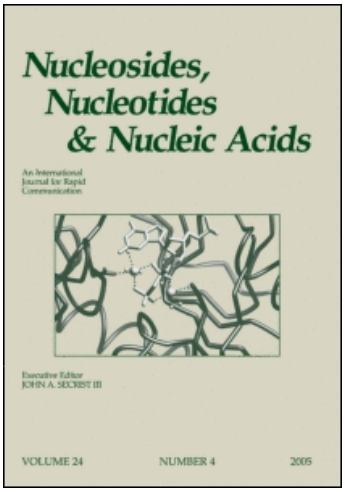

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A UNIVERSAL GLASS SUPPORT FOR OLIGONUCLEOTIDE SYNTHESIS

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Abstract: A single type of controlled pore glass derivatized with 3-anisoyl-2'(3')-O-benzoyluridine 5'-O-succinyl residues can be used as the support in solid phase syntheses of either oligoribo- or oligodeoxyribonucleotides.

Current practice in solid phase synthesis of oligodeoxyribonucleotides requires four separate supports, each prederivatized with the appropriate 3' terminal nucleoside of a prospective chain.[1] This strategy has been carried over into the less well-developed field of oligoribonucleotide synthesis,[2,3] so that a total of eight different kinds of support are now in use for the construction of all possible oligonucleotides. The present work deals with the concept of synthesizing both oligoribo- and oligodeoxyribonucleotide chains on a single glass support bearing 2'(3')-O-benzoyluridine 5'-O-succinyl ligands.[4] With this material, addition of the first 3'-nucleotide monomer in the initial chain-building cycle creates a 3'-3'(2') internucleotide bond to the free hydroxyl of the support-bound uridine. After release and deprotection of the finished oligomer, the uridine 3'(2')-phosphate that remains at its 3' end can be removed as the 2',3'-cyclic phosphate, by promoting attack of the neighboring 2'(3')-hydroxyl group on the internucleotide linkage.[5,6] The overall scheme is depicted in Figure 1 for one of the two isomers of benzoyluridine attached to the glass.

A new, one-flask method has been developed for preparing the support. First, the 5' hydroxyl of 2',3'-O-methoxybenzylideneuridine is acylated with succinic anhydride in the presence of dimethylaminopyridine. The free carboxyls of the resulting succinate half-esters are activated with p-toluenesulfonyl nitrotriazole and allowed to react with

controlled pore glass bearing long-chain alkylamine groups; in this way, stable amide linkages are formed between the nucleoside 5' succinyl residues and the support. The material is then treated with anisoyl chloride; this procedure blocks the N-3 positions of the uracil moieties[5,7] and prevents unwanted substitution at any other residual reactive centers on the glass during the addition of nucleotide monomers.[8] The final step consists of treating the derivatized glass with mild acid to open the 2',3'-O-methoxybenzylidene functions, generating 2'(3')-O-benzoates and concomitantly exposing the uridine 3'(2') hydroxyls as attachment points for subsequent oligonucleotide syntheses.

The effectiveness of the uridine linker in oligoribonucleotide synthesis was tested by constructing a set of four model tetramers containing different terminal nucleosides. A-A-A-U, A-A-A-C, A-A-A-A, and A-A-A-G were built up on the glass by the phosphite triester method using, as monomer units, the 3'-(2-cyanoethyl N,N-diisopropyl phosphoramidites)[9] of suitably blocked 2'-O-(o-nitrobenzyl)ribonucleosides.[10,11] After a typical synthesis (A-A-A-U), two small samples of the glass were treated separately with pyridine-15 M NH_4OH (1:2, v/v), one at 25° for 3 days, the other in a pressure tube at 50° for 16 h; both sets of conditions release the oligomers and also remove the cyanoethyl groups from the internucleotide linkages and the N-acyl

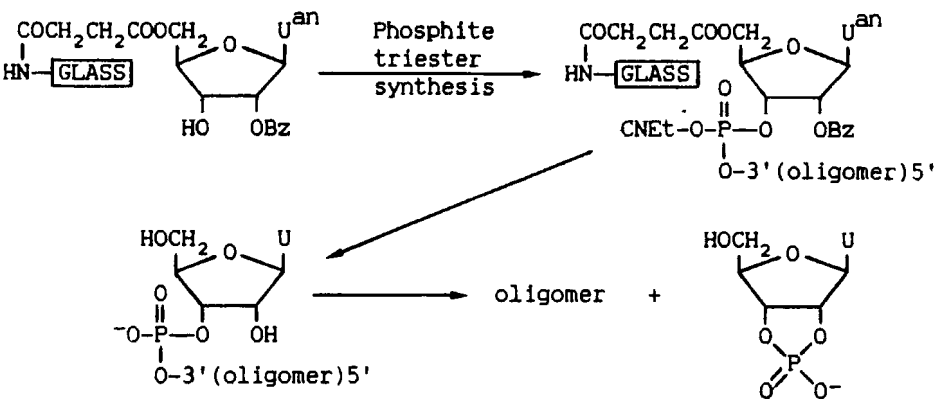

FIGURE 1

protection from the bases. The two mixtures were then filtered, evaporated, and treated with 80% acetic acid to take off 5'-dimethoxytrityl groups. Analysis by ion-exchange HPLC showed the presence of the nitrobenzyl-protected oligomers A(NBzl)-A(NBzl)-A(NBzl)-U(NBzl)3'-2'(3')U and A(NBzl)-A(NBzl)-A(NBzl)-U(NBzl) in the room temperature sample. Only A(NBzl)-A(NBzl)-A(NBzl)-U(NBzl) was found in the sample heated at 50°; its identity was confirmed by deprotection to A-A-A-U. Similar results were obtained in analogous experiments with the other three oligoribonucleotides, indicating that cleavage of the uridylyl residue from any of its four potential neighbors can be effected by heating in ammonia under the above conditions. Release of the oligomers from the glass, deprotection of their phosphates and bases, and removal of their terminal uridylyls were therefore carried out rapidly in a single step; subsequent deblocking of the 2' and 5' hydroxyls yielded the desired oligoribonucleotides in fully deprotected form.

In the case of oligodeoxyribonucleotides, uridylyl functions at their 3' ends have proved to be significantly more resistant to alkaline hydrolysis than those of their ribo- counterparts, presumably because the lack of a substituted oxygen atom at C2' makes the deoxyribonucleoside terminus a poorer leaving group. However, the cleavage can be carried out rapidly and efficiently, either by catalysis with lead (II) ion at neutral pH,[5,6] or by the action of alkali hydroxides. We have avoided exposing oligonucleotides to strong alkali because such treatment causes deamination of cytosine moieties. For instance, heating pdC with 0.3 M aqueous NaOH for 72 h at 45° results in 20% conversion to pdU, and with d(C-C-C-C) under the same conditions there is 55% loss of the tetramer. Recently, Cosstick and Eckstein,[12] in adapting our strategy to the solid support synthesis of oligodeoxyribonucleotides with alternating phosphorothioate linkages, employed 0.1 M sodium hydroxide in dioxane-water (1:4, v/v) at 50° for 15 h to effect removal of the terminal uridine phosphates and were unable to detect deoxyuridine-containing components in enzyme digests of their oligomer products. Our experiments indicate that, under the alkaline conditions used by these authors, 1-2% of pdC is converted into pdU. It is clear that even these low levels of deamination, randomly occurring at cytosine in long molecules containing a number of such residues, would

significantly diminish the proportion of molecules in the population that possess the correct sequence.

We found that hot ammonia cleaved the uridylyl termini from a series of synthetic oligodeoxyribonucleotides without causing detectable deamination of dC, but the treatment needed was more vigorous than that used for the equivalent oligoribonucleotides. For example, 15 M ammonia at 60° removes the terminus in 16 h for oligomers with dG or dA next to the uridine residue, or in 24 h for those with dT adjacent to U. Molecules having a penultimate dC residue, such as d(C-C-C-C)3'-2'(3')U and d(G-C-G-A-G-T-A-T-G-A-C-G-C)3'-2'(3')U required treatment at 65° for 24 h to detach the terminal ribonucleotide. Extended heating in concentrated ammonia is commonly recommended for deblocking of synthetic oligodeoxyribonucleotides, so, as with oligoribonucleotides, alkaline deprotection of the molecules and removal of the terminal uridylyl residues can be carried out simultaneously. The methodology reported in this communication therefore constitutes a simple system for obtaining both oligoribo- and oligodeoxyribonucleotides using a single, easily prepared glass support.

EXPERIMENTAL SECTION

Materials and Methods

2',3'-O-Methoxybenzylideneuridine was prepared by the published method[13] and purified by column chromatography on Merck 60 silica gel, using chloroform-methanol-pyridine (96.5 : 3.0 : 0.5 v/v) as the eluting solvent. Long-chain alkylamine controlled pore glass (CPG/LCAA; pore diam., 50 nm; particle size, 125-177 μm; available amines, 0.038 mmol/g) was purchased from Pierce Chemical Co. The following reagents were obtained from the suppliers indicated: anisoyl chloride, purum grade, and succinic anhydride and triethylamine, both puriss. grade (Fluka); N,N-diisopropylethylamine, 1-(p-toluenesulfonyl)-3-nitro-1,2,4-triazole and 4-dimethylaminopyridine (Aldrich). 2-Cyanoethyl N,N-diisopropyl phosphoramidochloridite and the 3'-(2-cyanoethyl N,N-diisopropyl phosphoramidites) of the nucleosides [$(MeO)_2$Tr]dbzA, [$(MeO)_2$Tr]dbzC, [$(MeO)_2$Tr]dibG, and [$(MeO)_2$Tr]dT were products of American Bionetics. Pressure tubes (No. 8648-03) were manufactured by Ace Glass Inc.

Pyridine, triethylamine and N,N-diisopropylethylamine were dried by storage over calcium hydride.

HPLC of oligonucleotides was carried out with a column (1 X 25 cm) of cross-linked polyethyleneimine on microparticulate silica.[14] The solvent system consisted of 120 ml of aqueous 0.05 M KH_2PO_4 containing 30% (v/v) MeOH and a linear gradient of 0-0.4 M $(NH_4)_2SO_4$ at pH 6.0, except for chromatography of the tridecamer where the gradient was 0-0.75 M; flow rate was 1.5 ml/min. Nitrobenzyl-protected oligoribonucleotides were chromatographed at 30° on a 0.46 X 15 cm column of the same ion-exchange material, using 40 ml of aqueous 0.05 M KH_2PO_4 containing 30% (v/v) MeOH and a linear gradient of 0-0.1 M $(NH_4)_2SO_4$ at pH 6.0 with a flow rate of 0.5 ml/min.

Routine base composition analyses of oligonucleotides were carried out using spleen phosphodiesterase as previously described.[15] However, in order to facilitate identification of their 3' termini, the oligomers in the present study were also degraded using snake venom phosphodiesterase. In each case, a sample of oligomer (1.3 A_{260} units) in 1.5 ml of 0.01 M Tris.HCl-0.001 M $MgCl_2$ (pH 8.7 at 20°) was treated with venom phosphodiesterase (0.3 unit) at 25°. The mixture was monitored spectrophotometrically at 260 nm until its absorbance stopped increasing (ca. 2 h). Each oligodeoxyribonucleotide digest was then chromatographed on a column (0.4 X 20 cm) of Dowex 1 ion-exchange resin (AG1-X2, -400 mesh, Bio-Rad Laboratories) using solvent A: 200 ml of 10% EtOH (v/v) containing a linear gradient of 0.05 M NaCl-0.005 M NH_4OH to 0.5 M NaCl-0.05 M NH_4OH at 18 ml/hr; for digests of oligoribonucleotides the eluting solvent was B: 200 ml of 20% EtOH (v/v) containing a linear gradient of 0.05 M NaCl-0.01 M NH_4OH to 0.5 M NaCl-0.1 M NH_4OH.

Whenever possible, reactions involving nitrobenzyl-protected nucleosides and nucleotides were kept under nitrogen, and in yellow light or in the dark. These precautions minimize the slow loss of o-nitrobenzyl groups caused by ambient long-wave ultraviolet light and also help to prevent the photo-oxidation of nitrobenzyl to nitrobenzoyl that can occur in solutions at high pH.[11]

Preparation of the Support

Our original preparation of the support[5] allowed us the option of using any one of a number of acyl groups to protect the 2'(3') hydroxyls of the uridine ligands. Since the benzoyl group has proven to be completely satisfactory in that role, the following simplified procedure for obtaining the derivatized glass has been adopted: 2',3'-O-methoxy-

benzylideneuridine (362 mg, 1 mmol) was rendered anhydrous by addition and evaporation of dry pyridine (3 X 3 ml). The residue was dissolved in dry pyridine (3 ml), and succinic anhydride (75 mg, 0.75 mmol) and 4-dimethylaminopyridine (92 mg, 0.75 mmol) were added. The reaction mixture was allowed to stand overnight, then treated with anhydrous triethylamine (0.21 ml, 1.5 mmol) and p-toluenesulfonyl nitrotriazole (400 mg, 1.5 mmol). After 10 min, 1 g of controlled pore glass was added and the mixture was set aside for 48 h. The derivatized glass was then washed in situ with small portions of dry pyridine (60 ml total), methanol (60 ml), and chloroform (60 ml). Residual chloroform was removed by air drying followed by vacuum desiccation for 2 h. The uracil N-3 positions were protected by treating the glass with anisoyl chloride (0.2 ml) and anhydrous triethylamine (0.25 ml) in dry pyridine (4 ml) for 24 h. The dark mixture was filtered, and the glass was washed with dry pyridine (60 ml), pyridine-water (9:1 v/v, 60 ml), methanol (60 ml), and chloroform (60 ml), then dried. In preparation for oligonucleotide synthesis, samples of the support were treated with 80% acetic acid (1 ml per 100 mg of glass) for 6 h at 25°, washed with pyridine, methanol, and chloroform, and stored in a vacuum desiccator over P_2O_5.

A small quantity (30 mg) of the derivatized glass was allowed to stand in 1-propanol-conc. NH_4OH (1:4 v/v, 5 ml) for 16 h, then the mixture was filtered. The filtrate was concentrated and chromatographed on Whatman 3MM paper in 2-propanol-conc. HCl-H_2O (170:41:39 v/v). The only detectable nucleoside was uridine and the amount obtained indicated a loading on the glass of 0.038 mmol/g.

Ribonucleotide Synthetic Units

For oligoribonucleotide syntheses, nucleoside precursors were the 5'-O-dimethoxytrityl 2'-O-(o-nitrobenzyl) derivatives of 6-N-benzoyl-adenosine, 3-anisoyluridine, 4-N-benzoylcytidine, and 2-N-isobutyryl-guanosine. We have briefly described the preparation of the adenosine monomer and its use in the high-yield solution phase construction of oligoribonucleotides;[11] a detailed report of the synthesis of all four nucleoside units will be published shortly.

A 0.3 mmol sample of each of the above ribonucleosides was allowed to react with 2-cyanoethyl N,N-diisopropyl phosphoramidochloridite in the presence of N,N-diisopropylethylamine, using the method developed by

Sinha et al.[9] for phosphorylation of deoxyribonucleosides and the following modified isolation procedure. The crude ribonucleoside 3'-(2-cyanoethyl N,N-diisopropyl phosphoramidite) obtained after concentration of each filtered reaction mixture was dissolved in nitrogen-saturated ethyl acetate (20 ml) and washed at 0° with 2 X 15 ml portions of 10% aqueous Na_2CO_3 (for rA and rG) or $NaHCO_3$ (for rC and U). The dried (Na_2SO_4) ethyl acetate layer was then evaporated to a glass, which was taken up in CH_2Cl_2 (1 ml) and precipitated with hexane (25 ml). The resulting solid was triturated three times with hexane and the supernatant solutions were decanted. The entire precipitation-trituration procedure was then repeated. Finally, the residue was dissolved in CH_2Cl_2 (3 ml) and evaporated in vacuo to a foam. Yields were in the range 80 to 90% and the material was used for oligomer synthesis without further purification.

Solid-Phase Oligonucleotide Synthesis

Oligonucleotides were constructed manually on 50 mg samples of the glass held in a syringe, with some modification of the standard methodology.[1] For example, detritylation was carried out with 5% dichloroacetic acid in CH_2Cl_2 and the capping procedure was omitted. For each condensation, the phosphoramidite and tetrazole concentrations were 0.1 M and 0.5 M, respectively, in acetonitrile; the duration was 10 min for ribonucleotides and 7 min for deoxyribonucleotides. The coupling yield of the first monomer was quantitative in the deoxyribo-series (as determined by trityl assay), and subsequent average addition yields were 96%. For oligoribonucleotides, average yields, including those of the first additions, were 92%.[16] Using the above methods, oligomers with the following sequences were synthesized: r(A-A-A-U), r(A-A-A-A), r(A-A-A-C), r(A-A-A-G), d(T-T-T-T), d(A-A-A-A), d(C-C-C-C), d(T-T-T-G), and d(G-C-G-A-G-T-A-T-G-A-C-G-C).

Release and Deprotection of Oligonucleotides

A 3 mg sample of glass bearing one of the synthesized oligoribo-nucleotides (for example, A-A-A-U) was treated with 3 ml of pyridine-15 M NH_4OH (1:2, v/v) in a closed flask at room temperature for 3 days. A second sample of the same glass was placed in a 25 ml pressure tube, treated with 24 ml of the pyridine-ammonia mixture, and heated at 50° overnight. Each of the suspensions was then filtered; the filtrates were evaporated to dryness and treated with acetic acid-water (4:1 v/v,

3 ml) for 30 min at 25°. The acid was removed by coevaporation with water, the residues were dissolved in EtOH-H_2O (2:3, v/v), and the two solutions were examined by HPLC. The partially-protected oligoribonucleotide produced in the mixture heated at 50° was deprotected at pH 3.5 with long-wave ultraviolet light as previously described[11], isolated by HPLC, and characterized by digestion with snake venom phosphodiesterase. The products pU, pA, and A were formed in the ratio 0.94:2.0:1.0.

Analogous sets of release and deprotection experiments were performed with the other model oligoribonucleotides A-A-A-A, A-A-A-C, and A-A-A-G.

For the oligodeoxyribonucleotides synthesized on the support, 3 mg samples of glass were heated in 15 M NH_4OH (24 ml) for 16 h and 24 h at 50°, 60°, and 65°; the resulting deprotected oligomers were compared with the corresponding products generated by room temperature treatment.

Prolonged heating in ammonia causes the glass to dissolve; upon evaporation, the resulting mixture of silicates and amines forms a poorly soluble residue that can trap significant amounts of deprotected oligonucleotides. The oligomers can be quantitatively recovered by stirring the precipitate for 2 h with EtOH-H_2O (2:3 v/v) containing a few drops of conc. ammonia. Nevertheless, the presence of dissolved silica leads to difficulties in isolating oligomers when larger samples of glass are processed. To avoid these problems, it is advisable to release the oligonucleotides into solution by overnight treatment of the support, at room temperature, with the appropriate ammonia mixture. The glass is then removed before carrying out the heating step in fresh ammonia. However, for large scale preparations, longer heating times may be required.

Deamination of Cytosine Moieties in Alkali

Deoxycytidine 5'-phosphate (3 A_{260} units) was heated at 45° in 0.3 M aqueous NaOH (1.5 ml) for 72 h. One third of the solution was neutralized with acetic acid and chromatographed on the Dowex 1 nucleotide analysis column, using solvent B. The extent of conversion of pdC (retention vol.: 47 ml) to pdU (retention vol.: 84 ml) was determined spectrophotometrically. A second sample of pdC was treated with 0.1 M NaOH in dioxane-water (1:4, v/v) at 50° for 72 h and analyzed in the same manner.

The oligonucleotide d(C-C-C-C) was also treated with 0.3 M NaOH at 45° for 72 h; in this case, the products were analyzed using solvent C: 200 ml of 20% EtOH (v/v) containing a linear gradient of 0.2 M NaCl-0.04 M NH_4OH to 0.8 M NaCl-0.16 M NH_4OH. Loss of the oligomer (retention vol.: 45 ml) was measured by comparing the area of the d(C-C-C-C) peak with that of an untreated control. Two other peaks, representing material containing one or more dU residues, emerged at 68 and 91 ml.

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