# An improved procedure for derivatization of controlled-pore glass beads for solid-phase oligonucleotide synthesis

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## ABSTRACT

**A simplified and economical method for the attachment of 2'-deoxyribo, ribo and arabinonucleosides onto long-chain alkylamidopropanoic acid controlled-pore glass (LCAAP-CPG, P-3) is described. In this procedure, 5'-O-tritylated nucleosides are coupled directly to LCAAP-CPG in excellent yields using 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide (DEC) as coupling reagent. The conventional and time-consuming preparation of nucleoside-3'-O-succinates is no longer required.**

## INTRODUCTION

Extensive research has been directed towards the development of supports containing covalently bound nucleosides for use in oligonucleotide synthesis (1). Among the rigid, microporous polymer supports, long-chain alkylamine controlled-pore glass (2) (LCAA-CPG) has become the most popular for DNA and RNA synthesis (1, 3). Its long-chain alkyl spacer makes the support bound terminal nucleoside more accessible to coupling reagents and its pore size is conducive to the synthesis of very long DNA and RNA oligomers (2, 4). The recent synthesis by Ogilvie *et al.* (5) involving LCAA-CPG is particularly noteworthy because it produced a 77-unit long transfer-RNA sequence, the longest RNA solid-phase synthesis published to date.

The conventional method of derivatizing LCAA-CPG is outlined in Scheme 1 (method A). It involves (i) the synthesis of nucleoside-3'-O-succinates (***2***); (ii) dicyclohexylcarbodiimide (DCC) mediated conversion of ***2*** into pentachlorophenyl or *p*-nitrophenyl esters of nucleoside-3'-O-succinates (***3*** and ***4***); and (iii) condensation of ***3*** or ***4*** with the primary amino groups of LCAA-CPG. Although this methodology is straightforward, the synthesis of nucleoside-3'-O-succinate esters is lengthy and has to be repeated for each nucleoside. Furthermore, the rate of coupling of nucleoside succinate esters to LCAA-CPG is slow and generally yields moderate loadings of support-bound nucleoside (10−25 μmol/g of CPG). These and other problems associated with LCAA-CPG derivatization were largely resolved by Pon *et al.* (6). by introducing 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide (DEC) to attach 3'-O-succinyl-2'-deoxyribonucleosides (***2***) directly to LCAA-CPG. This procedure eliminates the need to prepare succinate esters ***3/4*** and provides supports with a high degree of nucleoside attachment (30−65 μmol/g).

In this report we describe an alternative derivatization procedure in which nucleosides (2'-deoxyribo, ribo, and arabinonucleosides) are coupled *directly* to long-chain alkylamidopropanoic acid CPG (LCAAP-CPG, ***1***) *via* the 2'- or 3'-hydroxyl groups. Thus, the time-consuming preparation of nucleosides succinates ***2***, ***3*** or ***4*** is no longer required.

## MATERIALS AND METHODS

### Materials

Samples of LCAA-CPG (batch I and II, particle size 125−177 μm, pore diameter 500 Å) with a primary amino loading of about 100 μmol/g were obtained from Pierce (Rockford, IL). 4,4'-Dimethoxytrityl chloride, 4-dimethylaminopyridine, succinic anhydride, ninhydrin, tetrazole, 2-nitrophenol, and pentachlorophenol were obtained from Aldrich Chemical Co., Milwaukee, WI. Trichloroacetic acid, AnalaR grade (minimum assay 99%), was obtained from BDH Canada. Pyridine and 2,4,6-trimethylpyridine were distilled from calcium hydride and dried over activated 4A molecular sieves prior to use. Tetrahydrofuran was distilled from sodium benzophenone ketal under nitrogen as needed. Ultraviolet and visible spectra were recorded on a Hewlett-Packard HP8452 spectrophotometer.

### Preparation of Nucleosides

5'-O-(Dimethoxytrityl)-N4-benzoyldeoxycytidine (***1a***) was prepared according to published procedures (7). Ribonucleosides ***1b−e*** were prepared according to the procedures reported by Ogilvie *et al.* (8 , 9 9. Ogilvie,K.K., Schifman,A.L., and Penney,C.L. (1979) Can. J. Chem., 57, 2230.

Arabinonucleosides ***1f−h*** were prepared according to a published procedure (10). Compounds ***2−3*** and ***5−6*** were prepared according to the procedures reported by Pon *et al.* (6) and Usman *et al.* (3), respectively.

### Dimethoxytritylation of LCAA-CPG

DMTCl (845 mg, 2.50 mmol), $AgNO_3$ (422 mg, 2.50 mmol), pyridine (1.0 mL, 13 mmol), and THF (5.0 mL) were stirred vigorously using a vortex mixer. After 5 min of stirring at room

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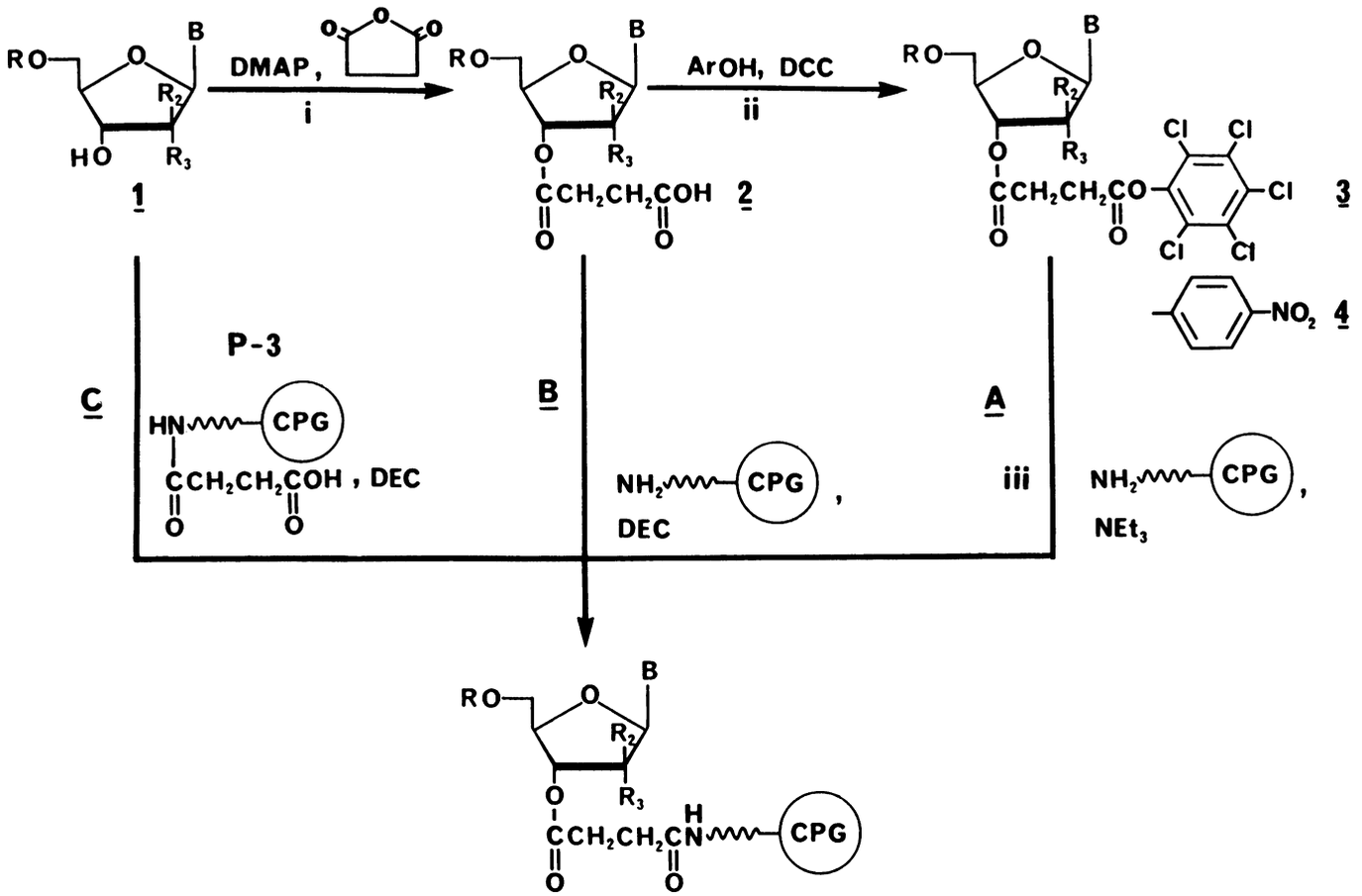
SCHEME 1




| | | | | |
|---|---|---|---|---|
| 1,a. | $R_1$= DMT | $R_2$= H | $R_3$= H | B= $Cy^{Bz}$ |
| b. | $R_1$= MMT | $R_2$= H | $R_3$= OH | B= Ur |
| c. | $R_1$= MMT | $R_2$= H | $R_3$= OH | B= $Cy^{Bz}$ |
| d. | $R_1$= MMT | $R_2$= H | $R_3$= OH | B= $Ad^{Bz}$ |
| e. | $R_1$= MMT | $R_2$= H | $R_3$= OH | B= $Gu^{Bz}$ |
| f. | $R_1$= MMT | $R_2$= OH | $R_3$= H | B= Ur |
| g. | $R_1$= MMT | $R_2$= OH | $R_3$= H | B= $Cy^{Bz}$ |
| h. | $R_1$= MMT | $R_2$= OH | $R_3$= H | B= $Ad^{Bz}$ |

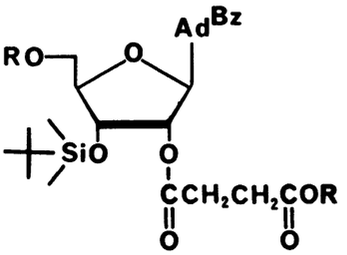
2, 3

$R_1$= DMT $R_2$= H $R_3$= H B= $Cy^{Bz}$

temperature, the mixture was centrifuged and the supernatant decanted. LCAA-CPG (15–25 mg) was taken in a vial to which the above supernatant (0.5 mL) was added. After 20 min of mixing, the slurry was filtered and the beads washed extensively with dimethylformamide, THF and then ether.

### Trityl Analysis

The amount of DMT, or 5'-O-tritylated (MMT, DMT) nucleosides covalently bound to CPG was determined quantitatively by measuring the trityl cation released after acid treatment of the CPG beads. Typically, an accurately weighed sample of CPG (5–10 mg) was treated with 10 mL of 5% trichloroacetic acid in 1,2-dichloroethane. After mixing, the



5, R= H
6, R= pentachlorophenyl

**Table 1.** Nucleoside Loadings Obtained by Coupling Nucleosides to LCAAP-CPG (**P-3**) and LCAA-CPG (**P-2**)

| Entry | Method | CPG Batch | Nucleoside | Nucleoside Coupling Step (h) | **P-4** (μmol/g) | Piperidine Step (h) | **P-5** (μmol/g) |
|---|---|---|---|---|---|---|---|
| 1 | C | II | dC ***1a*** | 24 | 39 | 12 | 37 |
| 2 | C | II | dC ***1a*** | 24 | 39 | 36 | 36 |
| 3 | C | II | dC ***1a*** | 24 | 39 | 168 | 23 |
| 4 | C | I | U ***1b*** | 72 | 34 | 0.1 | 34 |
| 5 | C | I | U ***1b*** | 72 | 34 | 24 | 30 |
| 6 | C | II | U ***1b*** | 72 | 40 | 24 | 30 |
| 7 | C | I | C ***1c*** | 72 | 28 | 0.1 | 29 |
| 8 | C | I | C ***1c*** | 72 | 28 | 24 | 24 |
| 9 | C | II | C ***1c*** | 72 | 33 | 24 | 23 |
| 10 | C | I | A ***1d*** | 72 | 32 | 0.1 | 33 |
| 11 | C | I | A ***1d*** | 72 | 32 | 24 | 26 |
| 12 | C | II | A ***1d*** | 72 | 36 | 24 | 24 |
| 13 | C | I | G ***1e*** | 72 | 31 | 0.1 | 31 |
| 14 | C | I | G ***1e*** | 72 | 31 | 24 | 26 |
| 15 | C | II | G ***1e*** | 72 | 36 | 24 | 25 |
| 16 | C | II | aU ***1f*** | 20 | 37 | 12 | 36 |
| 17 | C | II | aC ***1g*** | 72 | 23 | 16 | 20 |
| 18 | C | II | aA ***1h*** | 20 | 27 | 12 | 29 |
| | | | | | | *Loading* μmol/g | |
| 19 | A | II | dC ***3a*** | 36 | | 17 | |
| 20 | B | II | dC ***2a*** | 168 | | 35 | |
| 21 | A | II | A ***6*** | 96 | | 15 | |
| 22 | B | II | A ***5*** | 96 | | 13 | |

absorbance was measured at 504 nm (DMT) or 478 nm (MMT), and the amount of trityl cation determined by using extinction coefficients of 76 mL $cm^{-1}$ $\mu mol^{-1}$ (DMT) or 56 mL $cm^{-1}$ $\mu mol^{-1}$ (MMT). This assay was reproducible within ± 5–8%.

### Ninhydrin Test

Solutions of (a) 0.28M ninhydrin in absolute ethanol, (b) 80% phenol in ethanol, and (c) 0.5 mM KCN in pyridine were prepared according to the published procedure (11).

An accurately weighed sample of LCAA-CPG (3.0–12.0 mg) was added to a 10 mL volumetric flask. Distilled water (0.2 mL), reagent (b) (1 mL), and reagent (c) (1 mL) were added and the mixture was heated over a boiling water bath for 5–10 min. Ninhydrin solution (a) (0.2 mL) was then added to the mixture and heating was continued for 3 more minutes. During this time a purple-blue color developed if primary amino groups were present. The solution was cooled to room temperature, diluted to 10 mL with 60% ethanol, and its optical density determined at 570 nm with a UV/VIS spectrophotometer. Water (0.2 mL), subjected to the same procedure, served as the reagent blank.

### Acid activation of LCAA-CPG—Preparation of P-2

LCAA-CPG (4.5 g) was slowly stirred in a solution of 3% trichloroacetic acid in dichloromethane (50 mL) at room temperature for 3–4 h. The LCAA-CPG was filtered off and washed first with 9:1 triethylamine:diisopropylethylamine (50 mL), and then with $CH_2Cl_2$ and ether. The activated support was dried under vacuum over $P_2O_5$ before use.

### Succinylation Reaction–Preparation of P-3

To a dried 6 mL Hypovial (Pierce) containing acid activated LCAA-CPG (**P-2**) (1.0 g), succinic anhydride (2 mmol, 0.20 g) and 4-dimethylaminopyridine (4-DMAP, 0.33 mmol, 40 mg) was added, *via* syringe, anhydrous pyridine (6 mL) and the vial was shaken at room temperature for 16–20 h. The CPG was filtered off and washed successively with pyridine, $CH_2Cl_2$, and ether. **P-3** was dried under vacuum over $P_2O_5$ before use.

**Table 2.** Determination of Amino Loadings on LCAA-CPG

| LCAA-CPG solid support | Amino loadings in μmol/g of solid support | |
|---|---|---|
| | DMTCl/$AgNO_3$ | amidite/tetrazole |
| batch I (not activated) | 54 | 38 |
| batch I (acid activated) | 86 | 54 |
| batch II (acid activated) | 99 | 66 |
| Pon *et al.* (6) (not activated) | – | 6 |
| Pon *et al.* (6) (acid activated) | – | 71 |
| Gaur *et al.* (14) (not activated) | 31 | – |

### Nucleoside / Pentachlorophenol Coupling—Preparation of P-4

All reactions were carried out in septum-fitted, oven-dried, and argon-purged 6 mL Hypovials (Pierce).

*N4-benzoyl-5'-O-(dimethoxytrityl)deoxycytidine*

A mixture of ***1a*** (0.10 mmol, 62 mg), **P-3** (500 mg), 4-DMAP (0.05 mmol, 6 mg), triethylamine (40 μL), DEC (1.0 mmol, 192 mg), and anhydrous pyridine (6 mL) was shaken at room temperature for 24 h. Pentachlorophenol (0.25 mmol, 67 mg) was added and the mixture was shaken for an additional period of 16 h. The CPG was filtered off and washed successively with pyridine, $CH_2Cl_2$, and ether.

*Ribonucleosides*

The general procedure can be illustrated by the derivatization of **P-3** with 5'-O-(monomethoxytrityl)uridine (***1b***). A mixture of

*1b* (0.10 mmol, 52 mg), **P-3** (500 mg), 4-DMAP (0.05 mmol, 6 mg), triethylamine (40 μL), DEC (1.0 mmol, 192 mg), and anhydrous pyridine (6 mL) was shaken at room temperature for 72 h. Pentachlorophenol (0.25 mmol, 67 mg) was added and the mixture was shaken for an additional period of 16 h. The CPG was filtered off and washed successively with pyridine, $CH_2Cl_2$, and ether.

*Arabinonucleosides*

The general procedure can be illustrated by the derivatization of **P-3** with 5′-O-(monomethoxytrityl)arabinouridine (*1f*). A mixture of *1f* (0.20 mmol, 103 mg), LCAAP-CPG **P-3** (1.0 g), 4-DMAP (0.10 mmol, 12 mg), triethylamine (80 μL), DEC (2.0 mmol, 383 mg), and anhydrous pyridine (6 mL) was shaken at room temperature for 20 h. Pentachlorophenol (0.50 mmol, 134 mg) was added and the mixture was shaken for an additional period of 16 h. The CPG was filtered off and washed successively with pyridine, $CH_2Cl_2$, and ether.

### Piperidine Treatment – Preparation of P-5

Typically, **P-4** (500 mg) was treated with reagent grade piperidine (5 mL) and the slurry was shaken for 5 min-24 h (see Table 1). The CPG was filtered off, washed successively with $CH_2Cl_2$ and ether, and dried under vacuum.

### Acetic Anhydride/4-DMAP Capping Step

Stock solutions of 0.5M acetic anhydride in tetrahydrofuran (THF) and 0.5M 4-DMAP/2,4,6-trimethylpyridine in THF were prepared separately. The capping step involved mixing the support **P-5** (500 mg) with equal parts of the two stock solutions (3 mL each). The slurry was shaken for 2 – 3 h and then washed successively with pyridine, $CH_2Cl_2$, THF, and ether. Portions of this CPG can be re-capped on the DNA synthesizer just prior to synthesis.

### Derivatization method A: using [(pentachloropheny)succinyl] nucleosides *3* and *6*

*3*: Acid activated LCAA-CPG (**P-2**) (300 mg), nucleoside *3* (0.09 mmol, 88 mg), and triethylamine (30 μL) in anhydrous pyridine (2.0 mL) were combined in a sealed, argon-purged 6 mL Hypovial and the vial was shaken at room temperature for 36 h. The CPG was filtered off and washed successively with pyridine, $CH_2Cl_2$, and ether.

*6*: Acid activated LCAA-CPG (**P-2**) (500 mg), nucleoside *6* (0.15 mmol, 170 mg), and triethylamine (40 μL) in anhydrous pyridine (3.0 mL) were combined in a sealed, argon-purged 6 mL Hypovial and the vial was shaken at room temperature for 4 days. The CPG was filtered off and washed successively with pyridine, $CH_2Cl_2$, and ether.

### Derivatization method B: using nucleoside succinates *2* and *5*

*2*: A mixture of *2* (0.10 mmol, 70 mg), **P-3** (250 mg), 4-DMAP (0.13 mmol, 16 mg), triethylamine (40 μL), DEC (0.5 mmol, 96 mg), and anhydrous pyridine (5 mL) were combined in a sealed, argon-purged 6 mL Hypovial. The mixture was sonicated (ultrasound water bath) for 1 – 2 min and the Hypovial shaken at room temperature for one week. The CPG was filtered off and washed successively with pyridine, $CH_2Cl_2$, and ether.

*5*: A mixture of *5* (0.18 mmol, 150 mg), **P-3** (500 mg), 4-DMAP (0.09 mmol, 10 mg), triethylamine (50 μL), DEC (1.75 mmol, 340 mg), and anhydrous pyridine (5 mL) were combined in a sealed, argon-purged 6 mL Hypovial. The mixture was sonicated (ultrasound water bath) for 1 – 2 min and the Hypovial shaken at room temperature for 4 days. The CPG was filtered off and washed successively with pyridine, $CH_2Cl_2$, and ether.

### Solid-phase synthesis

Solid-phase syntheses of deoxyribonucleotides and arabinonucleotides were performed on an Applied Biosystems 381A DNA Synthesizer. $(araU_p)_7araU$ and $(araA_p)_7araA$ were prepared using a modified version (unpublished results) of the synthesis cycle reported by Damha *et al.* (10). The synthesis of $(dT_p)_7dC$ was carried out using the synthesis cycle provided by the Applied Biosystems 381A synthesizer.

## RESULTS AND DISCUSSION

### Improved CPG Derivatization Procedure (Method C)

*The Chemistry*

Ideally, derivatization of LCAA-CPG beads suitable for oligonucleotide synthesis should avoid multi-step modifications of the 3′-terminal nucleosides. A support bearing a terminal carboxylic acid would be most suitable since the carboxyl moiety could be esterified directly with one of the secondary hydroxyl groups of nucleosides. Earlier attempts to couple nucleosides directly to carboxyl-functionalized CPG using either DCC/4-dimethylaminopyridine (4-DMAP) (10, 12) or phosphorus oxychloride (12) as condensing reagents did not produced satisfactory results. Only poor nucleoside loadings in the range of 1 to 10 μmol/g were obtained with the DCC/(4-DMAP) system. The phosphoryl succinate mixed anhydride, formed by reaction of the carboxyl beads with phosphorous oxychloride, produced loadings of 18 – 36 μmol/g with 5′-O-mono-methoxytritylthymidine. Ribonucleoside derivatives gave much lower loadings, generally about 10 μmol/g.

We recognized that the combination of the DEC/4-DMAP coupling procedure developed by Pon *et al.* (6) and a carboxyl-functionalized CPG could provide a more efficient method for the direct attachment of nucleosides onto CPG beads. The methodology developed is similar to that previously described for the derivatization of silica gel supports (12, 13) and is outlined in Scheme 1 (method C). Beads of controlled-pore glass (particle size 125 – 177 μm, pore diameter 500 Å) derivatized with a long-chain alkylamine with a primary amino loading of about 100 μmol/g were obtained from Pierce Chemical Co. (Rockford, IL). The initial step involved treating the beads (**P-1**) with a solution of 3% trichloroacetic acid (TCA) in 1,2-dichloroethane at room temperature for 2 – 3 h. This step has been shown to liberate amino (or hydroxyl) groups thus providing the maximum number of reactive sites on the surface (6) (*vide infra*). The activated LCAA-CPG beads (**P-2**) were then derivatized with succinic anhydride/4-DMAP (2, 3, 6) to provide LCAAP-CPG beads (**P-3**). The next step in the derivatization required condensing a 5′-O-tritylated nucleoside (*1a – h*) to LCAAP-CPG (**P-3**) with DEC and 4-DMAP in pyridine (6). *It is of no consequence whether the protected ribonucleosides and arabinonucleosides are coupled to* P-3 *via the 2′- or 3′-hydroxyl group, or both, since they will be liberated in the final aqueous ammonia treatment in oligonucleotide synthesis.* Following nucleoside coupling, residual carboxyl groups were converted to amides by addition first of pentachlorophenol/DEC and then piperidine. Residual carboxyl groups on the support must be eliminated since

they may be transformed to acylphosphate mixed anhydrides during the nucleoside coupling and oxidation steps of nucleotide synthesis. Although the carboxylic acid groups may be regenerated by hydrolysis of the acylphosphate anhydrides during the water/iodine oxidation step, the phosphitylation of carboxyl groups would compete with the phosphitylation of 5'-hydroxyls of growing nucleotide chains. As a result, lower than expected coupling yields would be observed. Finally, the resulting beads (**P-5**) were treated with acetic anhydride and 4-DMAP to esterify additional ribose or arabinose hydroxyl groups and to block any underivatized sites which would react and generate by-products during oligonucleotide synthesis.

In this fashion, supports of N4-benzoyl-5'-O-DMT-2'-deoxycytidine (***1a***), four common ribonucleosides (***1b–e***) and three arabinonucleosides (***1f–h***) were fully derivatized in 3–5 days by simply mixing reagents together and filtering and washing the CPG beads. From the data collected in Table 1 it is apparent that all of the nucleoside loadings fall within a satisfactory 20–40 μmol/g range.

Many attempts were made to optimize reaction conditions and maximize the amount of nucleoside covalently bound to the support. The results of these studies are summarized below.

*Determination of Amino Loadings on LCAA-CPG* **P-1** *and* **P-2**
The amount of LCAA-CPG reactive sites were determined before and after treatment with 3% TCA/ 1,2-dichloroethane by two spectrophotometric methods. The first method is based on the reaction of LCAA-CPG with a mixture of 4,4'-dimethoxytrityl chloride (DMTCl), silver nitrate, and pyridine. After removing the excess reagents, the support is treated with trichloroacetic acid to release the DMT cation which is determined quantitatively by measuring its absorbance at 504 nm ($\epsilon$= 76 mL $cm^{-1}$ $\mu mol^{-1}$ in 1,2-dichloroethane). This approach is a modification of the method reported recently by Gaur *et al.* (14). The major difference between the reported procedure and ours is the use of silver nitrate (15) instead of tetra-*n*-butylammonium nitrate (14) as the catalyst. The second method is based on the reaction of LCAA-CPG with 5'-O-dimethoxytritylthymidine-3'-O-diisopropyl-β-cyanoethyl phosphoramidite (***7***) in the presence of tetrazole (6). The amount of phosphoramidite reaction with the support is determined spectrophotometrically by the DMT assay method described above. By these two methods, the number of reactive (or accessible) sites on LCAA-CPG supports from two different sample lots (*i.e.*, I and II, Pierce Chemical Co.) were determined and the results are shown in Table 2. For comparison, the loadings determined by Pon *et al.* (6) and Gaur *et al.* (14) on other LCAA-CPG samples (Pierce Chemical Co.) are shown together with our results. It is apparent from Table 2 that the number of reactive groups on the surface of acid unactivated (**P-1**) and activated (**P-2**) supports varied significantly among the different CPG batches. This may be due to slight variations in either the chemical structure of the long chain alkyl spacer (Figure 1), amino group loading, or in the pore size among the different batches of CPG. The data presented in Table 2 also show that the number of reactive groups determined on activated and unactivated LCAA-CPG supports were lower (6–99 μmol/g) than that reported by the manufacturer (100 μmol amino groups/g of support, based on the determination of nitrogen or acid-base titration). These results can be rationalized by assuming that some of the reactive groups on LCAA-CPG are sterically inaccessible to the DMTCl or amidite (***7***) reagents and yet can be titrated by acid (14). The limited reactivity of the majority of the sites on LCCA-CPG was a phenomenon observed in several other reactions described below.

Sites on LCAA-CPG reacted more efficiently with DMTCl/$AgNO_3$ than with the thymidine 3'-O-phosphoramidite/tetrazole reagents. For example, 99 μmol of sites/g of activated LCAA-CPG (batch I) reacted with DMTCl whereas only 66% of these underwent reaction with the amidite reagent (***7***). The dimethoxytrityl loading achieved by Gaur *et al.* (14) on unactivated LCAA-CPG (31 μmol/g) was roughly half the loading achieved on our supports (54 μmol/g). The nature of the nitrate catalysts used by Gaur *et al.* (*i.e.*, tetra-*n*-butylammonium nitrate) may have been the cause for the lower degree of tritylation observed. Reddy *et al.* (16) have found silver nitrate to be a superior catalyst to tetra-*n*-butylammonium nitrate for the dimethoxytritylation of nucleosides bound to CPG supports.

As first noted by Pon *et al.* (6), the amount of reaction between the acid activated LCAA-CPG and thymidine phosphoramidite (***7***)/tetrazole was significantly greater relative to LCAA-CPG in its normal, unactivated form (Table 2). The same trend was observed in the reaction between these supports and dimethoxytrityl chloride (Table 2). Pon *et al.* have attributed this anomaly to the presence of either 'masked' amino or hydroxyl groups on the surface of LCAA-CPG which are only accessible for reaction after the acidic deblocking. To determine the nature of the functional groups being unmasked by the acidic treatment, various portions of acid-activated and unactivated LCAA-CPG supports were treated with ninhydrin under standard conditions (11, Scheme 2). Figure 2 demonstrates that the amount of Ruhemann's purple released from acid-treated LCAA-CPG is roughly double the amount released from LCAA-CPG in its normal, unactivated form. These results are consistent with the hypothesis (6) that the acidic treatment liberates *primary* amino groups since reaction of ninhydrin with *secondary* or *tertiary* amino groups does not produce the purple-blue product.

Interestingly, when LCAA-CPG in its normal, unactivated form was treated successively with i) acetic anhydride/ 4-DMAP/ collidine/30 min; ii) acetonitrile; iii) 3% thrichloroacetic acid/

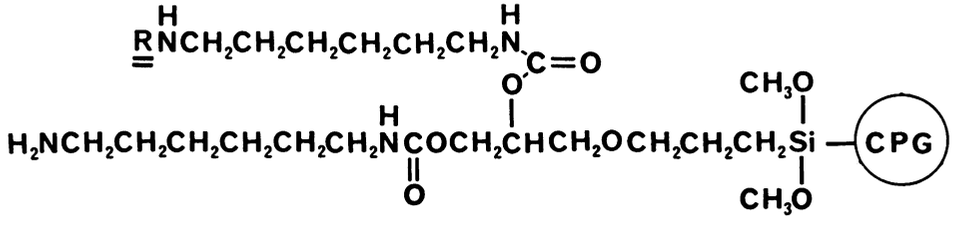



**Figure 1.** Reported structure of the alkylamine ligand on LCAA-CPG (17). The structure of the acid labile protecting group R is unknown.

1,2-dichloroethane/ 4 h; iv) acetonitrile; and v) ninhydrin under the standard conditions, the Ruhemann's purple was not produced (Scheme 2). This result indicates that the masked amino groups are of the type -NHR and are acetylated during step (i) generating -NR(Ac) groups. Deblocking of the unknown protecting group R during the acidic treatment (iii) would yield terminal -NHAc groups which cannot react with ninhydrin to form the characteristic purple-blue color. The structure of the long-chain alkylamine spacer on LCAA-CPG reported by Sproat and Gait (17) is consistent with our results (Figure 1). The masked amino groups are likely to be part of the protected hexamethylene diamine groups on the alkyl spacers (18).

*Preparation of LCAAP-CPG* **P-3**

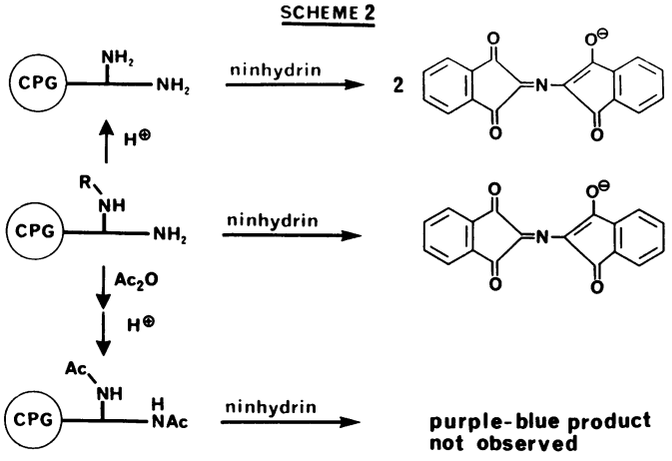
Completion of the reaction of acid-activated LCAA-CPG (**P-2**) with excess succinic anhydride/ 4-DMAP was qualitatively monitored by the ninhydrin test (11). Generally, a large excess of succinic anhydride was added to LCAA-CPG **P-2** so that a second addition was never required.

The number of reactive (or accessible) carboxyl groups on the surface of LCAAP-CPG **P-3** was calculated by determination of the amount of *p*-nitrophenol bound to **P-3** by DEC. The amount of *p*-nitrophenoxide ions released after a piperidine treatment was measured by visible absorption spectrophotometry (410 nm) using 15.7 $mL/cm^{-1}$ $mmol^{-1}$ as the extinction coefficient of *p*-nitrophenoxide (13). The number of reactive carboxylic acids calculated on batch I and II were 46 and 28 μmol/g, respectively. The same values were obtained when *p*-nitrophenol was replaced by pentachlorophenol ($\lambda = 329$ nm; $\epsilon =$ 5.2 $mL/cm^{-1}$ $\mu mol^{-1}$) in the above experiments. It is then apparent that the amount of carboxyl groups which react with the phenol derivatives occupies only a fraction of the number of potential sites available (> 100 μmol/g). Nevertheless, these results demonstrated that nucleosides could, in principle, be attached to LCAAP-CPG in up to 30–50 micromoles per gram of support.

*Coupling of Nucleosides to LCAAP-CPG* **P-3**

Nucleosides were covalently bound to **P-3** by simply shaking DEC with a mixture of a nucleoside (***1a–h***), **P-3**, and 4-DMAP

SCHEME 2



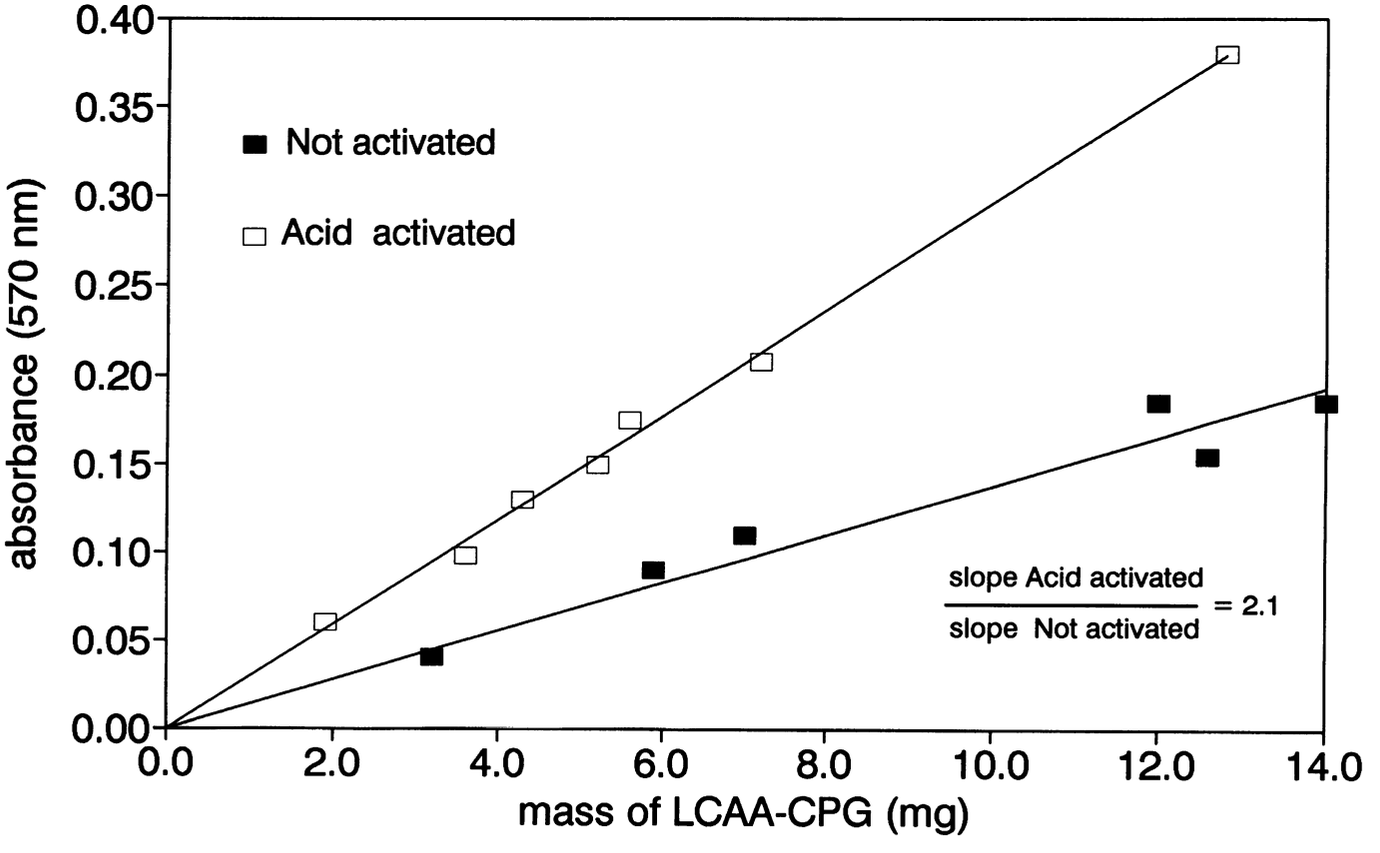




**Figure 2.** Visible absorbance at 570 nm for Ruhemann's purple liberated in solution from acid activated and unactivated LCAA-CPG. The ninhydrin-promoted reaction was carried out as described in the experimental section.

in triethylamine/pyridine for 12–72 h at room temperature. Pentachlorophenol was then added *in situ* to esterify any remaining carboxyl groups and the solid material was separated by filtration and washed repeatedly with organic solvents. Solvents such as ethanol or methanol should be avoided since these were found to cleave the pentachlorophenyl succinate esters (19). Nucleoside loadings were determined at this stage by detritylation of 5–10 mg samples with 5% thrichloroacetic acid/1,2-dichloroethane solution followed by spectrophotometric quantitation of the MMT ($\epsilon$= 56 mL $cm^{-1}$ $\mu mol^{-1}$) and DMT ($\epsilon$= 76 mL $cm^{-1}$ $\mu mol^{-1}$) cation absorbances at 478 and 504 nm, respectively. The results are shown in Table 1. With the exception of one support (araC-CPG, 20 $\mu$mol/g), all nucleoside loadings fall in the range of 27–40 $\mu$mol/g. Therefore, a large fraction of the available carboxyl groups on **P-3** are esterified during nucleoside coupling.

The nucleoside coupling reactions were initially allowed to proceed for 3 days. We have improved upon the procedure and have reduced the total time for nucleoside coupling to 24 h. The loadings were consistently in the 20–40 $\mu$mol/g range (Table 1).

*Piperidine Treatment*

On standing at room temperature, support **P-4** slowly releases pentachlorophenol, indicative of spontaneous hydrolysis of the pentachlorophenyl succinate esters. Therefore, it is essential to treat **P-4** with piperidine *immediately* after nucleoside/pentachlorophenol coupling to convert the pentachlorophenyl esters to inert succinylpiperidines (Scheme 3). Although the piperidine amidation step can be carried out *in situ* following the pentachlorophenol esterification step, we carried out all amidation reactions *after* work-up and isolation of LCAAP-CPG **P-4**. This enabled us to monitor spectrophotometrically the release of pentachlorophenol induced by piperidine and thus, confirm that both pentachlorophenol esterification and piperidine amidation reactions had occurred. As a control, and a check on the overall reaction kinetics, the amidation reaction was studied spectrophotometrically by treatment of support **P-7** (bearing solely terminal pentachlophenyl esters, Figure 3) with a 0.1% piperidine/ether solution. The results presented in Figure 3 demonstrate that the amidation reaction is fast ($t_{1/2}$ = 2.0 min, r.t.) and follows, as expected, pseudo first-order kinetics.

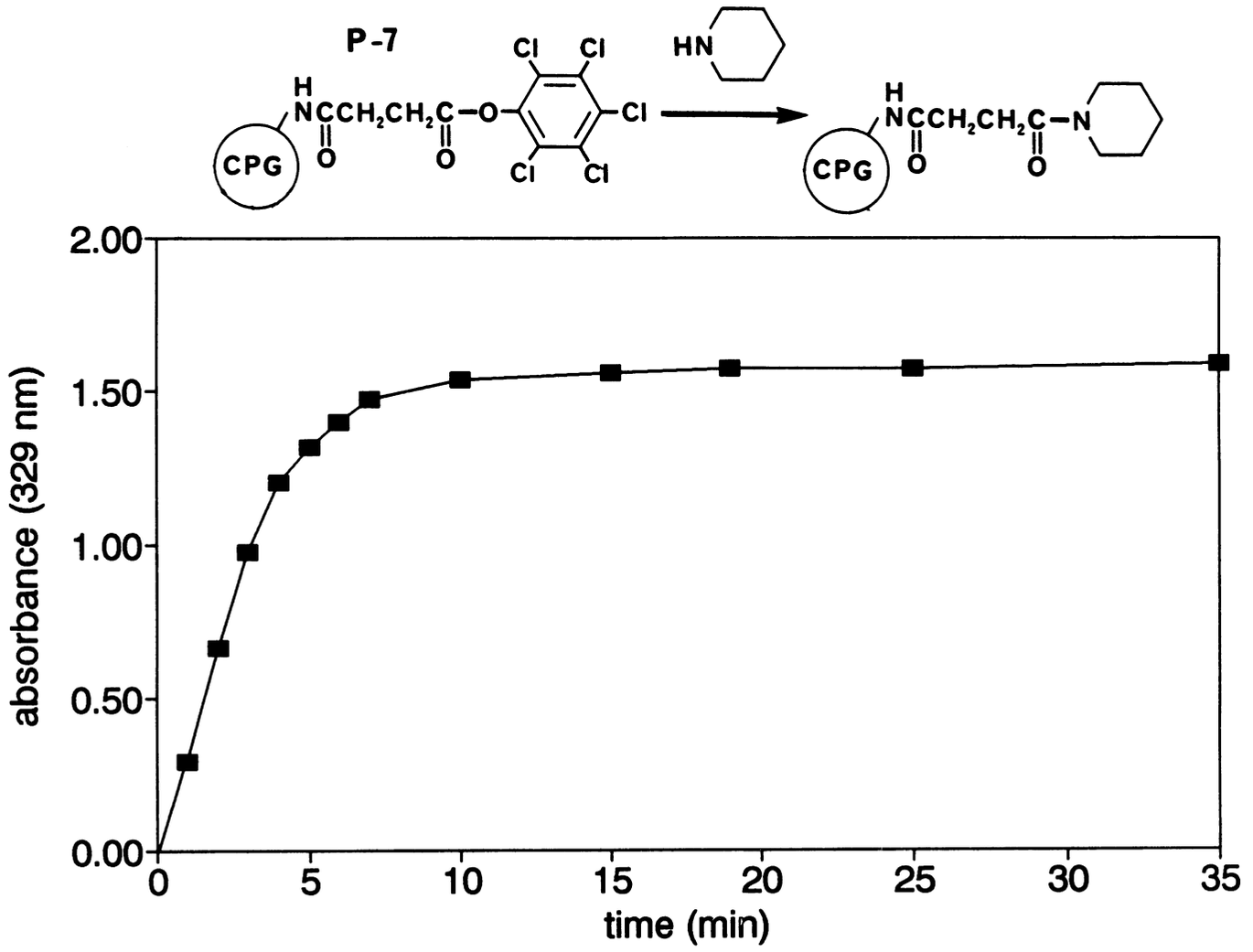
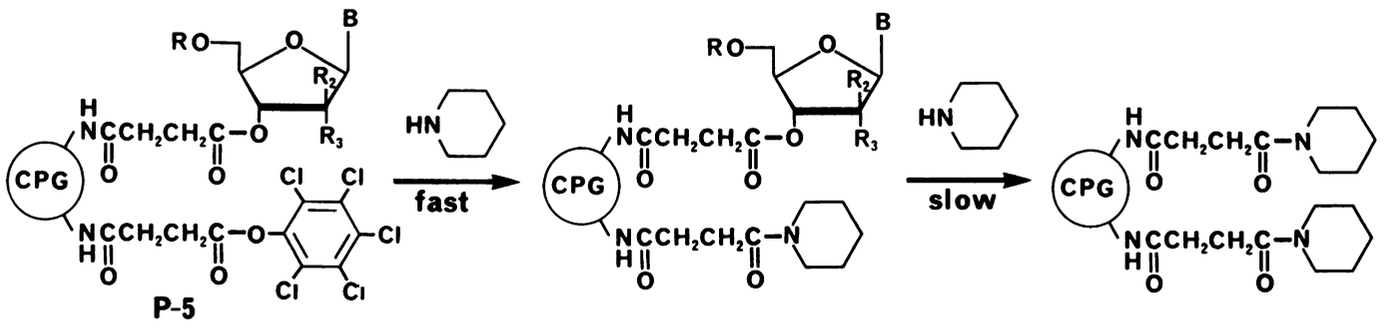



**Figure 3.** Cleavage kinetics of pentachlorophenol attached covalently to LCAAP-CPG. The cleavage (amidation) reaction is promoted by piperidine as indicated in the scheme above. In this experiment a weighed quantity of **P-7** (with a loading of 46 $\mu$mol of pentachlorophenylesters/g of support) was taken in a UV cuvette to which a solution of 0.1% piperidine/ether was added. The cuvette was stoppered and the visible absorbance at the $\lambda_{max}$ (329 nm) was monitored as a function of time.

SCHEME 3




Having established the necessary conditions for the amidation reaction, the supports (**P-4**) were treated with piperidine (5 min-24 h) and washed extensively to afford **P-5**. Prolonged treatment of support **P-4** with piperidine results in cleavage of *both* pentachlorophenyl succinate esters and nucleoside succinate esters (Scheme 3). For example, the loading of uridine on **P-4** dropped from 36 to 24 μmol/g (*i.e.*, 32%) after a 24 h treatment with piperidine (entries 4 and 5, Table 1). When this treatment was reduced to 5 min, the more labile pentachlorophenyl esters were cleaved quantitatively without affecting the nucleoside succinate esters (20). The 2′-deoxyribonucleoside succinate esters bound to **P-4** were more stable to piperidine than the arabino and ribo counterparts (Table 1).

*Acetic Anhydride Treatment (Capping)*

Due to the sensitivity of the pentachlorophenyl esters on **P-4**, it is critical that 'capping' of hydroxyl groups be carried out *after* the amidation step. Pentachlorophenyl esters on the surface of **P-4** were rapidly converted to carboxyl groups when exposed to the capping reagent (*i.e.*, $Ac_2O$/4-dimethylamino-pyridine/collidine/THF) and moisture. In this case it was necessary to convert the carboxyl groups to amides by the usual pentachlorophenol/DEC and piperidine steps.

We prefer and recommend that **P-5** be capped immediately after the piperidine treatment and again, just prior to the start of oligonucleotide synthesis. The second capping step is easily incorporated in the automated synthesizer and serves to re-cap sites that might be unmasked during storage of the support. In addition, it removes water from the support at the start of oligonucleotide synthesis.

## Comparison of Methods A, B, and C

In order to evaluate the current methods of CPG derivatization (A and B, scheme 1), supports bearing N4-benzoyl-5′-O-DMT-2′-deoxycytidine and the ribonucleoside N6-benzoyl-5′-O-MMT-3′-O-*t*-butyldimethylsilyladenosine were prepared using the same batch of LCCA-CPG. Reactions were carried out according to the literature procedures (2, 3) and the results are collected in Table 1. Method A gave consistently lower loadings of these and other nucleosides. On the other hand, both method B and our method consistently gave comparable loadings for deoxycytidine: 35 and 37 μmol/g, respectively. These values were substantially lower than the loading of 64 micromol per gram of support achieved by Pon *et al.* (6) using method B. We cannot account for this discrepancy. It may, however, be attributed to variations in either the pore size, amino group loading, or nature of the long chain alkyl spacer among the different CPG supports.

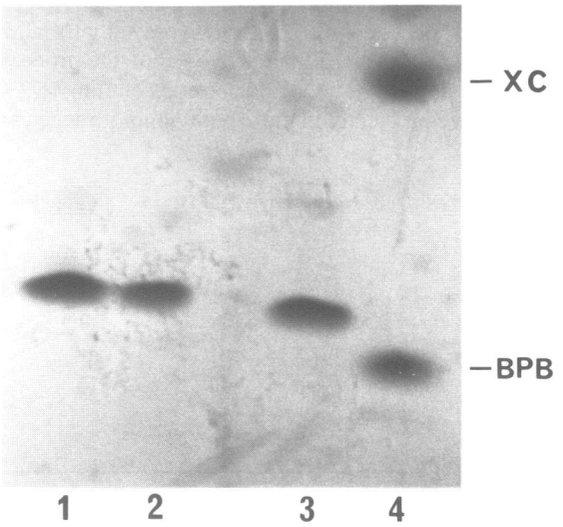



**Figure 4.** 24% Polyacrylamide/7 M urea electrophoresis gel showing the results of deoxyribonucleotide and arabinonucleotide synthesis using our newly derivatized CPG beads. All lanes show the crude product of synthesis obtained after the deblocking steps. Lane 1, ara(UUUUUUUU); lane 2, ara(AAAAAAAA); lane 3, 5′-d(TTTTTTTTC)-3′; lane 4, xylene cyanol (XC) and bromophenol blue (BPB) markers. The oligonucleotides were visualized by ultraviolet shadowing.

In the case of the ribonucleoside adenosine, our method gave a significantly higher loading (33 μmol/g) than the existing methods A (15 μmol/g) or B (13 μmol/g). This is most likely due to the reduced steric hindrance around the reactive site of 5′-O-MMT-N6-benzoyladenosine relative to the 3′-silylated derivatives ***5*** (method B) and ***6*** (method A).

## CONCLUSION

A fast and economical procedure for nucleoside derivatization of controlled-pore glass beads has been developed. This procedure eliminates entirely the time-consuming blocking steps (*i.e.*, succinylation, esterification, or silylation) of nucleoside hydroxyl groups and affords, within 3–5 days, supports with an excellent degree of nucleoside loadings (20–40 μmol/g). All of the required nucleosides are simple 5′-O-tritylated derivatives which can be prepared easily or obtained commercially. The higher loadings obtained (30–40 μmol/g) are quite useful for the synthesis of large amounts of oligonucleotides or *branched* oligoribonucleotides (21) where a high degree of CPG nucleoside attachment is required.

The improved derivatization procedure has been successfully applied in our laboratory to the synthesis of short oligomers of 2′-deoxyribo and arabinonucleotides. In each case, the crude oligomers obtained after the standard deprotection steps were of high purity (Figure 4) thereby demonstrating the utility of our CPG supports.

## ACKNOWLEDGEMENTS

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18. We have been unable to ascertain the structure of the ligand on LCAA-CPG with Pierce Chemical Co.
19. Treatment of **P-4** with excess ethanol results in the conversion of pentachlorophenyl esters to ethyl esters. The transesterification reaction also occurred with 5′-O-(dimethoxytrityl)-3′-O-(*tert*-butyldimethylsilyl)-2′-O-[(pentachlorophenyl)succinyl]uridine was treated with excess ethanol. In this case 5′-O-(dimethoxytrityl)-3′-O-(*tert*-butyldimethylsilyl)-2′-O-[(ethyl)succinyl]uridine was obtained ($^1$H, $^{13}$C-NMR).
20. The cleavage of pentachlorophenol was monitored spectrophotometrically while the cleavage of nucleosides from the supports was monitored by the trityl assay method.
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