nitromethane/water, and the use of acetic anhydride in N,N-dimethylaminopyridine as a capping reagent have been introduced.[2] These improved methods allow a dodecamer to be assembled in12 hours where each nucleoside addition cycle including all transformations and washes takes about 1 hour. At present, several oligonucleotides (6-15 long) have been synthesized by this procedure. In the present paper are described the syntheses and isolations of a hexamer, a nonamer and two dodecamers (see table). The oligonucleotides are first isolated as 5'-O-dimethoxytritylated compounds which are fully deprotected at the triester bond and at the purine/pyrimidine rings. They are purified on HPLC using reversed phase chromatography (C18) in a gradient system of 20-30% acetonitrile in 0.1 M triethylammonium acetate. The remaining 5'-O-dimethoxytrityl group is removed by 80% acetic acid in water and the fully deprotected oligomer is subsequently isolated in the same chromatographic system using a gradient of 10-15% acetonitrile.

RESULTS AND DISCUSSION

The solid phase support consists of 5'-O-dimethoxytrityl-N-benzoyl-2'-deoxynucleoside-3'-succinic acids[3] coupled to aminated silica. The silica, Porasil C, has been aminated with either triethoxysilylpropylamine[1,4] or dichlorodimethylsilane[4] followed by aminoethanol. This silica was then treated with 5'-O-dimethoxytrityl-N-benzoyl-2'-deoxynucleoside-3'-O-succinic acid in pyridine using dicyclohexylcarbodiimide (DCCI) as condensing reagent (figure 1). The degree of functionalization of T-silica was examined by 1) detritylation using $ZnBr_2$ in nitromethane and 2) ammonolysis, to give 5'-O-dimethoxytrityl-thymidine. The products were analyzed on HPLC and compared with reference samples. The dimethoxytrityl alcohol and 5'-O-dimethoxytrityl-thymidine content were the same for both types of silica (0.01 mmole/100 mg). The A, G, and C-silica were also analyzed and showed the same degree of functionalization as T-silica. The

Fig. 1

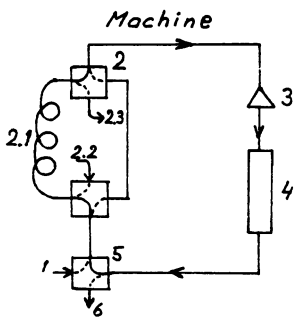


B = protected base, DMAP = 4-dimethylaminopyridine
DMTr = 4,4'-dimethoxytrityl, (P) = polymer support = silica I or II

extent of functionalization is comparable to a reported value[5] for Porasil C using dichlorodimethylsilane and it, therefore, can be assumed that the DCCI condensation is essentially a quantitative reaction. Such a high degree of functionalization allows us to make oligomers 9-15 nucleotides long using 150-200 mg of silica. The synthetic scheme is outlined in figure 1. Full cycles of operations and reaction times are given below. The "manual" machine used for the syntheses is shown in figure 2. The solid phase silica (150-200 mg), functionalized with either nucleoside T, C, A, or G, is loaded into the column.

1. A wash of dry tetrahydrofuran (THF) is pumped through the system with a flow of 1 ml/minute.
2. The 5'-O-dimethoxytrityl group is removed by a 1% water-nitromethane v/v mixture which has been saturated with $ZnBr_2$. This reagent continues to be fed in as long as the orange color of the trityl cation is observed. In general, the detritylation is complete within 15 minutes.
3. Excess reagent is removed by a wash cycle of THF (5 minutes).
4. The incoming 5'-O-dimethoxytrityl-nucleoside-3'-O-methyl-phosphochloridite in THF/sym-collidine is injected into the loop. A 10 times excess is used and recycled in the system for 10-15 minutes.
5. Excess reagent is then removed by a THF wash (5 minutes).
6. The phosphite triester is oxidized to the phosphate triester with a I_2/H_2O/THF solution (5 minutes).

Fig. 2



1 inlet
2 injection valve system
2.1 injection loop
2.2 inlet to loop with syringe
2.3 outlet from loop
3 pump
4 column
5 recycling valve
6 outlet

7. The oxidation reagent is then removed by a THF wash (10 minutes).
8. The cycle is completed by a capping of unreacted 5'-hydroxy groups using acetic anhydride/N,N-dimethylaminopyridine/THF. The reagent is in about 50 times excess and the reaction is complete within 5 minutes.
9. Finally the capping reagent is washed out with THF (5 minutes).

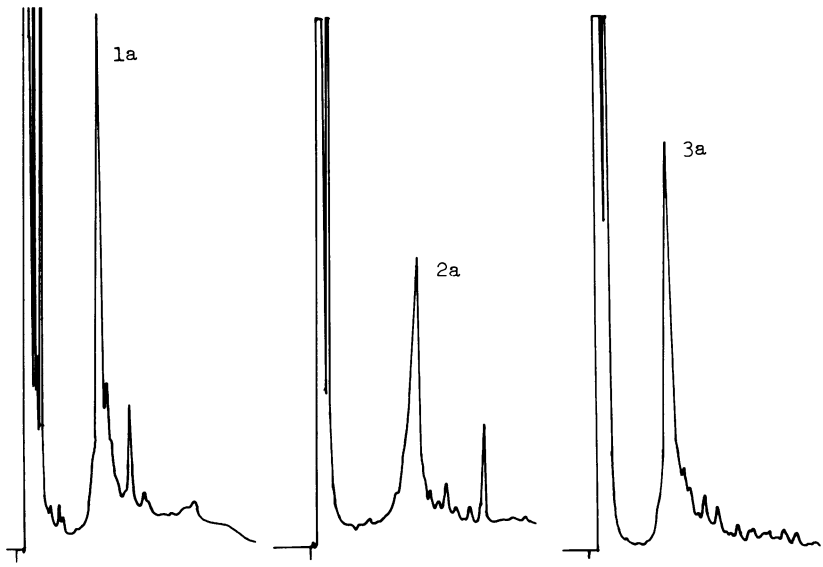
The use of $ZnBr_2$ as a detritylating agent has only been mentioned briefly[1,6]. It is reported to remove the dimethoxytrityl protecting group fast and selectively though the mechanism for such a reaction is unclear. In early experiments $ZnBr_2$ was found to be an inconsistent reagent; i.e., the detritylation was sometimes very efficient and at other times slow. Presumably this was due to different concentrations of the reagent in nitromethane. However, when 1% water was added, that mixture of $ZnBr_2$ (excess) and nitromethane gave a very powerful detritylating agent. For example, 200 mg of functionalized silica (in the column) could be detritylated with 12 ml of this $ZnBr_2$ solution. There was no indication of side reactions such as depurination from the use of this "wet" $ZnBr_2/CH_3NO_2$ reagent. Since phosphites as coupling reagents do not give quantitative yields it became necessary to find a reagent to inactivate the unreacted 5'-hydroxyl groups. This concept of a capping reagent is particularly important when purines are coupled to the support; 5'-O-dimethoxytrityl-N-benzoyl-2'-deoxyadenosine-3'-O-methylphosphochloridite and the corresponding deoxyguanosine derivative give 70-90% coupling, whereas the pyrimidine couplings are near quantitative. These yields have been determined from dimethoxytrityl alcohol content and analyzed on C18 using 70% methanol-water v/v. The most useful capping reagent is a mixture of acetic anhydride/N,N-dimethylaminopyridine[7] in THF. Silica (200 mg, 0.02 mmole of free hydroxy groups) is fully acetylated within 10 minutes with a 50 times excess of reagent. Should such a powerful reagent react with the nucleoside bases it could be easily removed in the subsequent ammonolysis of the oligonucleotide. A summary of the synthetic oligomers prepared by the solid phase method is given in the following table. Compounds 1,2 and 4

Table of oligonucleotides made by solid phase (P) and solution chemistry (S)

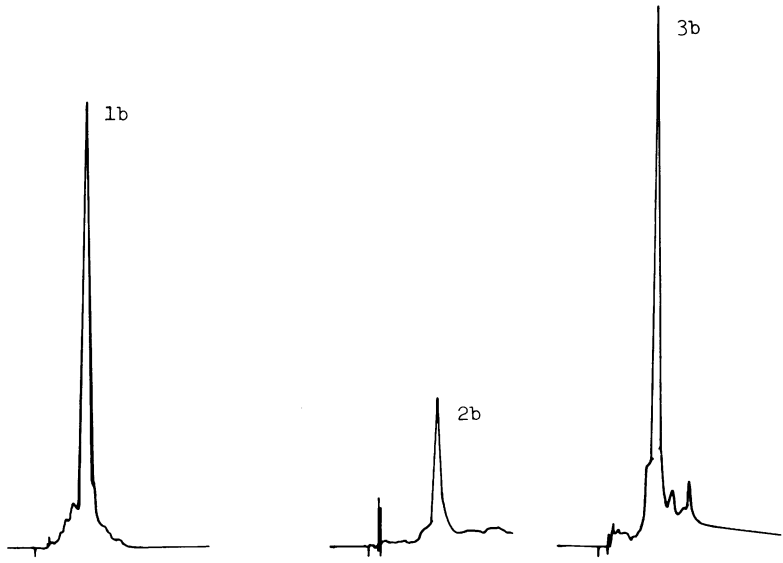
Compound	Method of Preparation	Acetonitrile % in 0.1 M $Et_3NH^+OAc^-$	Retention time min.	yield % DMTr-OH	yield % DMTr-compound	yield % fully deprotected
1a d[(DMTr)ACATCTGAG]	P, S	20-30%, 30 minutes	15.2	34	12	
1b d(ACATCTGAG)	P, S	10-15%, 30 minutes	8.8			4
2a d[(DMTr)TGTAAATGTACC]	P, S	20-30%, 30 minutes	19.2	16	5	
2b d(TGTAAATGTACC)	P, S	10-15%, 30 minutes	12.0			2
3a d[(DMTr)CTTTGAAATGAC]	P	20-30%, 30 minutes	14.4	14	3	
3b d(CTTTGAAATGAC)	P	10-15%, 30 minutes	10.4			1
4a d[(DMTr)TCTGAG]	P, S	28%	10.4	42	14	

were made by independent routes using standard triester synthesis[8]. The compounds were identical as the partially deprotected tritylated compound and as the fully deprotected oligomers. Yields for this method of synthesis have been determined in two different ways. The amount of tritylated compound attached to the support was measured as the amount of trityl alcohol obtained upon treatment with zinc bromide. They show coupling efficiency to be 70-100%. However, a drop in yield is encountered upon deprotection and cleavage of the compound from the support. The isolated yields are about 25% of the expected overall yields. This discrepancy has been observed by other workers, and was explained as a loss on C18 columns during isolation[9]. The amount isolated, however, was sufficient (in each case, greater than 10 O.D.). When a larger quantity was desired, a larger capacity column was used. Figure 3 shows the separation profiles for compounds 1-3 at the two stages of HPLC purification. Gel electrophoresis (20% acrylamide) shows that the solid phase oligonucleotides are of a higher purity than those from solution chemistry, all of which have been purified to a single peak by HPLC. Structural determination was performed according to the standard methods of sequencing by homochromatography.[10]

Fig. 3



HPLC profiles of dimethoxytritylated oligomers 1-3a with a flow of 1 ml/minute and a gradient of 20-30% acetonitrile in 0.1 M triethylammonium acetate over 30 minutes.



HPLC profiles of fully deprotected oligomers 1-3b with a flow of 1 ml/minute and a gradient of 10-15% acetonitrile in 0.1 M triethylammonium acetate over 30 minutes.

EXPERIMENTAL SECTION

The following chemicals were purchased from commercial sources: deoxycytidine, thymidine, deoxyadenosine and deoxyguanosine (Pharma-Waldhof), 4,4'-dimethoxytrityl chloride (Aldrich), triethoxysilylpropylamine, dimethyldichlorosilane, aminoethanol, nitromethane, zinc bromide, succinic anhydride, acetic anhydride, N,N-dimethylaminopyridine, tetrahydrofuran, benzoylcyanide, sym-collidine, triethylamine, thiophenol, n-butylamine (Fluka), Poracil C(37-75μ) silica (Waters). For the reversed-phase C18 column chromatography, analytical columns from Altex and HPLC, acetonitrile(Fluka, HPLC-grade).

Silica I. Functionalization of Silica Gel (Porasil C) with Dichlorodimethylsilane and Aminoethanol

The silica (Porasil C, 10 g) was dried at 200°C for 24 hours. After cooling in a desiccator, the silica was mixed with dry pyridine (75 ml), and dimethyldichlorosilane (0.1 mole, 13 g) was added. The mixture was shaken at room temperature for 30 minutes after which the pyridine solution was decanted off. The vessel was cooled to 0°C, and an excess of aminoethanol (0.3 mole, 18 g) was added. The mixture was then stirred for 24 hours at room temperature. The silica was filtered, washed with pyridine (200 ml) and ethyl ether (100 ml), and dried in a desiccator at room temperature. The silica was mixed with pyridine (75 ml), and trimethylsilylchloride (0.1 mole) was added. The mixture was shaken for 5 hours at room temperature. The silica was then filtered, washed with pyridine (100 ml) and ethyl ether (100 ml), and dried.

Silica II. Functionalization of Silica Gel (Porasil C) with Triethoxysilylpropylamine

To Porasil C (10 g) was added triethoxysilylpropylamine (15 ml) and toluene (50 ml), and the mixture was refluxed for 7 hours. The mixture was cooled. The silica was filtered off and washed with pyridine (2 x 100 ml). Trimethylsilylchloride (11 ml) was added and the mixture was shaken for 4 hours at room temperature. The silica was filtered, washed with pyridine (3 x 50 ml) and ethyl ether (3 x 50 ml), and dried in a desiccator.

General Procedure for the Preparation of 5'-O-Dimethoxytrityl-2'-Deoxynucleoside-3'-O-Succinic Acids

The 5'-O-dimethoxytrityl-2'-deoxynucleoside[11,12] (dT, dA^{Bz}, dG^{Bz} or dC^{Bz}, 2 mmole) and N,N-dimethylaminopyridine (2 mmole) were dissolved in pyridine (50 ml). To the clear solution was added in portions succinic anhydride (2.1 mmole) at room temperature. The mixture was stirred for 48 hours. Water (3 ml) was then added to consume unreacted succinic anhydride. The mixture was concentrated at reduced pressure and the pyridine removed by coevaporation with toluene. The residue was taken up in dichloromethane (200 ml), and the organic phase was washed with an aqueous solution (100 ml) of citric acid (4 mmole). The free 3'-O-succinic acid was mainly partitioned to the organic phase. The solution was dried over Na_2SO_4 and concentrated at reduced pressure. The solid residue was dissolved in dichloromethane (50 ml) and precipitated from n-hexane (300 ml). The nucleoside-3'-O-succinic acids were isolated in 70-80% yield. TLC analysis on silica (10% methanol-dichloromethane) showed the reaction product as a spot below the 5'-O-dimethoxy-2'-deoxynucleoside starting material (trace amount). The precipitated succinyl nucleosides were used without further purifications.

General Procedure for the Reaction of 5'-O-Dimethoxytrityl-2'-Deoxynucleoside-3'-O-Succinic Acid with Silica-I and Silica-II

Silica-I or Silica-II (6 g) in pyridine (10 ml) was treated with triethylamine (1 g) to obtain the silica free amine. Excess triethylamine was removed by coevaporation with pyridine. To the silica was added the deoxynucleoside-3'-O-succinic acid (5 mmole) in pyridine (100 ml) and dicyclohexylcarbodiimide (44 mmole , 9 g). The mixture was shaken at room temperature for 2 days. The silica was filtered, washed with pyridine (300 ml) and reacted with benzoylcyanide (0.1 mole, 13 g) in 50 ml of pyridine for 3 hours. The silica was again filtered, washed with pyridine (200 ml) and ethyl ether (200 ml), and dried. The degree of 5'-O-dimethoxytrityl-2'-deoxynucleoside incorporated into silica was determined by the following methods: 1) treatment with $ZnBr_2$ in nitromethane to detritylate and 2) ammonolysis, to cleave the 5'-O-dimethoxytritylnucleoside. Analyses were performed on HPLC using Lichrosorb silica. Detritylation of 100 mg of

T-silica yielded 0.012 mmole of trityl alcohol (1.5% methanol-dichloromethane, 2 ml/minute, R(t)=4.0 minutes). Treatment of a second 100 mg of silica with concentrated ammonia gave 0.011 mmole of 5'-O-dimethoxytrityl-thymidine (5% methanol-dichloromethane, 2 ml/minutes, R(t)=3.5 minutes). The A^{Bz}-, C^{Bz}-, and G^{Bz}-silica were similarly analyzed and found also to be functionalized 0.01 mmole/100 mg silica.

General Procedure for the Preparation of 5'-O-Dimethoxytrityl-2'-Deoxynucleoside-3'-O-Methyl Phosphochloridites

The baseprotected 5'-O-dimethoxytrityl-nucleoside[11,12] (5.5 mmole) in dry THF (20 ml) was added over 30 minutes to a stirred and cooled solution (-78°) of methyldichlorophosphite[13,14] (5.0 mmole, 0.48 ml) and sym-collidine (25 mmole, 3.3 ml) in THF (20 ml) under argon. After an additional 30 minutes, the reaction mixture was centrifuged. The clear supernatant was removed from the collidine hydrochloride and transferred to a round bottom flask. The solution was concentrated to a gum, and was then diluted to a convenient concentration (0.2 mmole/ml) with five equiv. of sym-collidine in THF.

Detritylating Reagent

To nitromethane (500 ml) and water (5 ml) was added $ZnBr_2$ (70 g), and the mixture was stirred at room temperature for 24 hours. A test on the facility of trityl removal was done. 5'-O-Dimethoxytrityl-N-benzoyl-deoxyguanosine-3'-p-chlorophenyl-β-cyanoethyl phosphate and b) 5'-O-dimethoxytrityl-N-benzoyl-deoxyadenosine-3'-p-chlorophenyl-β-cyanoethyl phosphate were both treated with this $ZnBr_2$ solution at room temperature. The reaction was followed by TLC (silica, 10% methanol-dichloromethane v/v). The detritylation was complete within 5 minutes. The mixture was checked periodically for two days; no evidence of depurination could be detected.

Capping Reagent

A solution of N,N-dimethylaminopyridine (20 mmole, 2.4 g) in THF (40 ml) was made. Acetic anhydride (10 mmole, 1 g) and sym-collidine (10 mmole, 1.2 g) were added to 10 ml of the stock solution. The capping solution darkened during the course of a day and was consequently remade every day. The capping activity

and selectivity, however, appeared unchanged after 24 hours storage at room temperature.

Removal of the Oligonucleotide from the Solid Support

The silica containing the synthesized oligonucleotides was washed with 1,4-dioxane after the last cycle and transferred to a vial. The methyl protecting group of the internucleotide phosphotriester was removed by treatment with thiophenol-triethylamine in dioxane at room temperature[1,15]. The reaction was fast and selective. (Reaction times of 10 minutes and 20 hours gave the same yield of isolated oligonucleotide). After 30 minutes, the reagent was removed, and the silica was washed with dioxane and water. Concentrated ammonia was added and the mixture heated at 50°C for 4 hours to deprotect A^{Bz} and C^{Bz}. At the same time the ester bond which linked the oligonucleotide to the support was hydrolyzed. The silica was removed by filtration and the 5'-O-dimethoxytrityl-oligonucleotide in ammonia was concentrated at reduced pressure. To the residue was added 2 ml of 1:1 butylamine-methanol v/v to remove the benzamide of G. After 48 hours at room temperature the mixture was again concentrated. The residue was taken up in 1 ml of 0.1 M triethylammonium bicarbonate (TEAB) and extracted with ethyl acetate and ethyl ether.

Isolation of the Partially Deprotected 5'-O-Dimethoxytrityl-nucleotides and the Fully Deprotected Oligonucleotides

The reaction mixture in 1 ml of 0.1 M TEAB was analyzed on HPLC using C18 columns[16]. A gradient system of 20-30% acetonitrile in 0.1 M triethylammonium acetate over 30 minutes was used for the separation of hexamers to dodecamers. The tritylated oligonucleotide could easily be identified and was isolated on either analytical or semipreparative columns. The fraction containing the desired compound was concentrated and treated with 80% acetic acid-water to remove the trityl protecting group. After complete deprotection (10-30 minutes), the solution was concentrated and residual acetic acid coevaporated with water. The fully deprotected oligonucleotide was analyzed and isolated by HPLC, using a gradient of 10-15% acetonitrile over 30 minutes at 1 ml/minute (see table).

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