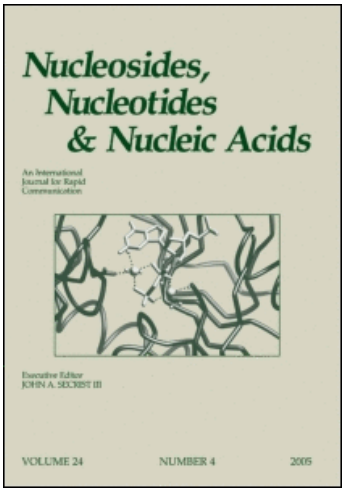

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A PHOSPHATE BOUND UNIVERSAL LINKER FOR DNA SYNTHESIS

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ABSTRACT A uridine-based linker immobilized onto polystyrene beads at the 5' terminus via a phosphodiester group and then used as a universal DNA synthesis support gives post synthesis DNA cleavage in 8 hrs or less without alkali metal salts. DNA produced with the new support was analyzed by HPLC, MALDI mass spectroscopy and PAGE. Each analysis showed DNA of equivalent quality to that produced with standard CPG supports, without contaminating materials resulting from linker or support backbone decomposition.

INTRODUCTION

Oligonucleotide synthesis on a universal support enables the addition of the 3' terminus of the desired DNA fragment by automated coupling of the corresponding phosphoramidite, instead of using different supports of predetermined composition. After synthesis is complete, workup affords the 3'-hydroxyl functionalized DNA while the 3'-phosphate remains attached to the universal support linker. Several strategies[1-5] have been reported to provide this simplification, which has become increasingly important with the advent of multiple well plate formatted syntheses. Most of these methods rely on a tetrahydropyran vicinal diol-based linker system, in which the phosphate bond to the 3'-terminus of the desired DNA fragment is cleaved concomitant with cyclic phosphate formation with the diol. Post synthesis cleavage protocols involve either strongly basic conditions with heating at elevated temperatures for long periods, or the use of alkali metal salts[4] which require additional steps for removal. These limitations have, so far, outweighed the added convenience of one support material for every sequence, and prederivatized supports are still predominantly preferred.

We have reformulated the tetrahydropyran vicinal diol-based linker system so that the linker remains firmly bound to the support during the deprotection and cleavage steps of DNA synthesis. Thus, only DNA that has been completely removed from the diol-phosphate moiety is present in the cleaved product.

MATERIALS AND METHODS

Acetic acid (HOAc), pyridine, methanol (MeOH), aqueous ammonia, ethyl acetate (EtOAc), dimethylformamide (DMF), dichloromethane (CH_2Cl_2) and acetonitrile (CH_3CN) were Omnisolve grade from VWR (San Francisco, CA); tetrahydrofuran (THF), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), *N*-methylimidazole, tetrabutylammonium fluoride (TBAF), triethylamine (TEA), acetic anhydride (Ac_2O) and magnesium sulfate ($MgSO_4$) were from Aldrich (Milwaukee, WI). DMT chloride, uridine and *tert*-butyldimethylsilyl chloride were obtained from Chem Impex (Wood Dale, IL); 2-cyanoethyl tetraisopropylphosphorodiamidite was obtained from Chemgenes Corp. (Waltham, MA); 1*H*-tetrazole was obtained from American International Chemical (Natick, MA). DNA syntheses were performed on a Biosearch 8750 synthesizer and an adapted Cavro robotic DNA synthesis system (in house design), with Beckman DNA amidites (Fullerton, CA); all other DNA synthesis reagents were the same as those previously reported[6]. Elemental analyses were performed by Desert Analytics (Tucson, AZ); MALDI mass spectroscopy and capillary zone electrophoresis (CZE) were performed by the Stanford University PAN facility (Stanford, CA).

Quantitation of DNA products was performed by determining the absorbance of aqueous solutions at 254 nm using a Beckman DU 640 spectrophotometer. When appropriate, resin loadings were determined by DMT cleavage assay, as follows: an exactly measured aliquot (20 mg) of the dried support material was treated with dichloroacetic acid in CH_2Cl_2 (3:97, 100 mL), and the absorbance was read at 498 nm; cell path length = 1.0 cm, e = 70,000 cm^{-1} M^{-1}. Anion Exchange HPLC analyses were performed by injecting 2-20 μL of the aqueous samples, depending on the concentration, onto a Dionex anion exchange column (4.6 x 250 mm); samples were eluted at 2 mL/min with aqueous buffers of (A) 0.025 M TRIS HCl and 0.01 M TRIS, and (B) 0.025 M TRIS HCl, 0.01 M TRIS, and 1.0 M NaCl using a linear gradient of 1:0 to 0:1 over 20 min, with UV detection at 260 nm.

Synthesis of the Universal Linker

2'-O-Acetyl-3'-O-(4,4'-dimethoxytrityl)uridine and 3'-O-Acetyl-2'-O-(4,4'-dimethoxy trityl)uridine (4). Uridine (16 g, 61 mmol) was dissolved in pyridine (200 mL), reduced to a tar by rotary evaporation, and redissolved in DMF (200 mL). Imidazole (9.8 g, 140 mmol) was then added and the reaction stirred until all solids had dissolved. *tert*-Butyldimethylsilyl chloride (11 g, 70 mmol) was added, and the solution was stirred for 2 h. The mixture was then poured into water (1 L) and stirred vigorously for 30 min. The white solid that formed was collected by filtration through a coarse sintered glass funnel, and washed on the filter with additional water (300 mL). The wet product was taken up in

CH_2Cl_2 (200 mL), the aqueous layer that separated was removed, and the organic phase was dried over $MgSO_4$ and concentrated to give 20 g (88% yield) of 5'-*O-tert*-butyldimethylsilyluridine (**1**) as a white foam. Intermediate **1** (13 g, 35 mmol) was dissolved in pyridine (200 mL) and the solution then reduced to 100 mL by rotary evaporation. DMT-chloride (12 g, 36 mmol) was added, and the solution allowed to sit at 25 °C for 48 h. Ac_2O (20 mL) and *N*-methylimidazole (2 mL, 25 mmol) were added, and the solution allowed to sit at 25 °C for an additional 24 h, then concentrated by rotary evaporation. The resultant residue was dissolved in EtOAc (300 mL), washed with 1 N citric acid (300 mL), brine (200 mL), dried over $MgSO_4$, and concentrated. The residual tar was dissolved in THF (100 mL), treated with 1 N TBAF in THF (50 mL), and allowed to sit at 25 °C overnight. The solvent was then removed by rotary evaporation, and the residue dissolved in EtOAc (200 mL), washed with saturated aqueous $NaHCO_3$ (200 mL), brine (200 mL), dried over $MgSO_4$, and concentrated to a tar. The product was loaded onto a silica gel column (30 x 5 cm) that was prewashed with MeOH - TEA - CH_2Cl_2 (1:1:99), and eluted with a gradient from 1:1:99 to 5:1:99 in 500 mL increments. Fractions were inspected by TLC on alumina backed silica eluted with MeOH - CH_2Cl_2 (1:19), and those containing pure product (R_f 0.14) were pooled and evaporated to provide 6.3 g (30% from **1**) of white foam; ^1H NMR ($CDCl_3$) δ 7.4-7.2 (m, 9H), 6.8-6.7 (dd, 4H), 6.1 (d, 1H), 5.6 (d, 1H), 4.6 (dd, 1H), 3.95 (d, 2H), 3.75 (d, 6H), 3.7-3.5 (m, 2H), 2.6 (q, 5H), 2.1 (s, 3H), 1.0 (t, 8H), with additional minor peaks corresponding to the other regioisomer; Anal calc'd for $C_{32}H_{32}N_2O_9$.0.8 TEA: C, 65.77; H, 6.96; N, 5.83. Found: C, 65.37; H, 7.11; N, 6.01.

5'-O-(N,N-Diisopropyl-2-cyanoethylphosphoramidite)-2'-O-acetyl-3'-O-(4,4'-dimeth oxytrityluridine and 5'-O-(N,N-Diisopropyl-2-cyanoethyl phosphoramidite)-3'-O-acetyl-2'-O-(4,4'-dimethoxy-trityl)uridine **5**. Compound **4** (3.0 g, 5.1 mmol) was dissolved in dry CH_3CN (200 mL), reduced to a tar by rotary evaporation, redissolved in dry CH_3CN (100 mL), and combined with a premixed solution of 2-cyanoethyl tetraisopropyl-phosphorodiamidite (3.0 g, 10 mmol) and tetrazole (180 mg, 2.6 mmol). The reaction was allowed to stand at 25 °C for 2 h, and then saturated aqueous $NaHCO_3$ (20 mL) was added. The mixture was reduced to a tar by rotary evaporation and redissolved in EtOAc (200 mL). The organic phase was washed with saturated aqueous $NaHCO_3$ (100 mL), dried over $MgSO_4$, and reduced to a tar by rotary evaporation. The residue was purified by chromatography on a silica gel column (20 x 2 cm), packed and eluted with 1% pyridine in EtOAc. TLC-pure fractions (aluminum backed silica plates eluted with 1% pyridine in EtOAc; R_f 0.8) were pooled and evaporated to give 3.5 g (87% yield) of white foam; ^{31}P NMR ($CDCl_3$) δ 150.223, 149.606, 147.740 (with a shoulder).

Synthesis of PEG Polystyrene Based Universal Support

The procedure of J. H. Adams et al.[7] as described for preparation of aminomethyl polystyrene and linker attachment was followed closely: PEG_{2000} (50 g, 25 mmol) was dissolved in CH_2Cl_2 - Pyridine (1:1, 200 mL), rotary evaporated to near dryness, redissolved in pyridine (300 mL), and the reaction volume reduced to about 100 mL. A solution of *p*-nitrophenyl chloroformate (2.5 g, 12 mmol) in CH_2Cl_2 (12 mL) was added dropwise to the vigorously stirred mixture. The reaction was stirred at 25 °C for 4 h, evaporated to a viscous state, and a suspension of 1% crosslinked aminomethyl polystyrene resin (6.2 g, 200-400 mesh, 2 mmol/g) and HOBt (2.0 g, 15 mmol) in CH_2Cl_2 - DMF (1:1, 100 mL) was added with rapid stirring. After 2 h, more DMF (50 mL) was added to the markedly swollen resin and the reaction stirred overnight. The resin beads were then separated by filtration, washed with DMF (3 x 200 mL), resuspended in DMF (200 mL), and capped with HOBt (3 g, 22 mmol) and Ac_2O (2 mL, 21 mmol). After 30 min the beads were filtered, washed with DMF (3 x 200 mL), CH_2Cl_2 (3 x 200 mL), MeOH (3 x 200 mL), and dried to give 14.5 g of ninhydrin negative off-white beads (57% PEG content). A portion of this resin (2 g) was washed with CH_3CN (40 mL), suspended in pyridine (40 mL), dried by evaporation, and universal linker amidite **5** (200 mg, 0.25 mmol) and 0.3 M *S*-ethyltetrazole in CH_3CN (10 mL) were added. The mixture was swirled for a few seconds to affect dissolution of the amidite, and allowed to stand at 25 °C for 10 min. The coupled resin was then filtered through a sintered glass funnel, washed with CH_3CN (3 x 30 mL), and oxidized with amidite oxidizer solution (10 mL)[6]. After 5 min, the resin was washed with CH_3CN (3 x 30 mL) and the unfunctionalized hydroxyls capped with a 2 hr treatment of Ac_2O - *N*-methylimidazole - THF (1:1:8). The resin was filtered, washed with CH_3CN (3 x 60 mL), and dried under vacuum. Loading was determined by DMT cleavage assay and found to be 42 μm/g.

Synthesis of Macroreticular Polystyrene Based Universal Support

Polymer Labs PL RP-S, 1000 Å pore size, 50-70 micron particle size, 5 g, were dried overnight, then gently shaken in dichloromethane (40 mL) with anhydrous ferric chloride (0.4 g) and chloromethylphthalimide (1 g). After 24 hours the beads were isolated by filtration and washed successively 3 times each with CH_2Cl_2, dioxan/12M HCl (95:5), dioxan/water (3:1) and methanol. The air dried white intermediate was suspended in dioxan/40% aq. methylamine (3:1) and gently shaken for 3 days at room temperature. The product amino beads were isolated by filtration, washed successively 3 times with DMF, CH_2Cl_2 and methanol and dried to give the amino resin (5 g). Triethylene glycol (3 g, 20 mmol) was dissolved in acetonitrile (50 mL) and rotary evaporated to dryness. This process was repeated with acetonitrile, and then with pyridine. A final concentration from

pyridine to half volume was performed, then a solution of p-nitrophenyl chloroformate (1 g, 5 mmol) in CH_2Cl_2 (10 mL) was added and the reaction stirred overnight. The dry resin (4 g) was then added, along with anhydrous HOBt (0.5 g), and the reaction gently shaken overnight. The resin was isolated by filtration then washed 3 times each with 40 mL DMF and 40 mL acetonitrile. The unfunctionalized amine groups were capped with a 5 min treatment of Ac_2O - *N*-methylimidazole - THF (1:1:8). After washing a resin sample was ninhydrin negative. A portion of the dried resin (2 g) was washed with CH_3CN (40 mL), suspended in pyridine (40 mL), dried by evaporation, and universal linker amidite **5** (200 mg, 0.25 mmol) and 0.3 M *S*-ethyltetrazole in CH_3CN (10 mL) were added. The mixture was swirled for a few seconds to affect dissolution of the amidite, and allowed to stand at 25 °C for 10 min. The coupled resin was then filtered through a sintered glass funnel, washed with CH_3CN (3 x 30 mL), and oxidized with amidite oxidizer solution (10 mL)[6]. After 5 min, the resin was washed with CH_3CN (3 x 30 mL) and the unfunctionalized hydroxyls capped with a 2 hr treatment of Ac_2O - *N*-methylimidazole - THF (1:1:8). The resin was filtered, washed with CH_3CN (3 x 60 mL), and dried under vacuum. Loading was determined by DMT cleavage assay and found to be 38 μm/g.

Procedure for Deprotection and Cleavage of DNA from the Universal Supports

Automated DNA synthesis[8] was concluded with the 5'-DMT group off, and the solid support containing the product was then expelled into a 2 mL plastic screw-top tube. Concentrated aqueous ammonia (0.5 mL) was added and the tube heated in an aluminum hot block at 60 °C for 3 hrs with the PEG polystyrene support, and 8 hrs with the macroreticular polystyrene support. Cleaved products were then evaporated to dryness for subsequent analysis.

RESULTS AND DISCUSSION

With many of the previously reported vicinal diol-based universal supports[1,2,4,5], there has been clear evidence[5] that cleavage of a bond between the vicinal diol linker and the support occurs first, and that much more forcing conditions are then needed to affect liberation of the desired 3'-hydroxyl oligonucleotide from the vicinal diol phosphate linker. See Figure 1. This has involved heating at elevated temperatures with strongly basic solutions for extended periods, as well as the use of alkali metal salts[4]. The initial presence of product DNA containing an unhydrolyzed 3' uridine phosphate in these systems was verified by attaching protected uridine alcohol **4** to the hydroxyl functionalized macroreticular polystyrene support by a hemisuccinate linkage[3] and then performing DNA synthesis of a 15-mer, followed by cleavage and deprotection in conc. ammonia at 60 °C

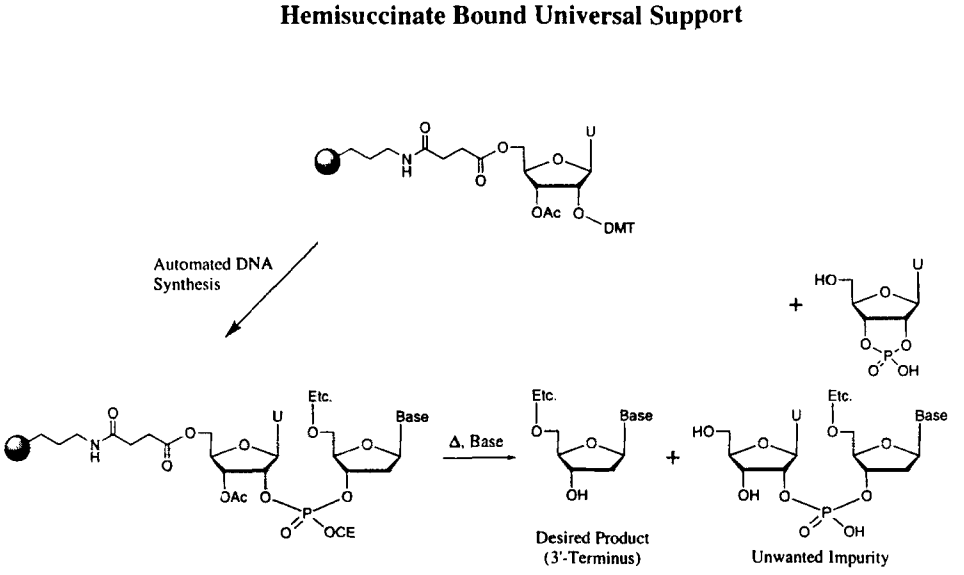

Figure 1. Cleavage products from DNA made on hemisuccinate linked universal support. "Etc." = synthesized oligonucleotide.

for 8 hrs. AX HPLC analysis of the product showed the desired compound as well as a major second peak having additional negative charge, and a MALDI mass spectrum also showed two major products, one with the correct mass for the desired compound, and another with 307 a.m.u. higher molecular weight. Minor peaks in this and subsequent mass spectra are sodium and potassium adducts. Both results are consistent with the contaminant being the desired oligo still attached to the cleaved uridine phosphate linker at the 3'-terminus. See Figure 2.

We describe the synthesis and use of a solid support in which the vicinal diol (uridine) is immobilized by a phosphate bond. The robust nature of this bond to alkaline cleavage conditions ensures that only product DNA containing a 3'-hydroxyl is liberated from the support, instead of a mixture of the desired product and a DNA fragment containing an unhydrolyzed uridine at the 3'-terminus. See Figure 3. A comparative synthesis and cleavage under identical conditions of the 15 mer above on the macroreticular polystyrene universal support and analysis of the product by both AX HPLC and a MALDI mass spectrum showed clean product, without any trace of the higher molecular weight contaminant discussed above. See Figure 4.

The synthesis of the linker **5** is shown in Figure 5. Commercially available uridine was treated with *tert*-butyldimethylsilyl chloride using established chemistry[9] to afford **1** as a

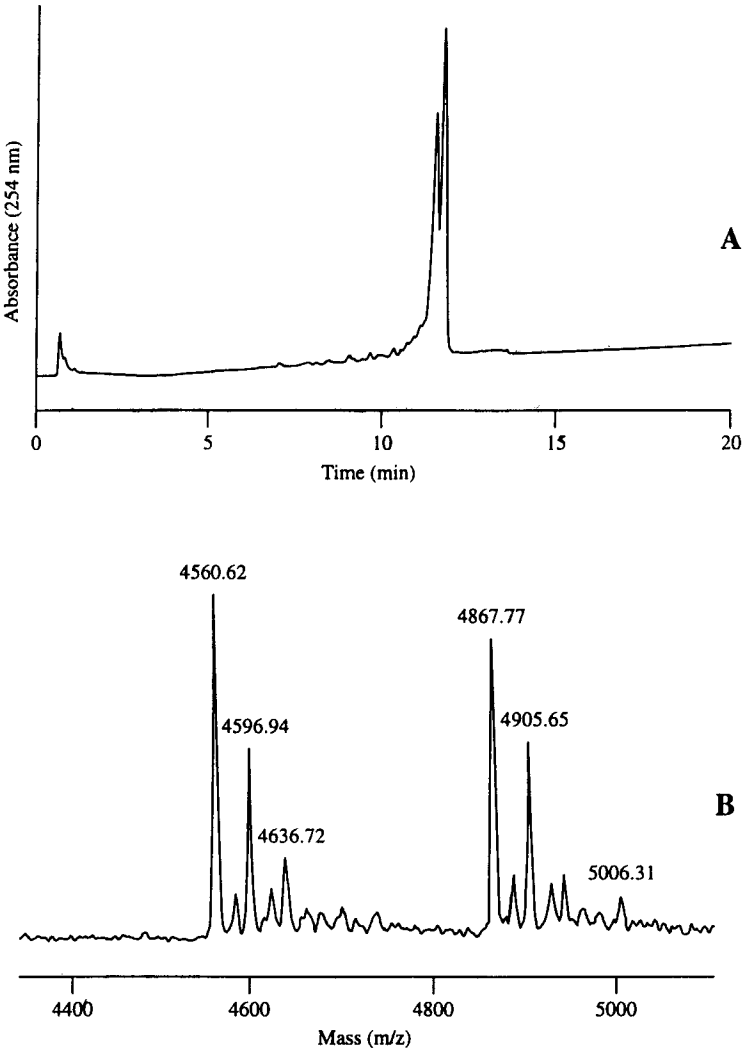

Figure 2. **A** = Anion Exchange HPLC of 15 mer 5'-ATACTAATCATGAGC-3' made on hemisuccinate linked universal support. **B** = MALDI mass spectrum of 15 mer, Mass Calc'd for 5'-ATACTAATCATGAGC-3' = 4562 a.m.u.; Mass Calc'd for 5'-ATACTA-ATCATGAGC-3'-uridine phosphate = 4869 a.m.u. Minor peaks are sodium and potassium adducts.

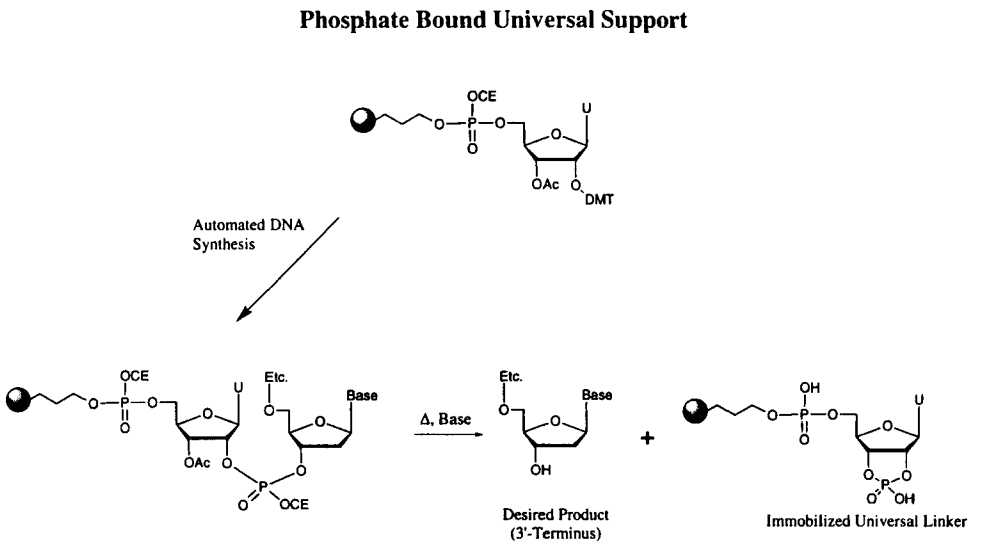

Figure 3. Cleavage products from DNA made on 5'-Phosphate linked universal support. "Etc." = synthesized oligonucleotide.

white powder. Either of the two remaining hydroxyls were functionalized by addition of 1 equiv of DMT chloride to give a mixture of 2' and 3'-O-DMT adducts **2** (intermediates **2** - **5** are depicted in Figure 5 as the 2'-O-DMT regioisomer for simplicity), and the remaining hydroxyl was then acetylated to give **3** (also a mixture of regioisomers). Cleavage of the TBDMS group with TBAF followed by chromatographic isolation provided **4**, which was a 2:1 mixture of 2' and 3'-O-DMT regioisomers, according to proton NMR. Acetate occupies the remaining free hydroxyl in either case. Compound **4** was converted to **5** by addition of 2-cyanoethyl tetraisopropylphosphorodiamidite and catalytic tetrazole. Compound **5** was also a mixture, now four compounds due to the diastereoisomerism of the phosphoramidite moiety effecting each regioisomer. Compound **5** was added to the hydroxyl supports by conventional activation with S-ethyl tetrazole, followed by oxidation and capping. Two novel hydroxyl bearing polystyrene supports derived from recently introduced methodology[7] were used to immobilize the phosphate derived from **5**: PEG-polystyrene[10] and macroreticular polystyrene[11]. The merits of each support material is discussed below.

Synthesis of deoxyoligonucleotides on the universal supports was accomplished by the amidite method[8]. For the PEG-polystyrene-based support, about 50% longer DMT removal times were required for good results. As well, the material swelled differentially in

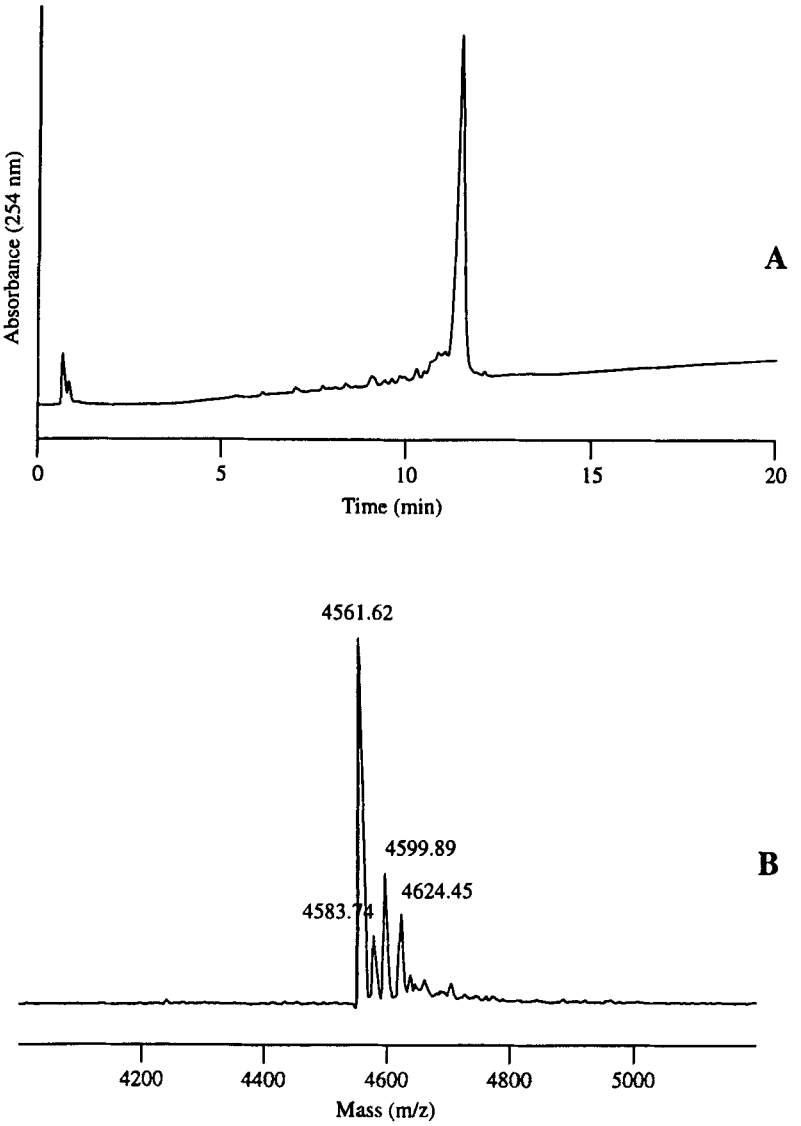

Figure 4. **A** = Anion Exchange HPLC of 15 mer 5'-ATACTAATCATGAGC-3' made on Phosphate linked universal support. **B** = MALDI mass spectrum of 15 mer, Mass Calc'd for 5'-ATACTAATCATGAGC-3' = 4562 a.m.u. Minor peaks are sodium and potassium adducts.

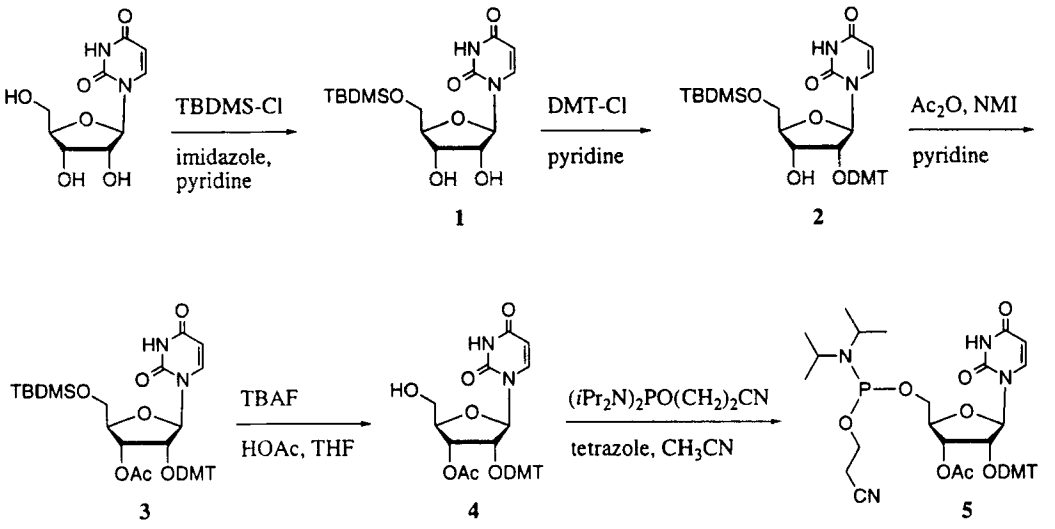

Figure 5. Synthesis of Universal linker amidite **5**. Intermediates **2** - **5** are a mixture of 2' and 3'-O-DMT regioisomers, here depicted as the 2'-O-DMT regioisomer for simplicity. (See Text).

different solvents, so that synthesis columns had to be packed with ample head space to prevent clogging during the synthesis. After automated DNA synthesis, deprotection of the product oligonucleotide and cleavage from the support was accomplished by heating the resin in concentrated ammonia at 60 °C for 3 h. Only a small amount of additional material was obtained on longer heating. Table 1 shows the results for four demonstration sequences 15 to 35 bases long made with the PEG-Polystyrene universal support, as well as control sequences made with CPG containing predetermined 3'-residues. Yields with the support were consistently 50 - 90% of that observed with the controls. Figure 6 compares the AX HPLC chromatograms of the 15- and 20-mer crude DNA products made on the new support with the same sequences made on standard prederivatized hemisuccinate CPG supports. A MALDI mass spectrum of an 18 mer 5'-TGTAAAACGA-CGGCCAGT-3' showed a clean product without significant higher molecular weight contaminants, and a CZE showed very little slower eluting contaminants, indicating support backbone integrity under the cleavage conditions (data not shown).

The macroreticular polystyrene based material was somewhat better behaved in solid phase DNA synthesis; no differential swelling was observed, and standard DMT removal times could be used during automated synthesis. Ammonia deprotection took longer, 8 hrs at 60 °C resulted in most of the DNA being cleaved; longer times only gave slightly more

TABLE 1. Comparison of synthesis yields for test sequences on PEG PS Universal Support and 1000 Å CPG controls on a 200 nmole scale.

Sequence	Length	Synthesis Yield (o.d.s) Universal Support	Standard CPG
5'-CGATCTGAATAGCTT-3'	15	13.7	14.5
5'-TCCAGCTCATCGAGGTCATA-3'	20	10.4	19.2
5'-CAGCGGAACCGCTCATTGCCAATGG-3'	25	9.4	18.4
5'-ACGCTGGACCCCGTCGTCTGCGCCGGCGACTTCGC-3'	35	38	42

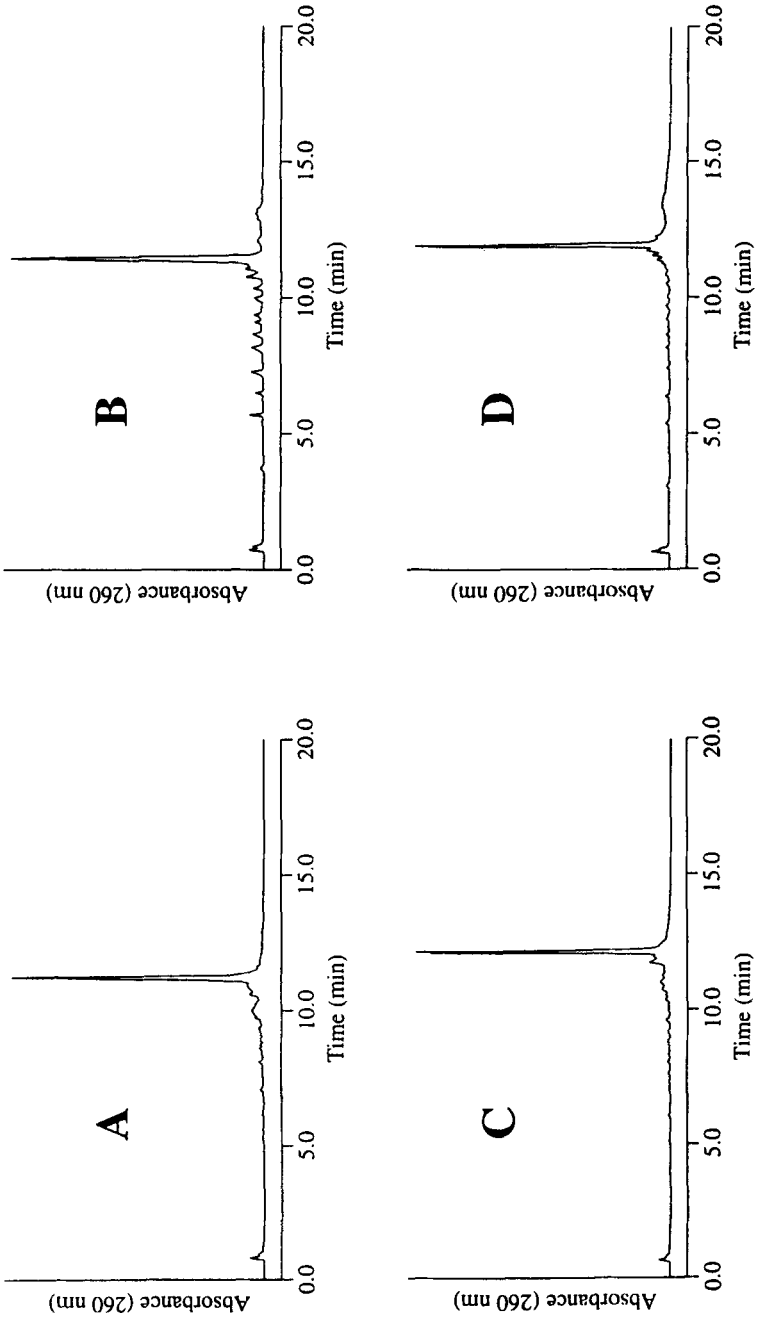

Figure 6. Anion Exchange HPLC of 15 and 20 mers on PEG-PS universal support and CPG controls. **A** = 5'-CGATCTGAATAGCTT-3' on PEG-PS universal support; **B** = same sequence on T 1000 Å CPG. **C** = 5'-TCCAGCTCATCGAGGTCATA-3'on PEG-PS universal support; **D** = same sequence on A 1000 Å CPG.

TABLE 2. Synthesis yields for test sequences with Macroreticular PS Universal support at a 200 nmole scale

Sequence	Length	Synthesis Yield (o.d.s)
5'-ATACTTATCATGAGC-3'	15	14.8
5'-GATGAGTTCGTGTCCGTACAACTGT-3'	25	18.7
5'-ACCCTGGACCCCGCTGTCTGCGCCGGCGACTTCGC-3'	35	21.5
5'-GCTGACAACGCTGCTGCTGCTGCTGCTACTGCTG-CTGAACGCATCCG-3'	50	33.5

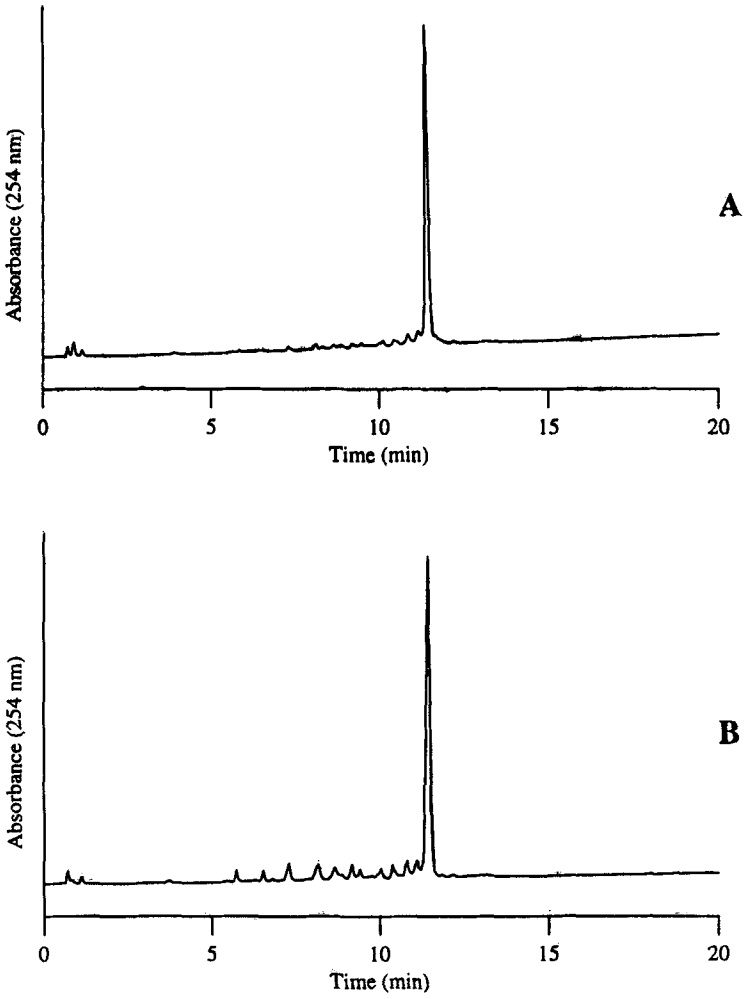

Figure 7. **A** = Anion Exchange HPLC of 15 mer 5'-CGATCTGAATAGCTT-3' on Macroreticular PS universal support; **B** = same sequence on T 1000 Å CPG.

material. One explanation for the faster cleavage times with the PEG-polystyrene variant is that the swellable nature of the material makes active sites on it more accessible to solvents, with reaction kinetics more closely resembling solution phase than the rigid macroreticular polystyrene counterpart and hence faster cleavage. Table 2 shows the sequences and yields for a series of oligonucleotides 15 - 50 bases long made with this material. AX HPLC of the 15-mer made on this material and a 1000 Å CPG control showed a favorable comparison. See Figure 7. A 20% PAGE of 1 - 2 o.d.s of the 15, 25, 35 and 50 mers

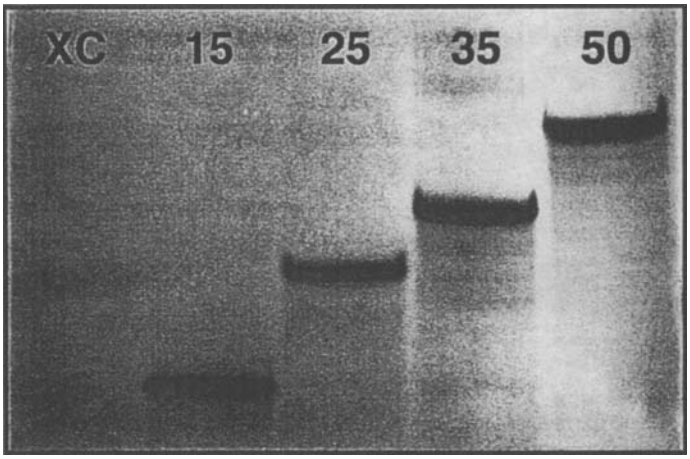

Figure 8. 20% PAGE of 15, 25, 35 and 50 mers made on Macroreticular PS universal support. Sequences given in Table 2. XC = Xylene Cyanol.

made on the macroreticular polystyrene showed very clean products in this analysis format. See Figure 8.

CONCLUSION

Our new formulation of the vicinal diol universal support offers several advantages besides the added convenience of using a single support material for every sequence. 3'-Linker cleavage times are shorter than with other vicinal diol universal supports at the same temperature and conditions (16 h)[5]. Most importantly, DNA products made with these new materials are free of 3'-modified contaminants, which would be deleterious to the performance of DNA fragments in PCR or ligation studies. Such contaminants are frequently hard to visualize by low resolution analysis techniques such as gel electrophoresis or reverse phase HPLC, particularly with longer fragments. Additionally, no non-volatile salts or other reagents besides aqueous ammonia are needed for deprotection and cleavage, so the crude DNA can be used for a number of applications without the necessity of post-synthetic desalting steps. These factors should enable higher throughput and shorter turnaround times when these new materials are used in conjunction with array based automated synthesizers or high throughput screening devices.

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