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Solid-phase synthesis of polynucleotides. IV. Usage of polystyrene resins for the synthesis of polydeoxyribonucleotides by the phosphotriester method

Ken-ichi Miyoshi, Rene Arentzen, Ting Huang and Keiichi Itakura

Division of Biology, Molecular Genetics Section, City of Hope Research Institute, Duarte, CA 91010, USA

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ABSTRACT

Contrary to the expectation, the Merrifield polystyrene resin, 2% cross-linked by divinylbenzene, is as efficient as the polyacrylmorpholide resin for the synthesis of polydeoxyribonucleotides using a phosphotriester method. On the Merrifield resin, the tetradecamer, dTpCpGpTpCpApApCpTpGpGpCpTpT, and the hexadecamer, dCpCpApGpTpCpApCpGpApCpGpTpTpGpT, were synthesized by the phosphotriester method using di and trinucleotide blocks as coupling units.

INTRODUCTION

In the previous papers[1,2], we have reported the practical synthesis of polydeoxyribonucleotides by the solid-phase method. In this synthesis, appropriate di and trinucleotide units (2), instead of monomer units, were coupled to the polyacrylmorpholide resin (1) using a phosphotriester approach (Figure 1). The chemical principles of this block coupling approach on the resin is very simple and rapid: 1) sequential addition of appropriately protected di or trinucleotide blocks, 2) masking of the unreacted 5'-hydroxyl group with acetic anhydride, and 3) removal of the dimethoxytrityl group from the polynucleotides bound on the support to afford a new 5'-hydroxyl function for the next coupling reaction. High coupling yields were maintained for the synthesis of even longer polynucleotides than icosadecamer[3] and the separation of the final product from a series of shorter polynucleotides having at least two or three charges less than the desired polymers was performed very easily by high performance liquid chromatography (HPLC) on Permaphase AAX.

One disadvantage of the acrylmorpholide resin may be high affinity to hydroxylic solvents, such as water and methanol.

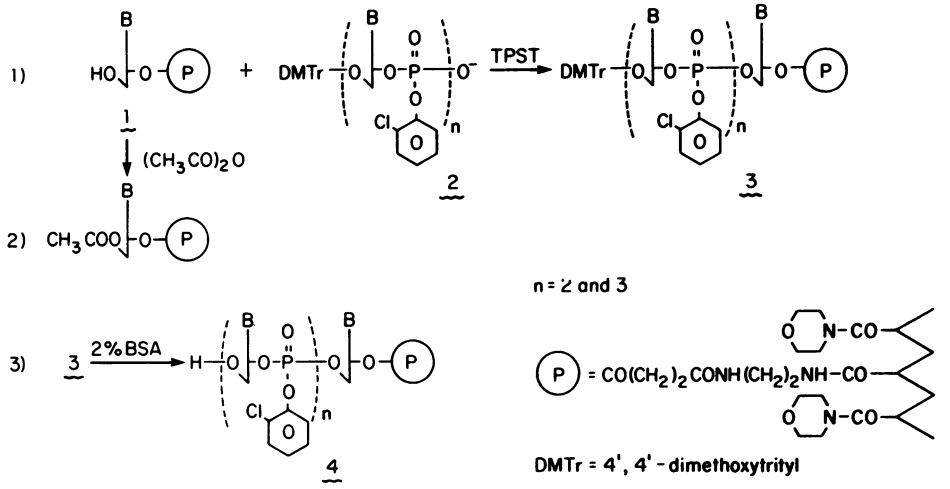

Figure 1 Block coupling strategy

Before each coupling step, the resin must be co-evaporated with dry pyridine to eliminate a small amount of these solvents inside the resin and high coupling yields were obtained constantly. This evaporation step would hamper the automation of the whole operation. As mentioned in the previous papers by us[1] and other investigators[4], polystyrene derivatives were thought to be inappropriate resins for the synthesis of polynucleotides by the solid-phase method for some reasons. We have re-investigated various polystyrene derivatives that should have low affinity to hydroxylic solvents, including Merrifield polystyrene (2% cross-linked by divinylbenzene), SM-2 (macroporous resin) and Kel F-g-styrene (0% cross-linked graft resin), and found that the coupling efficiency to form phosphotriester bonds on Merrifield resin is as high as on the polyacrylmorpholide resin. The details of this work will be described in this article.

RESULTS AND DISCUSSION

The chemical principles involved in the solid-phase synthesis of sequence-specific polydeoxyribonucleotides have been

reported in the previous papers[1,2].

Polymer Support and Resin-Nucleoside Linkage

The basic amino benzyl resins (7) were prepared by two methods of the published procedure[5,6] (Figure 2). The commercially available chloromethyl-polystyrene (5a, 1.06 mmole of Cl/g) was converted into the phthalimidomethyl-resin (6) by treatment with potassium phthalimide.[5] In the case of SM-2 or Kel F-g-styrene, the phthalimidomethyl-resin (6) was prepared

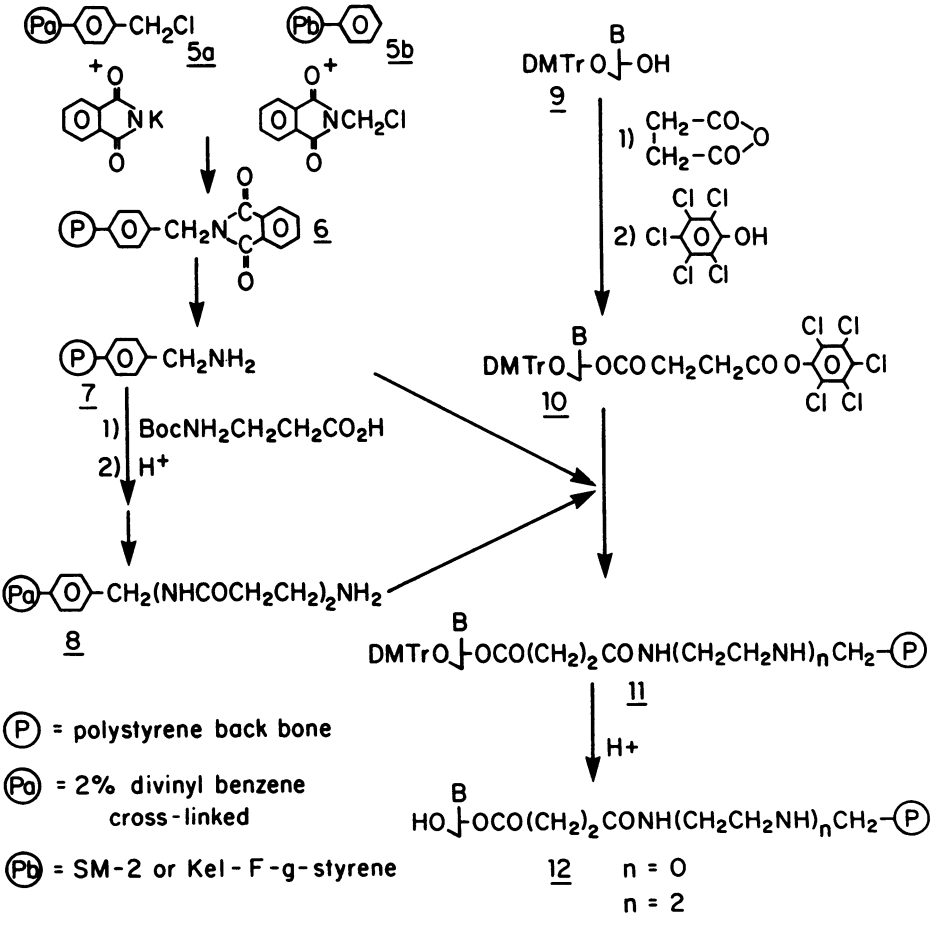

Figure 2 PREPARATION OF SUPPORT

by the reaction of 5b with N-(chloromethyl)phthalimide.[6] These phthalimidomethyl-resins were converted into the amino resins (7) with hydrazine (97%) in ethanol. The procedure to attach the first nucleoside residue to the amino resin (7) was exactly the same as that for polyacrylmorpholide resin.[1]

Coupling of the amino resin (7) with the activated ester (10) afforded the dimethoxytrityl resin (11, n=0). The amount of a nucleoside attached on the polymer (11, n=0) was estimated by the spectroscopic analysis of dimethoxytrityl group and the nucleoside liberated from the resin as previously described.[1] Because of the difficulty in removing the dimethoxytrityl group from the first nucleoside, SM-2 (macroporous resin) was not pursued further. The linkage between the first nucleoside and these resins is essentially a carboxyl ester bond, stable in pyridine and under acidic conditions, and should easily be cleaved by base treatment.

The coupling efficiency on these two resins was compared by the model coupling study using dithymidylic acid (2, n=2, B= thymine) in the presence of 2,4,6-triisopropylbenzenesulfonyl tetrazolide (TPST) in pyridine. On the Merrifield resin (2% cross-linked polystyrene), the coupling reaction went to as high as 90%. On the other hand, on Kel F-g-styrene (0% cross-linked polystyrene) the yields were rather poor (∿50%). A similar result was also obtained in the formation of internucleotidic phosphotriester bonds by the activation of deoxynucleoside 3'-phosphomonotriazolides with N,N-dimethylaminopyridine.[7] Note that our coupling yields on the Kel F-g-styrene resin using the phosphotriester method were in sharp contrast to the results reported on a similar resin using the phosphodiester method.[8] One significant difference between two resins is the amount of polystyrene grafted on the carrier (7.5% versus 30%), though the amount of functionalized benzene ring is similar. Consequently, we concentrated the following works on the Merrifield resin (12).

Polynucleotide Assembly

The essential features of the conventional coupling method using the 3'-phosphodiester component (2) and TPST were the same as reported[1,2]. The appropriately protected di and trinucleotides were sequentially coupled to the growing oligomer chain

from the 3'- to the 5'-ends.

General Procedure for the Synthesis

One cycle of the addition of an oligonucleotide block (2, n=2 and 3) to the polystyrene resin was essentially the same as described for the polyacrylmorpholide resin[1,2], except for the co-evaporation of the resin with pyridine (Table 1). In the case of the polystyrene resin, one operation of the co-evaporation was enough to remove hydroxylic solvents. The two polydeoxyribonucleotides, tetradecamer d(TCGTAACTGGCTT) and hexadecamer d(CCAGTCACGACGTTGT), were synthesized on the resin (12, n=0) by the sequential addition of di and trinucleotides, respectively.

The overall coupling yields were very similar to that on polyacrylmorpholide resin and the HPLC analysis on Permaphase AAX after removal of all protecting groups were described in Figure 3.

Comparison of the Merrifield Resin versus the Polyacrylmorpholide Resin

During the synthesis of polydeoxyribonucleotides on the Merrifield resin, the following significant differences from the polyacrylmorpholide resin were observed.

TABLE 1.

STEP	SOLVENT or REAGENT	AMOUNT	SHAKING TIME	NUMBER OF OPERATIONS
1	2% BSA	10ml	0.5	2
2	$CHCl_3$-MeOH (7:3 v/v)	10ml	1	2
3	Pyridine	10ml	1	2
4	Dimer or Trimer in Pyridine	5 equivalent	co-evaporation	1
5	TPST in Pyridine	10 equivalent /5ml	180	1
6	Pyridine	10ml	1	2
7	10% Solution $(CH_3CO)_2O$ in Pyridine	10ml	60	1
8	Pyridine	10ml	1	2
9	$CHCl_3$-MeOH (7:3 v/v)	10ml	1	3

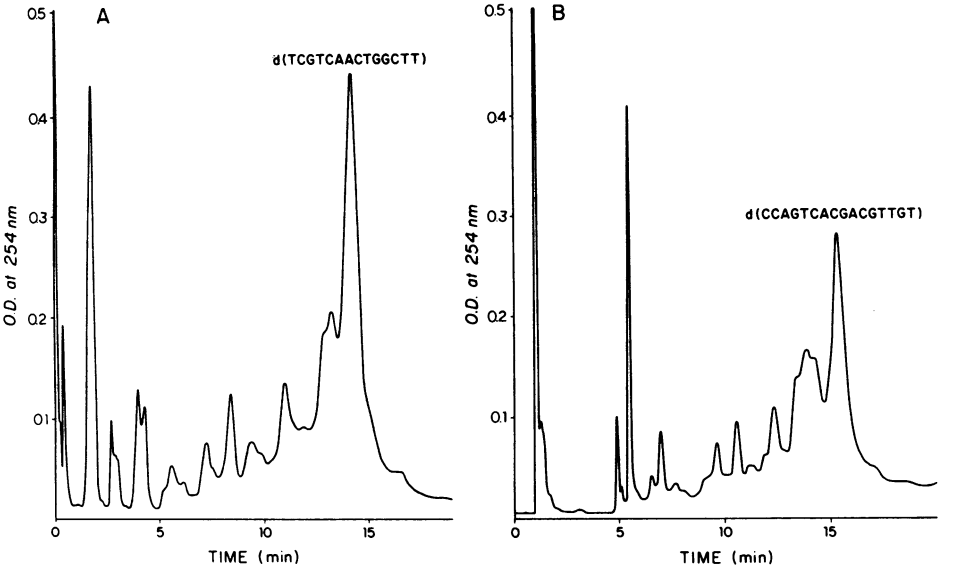

Figure 3

1) Yield of the first coupling reaction.

The significant lower yields (50∿60%) of the first addition of nucleotide blocks (2) to the resin (12, n=0) than the subsequent coupling yields (80∿90%) were observed. This might be due to the steric hindrance of the polystyrene backbone and could be overcome by the introduction of a spacer between the coupling site, the 5'-hydroxyl group of a nucleoside and the resin. Consequently, the spacer was inserted by the reaction of the amino resin (7, (Pa)) with *t*-butyloxycarbonyl β-alanine. The amino resin (8) bearing two units of β-alanine was prepared by repeating this reaction twice, and derivatized to the starting polymer support (12, n=2) in a similar way as described for the original resin (12, n=0). The coupling reaction of the first oligonucleotide blocks on the resins with and without the spacer were equally lower than the following coupling and, therefore, the lower yield should be caused by other factor(s).

2) Cleavage of nucleotide-resin linkage.

The linkage between the nucleotide and the resin is a carboxyl ester bond, and should be cleaved by treatment with

aqueous ammonia. This was the case in the polyacrylmorpholide resin, except for the first nucleoside.[9] On the other hand, a very small amount of the dimethoxytritylated oligonucleotides was released after treatment of the polystyrene resin with aqueous ammonia. It seems that the fully protected oligonucleotides have a high affinity to the polystyrene backbone in aqueous solution and that inorganic hydroxide ion can hardly attack the protected oligonucleotides.[10] Alternatively, the resin sample was shaken with 0.3 M tetramethylguanidium p-nitrobenzaldoximate[11] in dioxane-water (1:1 v/v) overnight, and then with an aqueous ammonia (28%) at 50°C for six hours. Under these conditions, the ester bond was cleaved quantitatively and all the base labile protecting groups were deblocked. In addition, the benzaldoximate gave less side reactions than aqueous ammonia in deblocking of aryl phosphotriester bonds.[11]

Conclusion

One arguement led to the speculation that polystyrene derivatives are not appropriate for the synthesis of oligonucleotides due to their non-polarity. These non-polar resins may not be fully permeated and, consequently, do not swell in the polar reaction media required for oligonucleotide synthesis. Contrary to the expectation, the Merrifield resin (2% cross-linked by divinylbenzene) is as efficient as the polyacrylmorpholide resin for the synthesis of polynucleotides using a phosphotriester method. The overall yields of five coupling operations to synthesize hexadecanucleotides on the two resins are very similar: 34% (Merrifield) and 40% (polyacrylmorpholide).

For the automation of the whole operation by machine, the polystyrene resin might have an advantage over the polyacrylmorpholide resin because of lower affinity to hydroxylic solvents. Indeed, one co-evaporation of the resin with dry pyridine is enough to obtain constant yields. On the other hand, three-time co-evaporation is necessary for the polyacrylmorpholide resin.

EXPERIMENTAL SECTION

Material and Methods

Material and methods not otherwise mentioned here have been

described in the previous papers.[1,2] Chloromethyl-polystyrene (2% cross-linked by divinylbenzene, 1.06 mmole Cl/g) and macroporous styrene (SM-2) were purchased from Bio-rad and Kel F-g-styrene (7.5% polystyrene by weight) was a gift from ICI, Australia. Potassium phthalimide and t-butyloxycarbonyl β-alanine were obtained from Aldrich, and N-(chloromethyl)phthalimide from Tridom/Fluka.

Phthalimidomethyl-resins (6) were prepared as published either from chloromethyl-resin (5a, 2% cross-linked polystyrene) and potassium phthalimide,[5] or Kel F-g-styrene and N-(chloromethyl)phthalimide.[6] The resulting phthalimidomethyl-resins were easily converted to aminomethyl resins (7) by treatment with hydrazine in ethanol.[5] 2% Cross-linked polystyrene (7) contains 1.00 mmole of N/g and Kel F-g-styrene (7) has 0.06 mmole of N/g by picric acid titration.[12] The confirmation of the polynucleotide sequence was performed by the published method.[13]

Preparation of the Resin with Two Units of β-Alanine (8)

A mixture of 7 (2% cross-linked polystyrene, 5 g), $BocNH_2$-CH_2COOH (0.95 g, 5 mmole) and DCC (1.1 g, 5 mmole) in CH_2Cl_2 (20 ml) was shaken for 2 hours. After the reaction, the mixture was filtered and the resin was washed successively with CH_2Cl_2, DMF and pyridine. The unreacted amino group was masked with $(CH_3CO)_2O$ in pyridine (25% v/v, 25 ml) for 30 minutes and the reaction mixture was filtered. The resin was washed with pyridine and CH_2Cl_2 and the Boc group was removed by treatment with CF_3COOH(TFA)-CH_2Cl_2 (1:4 v/v, 30 ml) for 25 minutes. The resin was collected and washed with CH_2Cl_2, triethylamine in CH_2Cl_2 (5% v/v) and CH_2Cl_2. The same reaction was repeated on the resulting resin using $BocNHCH_2CH_2COOH$ (0.57 g, 3 mmole) and DCC (610 mg, 3 mmole) in CH_2Cl_2. The unreacted amino group was masked with $(CH_3CO)_2O$ in pyridine and then the Boc group was removed by TFA in CH_2Cl_2 in the same way.

Attachment of Deoxynucleoside-3'-O-Pentachlorophenyl Succinate (10) to the Resin (7)

A mixture of the amino resin (7, (Pa), n=o, 5 g, 1.00 mmole/g), the activated ester (10, B=thymine, 1 mmole) and triethylamine (200 mg, 2 mmole) in 20 ml of DMF was shaken at room

temperature overnight. The resin was collected by filtration and washed with DMF and pyridine and treated with $(CH_3CO)_2O$-pyridine (2:3 v/v, 50 ml) for 2 hours to mask the unreacted amino group on the resin. The mixture was filtered and the resin was washed with pyridine and methanol, and dried *in vacuo* to give the dimethoxytritylated resin. Yield: 5.4 g (0.136 mmole of thymidine/g).

In a similar way, the spacer resin (12, (Pa), n=2, 0.11 mmole of thymidine/g) and Kel F-g-resin (12, (Pb), 0.05 mmole/g) were prepared from the activated ester (10, B=thymine, 890 mg, 1 mmole) and the amino resin (7 and 8, 5 g).

The Tetradecanucleotide, d(TCGTCAACTGGCTT)

Six cycles of nucleotide addition to the resin (12, n=0, 400 mg, 0.136 mmole of thymidine/g) was performed manually in

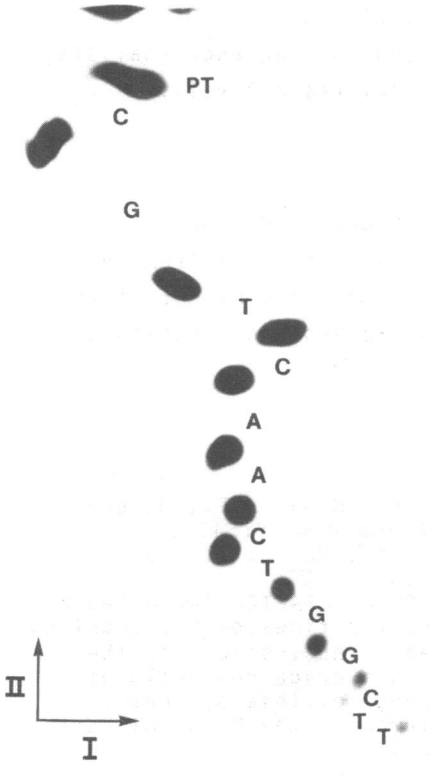

Figure 4a

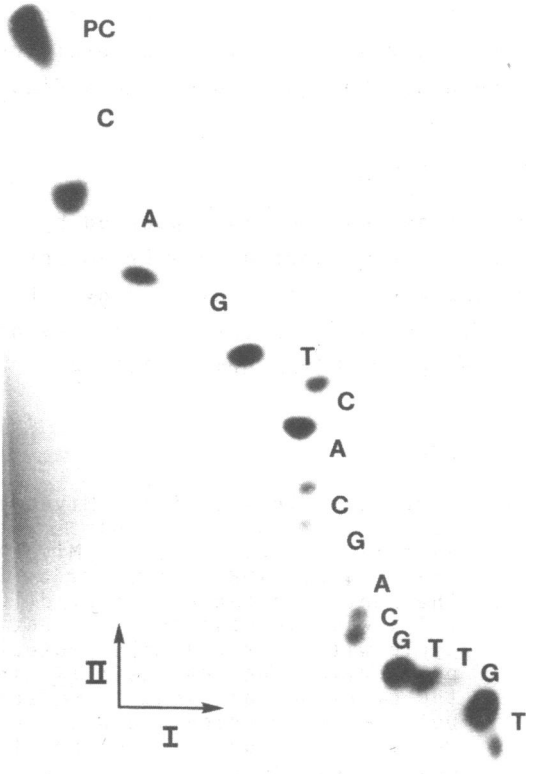

Figure 4b

a round-bottomed flask equipped with a sinister glass filter and a two-way stopper. Each coupling unit, the appropriately protected dinucleotide (2, n=2) except for the first cycle (2, n=1, B=thymine) was sequentially coupled to the resin in order of T-GC-TG-AC-CA-GT-TC. The yield of each step was 52, 94, 95, 81, 91, 86, and 86%, respectively. The unprotected tetradecamer was chromatographed on a Permaphase AAX column and the HPLC profile was shown in Figure 3a. After desalting of the major peak, the sample was further analyzed and purified on μBondapak C_{18} column. The sequence of the tetradecamer was confirmed by the standard method (Figure 4a).

The Hexadecanucleotide, d(CCAGTCACGACGTTGT)

Resin (12, n=0, B=thymine, 400 mg) was used for the synthesis. Five cycles of trinucleotide block (2, n=3, TTG-ACG-ACG-GTC-CCA) coupling was sequentially carried out in the presence of TPSTe. The coupling yield of each cycle was 61, 90, 86, 88 and 81%, respectively. HPLC profiles and the sequence analysis were described in Figure 3b and Figure 4b, respectively.

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REFERENCES

1. Part II of this series: Miyoshi, K.; Miyake, T.; Hozumi, T. and Itakura, K. (1980) Nucl. Acids Res. 8:5473.
2. Part III of this series: Miyoshi, K.; Huang, T. and Itakura, K. (1980) Nucl. Acids Res. 8:5491.
3. The hentriacontanucleotide, d(TGGTGCACCTGACTCCTGAGGAGAAGTC TGC), was synthesized by repeating ten times of the coupling cycle with appropriately protected trinucleotides on the polyacrylmorpholide resin (1) in an average 80% yield of the coupling reaction. The sequence of this synthetic polydeoxyribonucleotide was confirmed by the Maxam-Gilbert method. (Our unpublished results.)
4. Gait, M.J. and Sheppard, R.C. (1977) Nucl. Acids Res. 4: 1135.

5. Mitchell, A.R.; Erickson, B.W.; Ryabster, M.N.; Hodges, R.S. and Merrifield, R.B. (1976) J. Am. Chem. Soc. 98:7357.
6. Mitchell, A.R.; Stephen, B.H.; Erickson, B.W. and Merrifield, R.B. (1976) Tetrahedron Letters, 3795.
7. Miyoshi, K. and Itakura, K. (1980) Nucl. Acids Res., Spec. Ed., in press.
8. Potapov, V.K.; Veiko, V.P.; Koroleva, O.N. and Shabarova, Z.A. (1979) Nucl. Acids Res. 6:2041.
9. The first dimethoxytrityl nucleoside attached to the resin could not be removed completely by treatment of ammonia solution at 50°C overnight. After removal of the dimethoxytrityl group, the cleavage of a nucleoside was quantitative (see Reference 1.). There was no difficulty in the cleavage of the dimethoxytrityl oligonucleotides from the resin.
10. Tetrabutylammonium hydroxide was successfully used for the cleavage of a carboxyl ester bond on the polystyrene resin in aqueous tetrahydrofurane (private communication from Dr. Kolosov).
11. Reese, C.B.; Titmas, T.C. and Yan, L. (1978) Tetrahedron Letters, 2727.
12. Gisin, R.F. (1972) Anal. Chim. Acta 58:248.
13. Jay, E.; Bambara, R.; Radmanabhan, P. and Wu, R. (1974) Nucl. Acids Res. 1:331.