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RAPID SYNTHESIS OF OLIGORIBONUCLEOTIDES USING 2'-O-(*o*-NITROBENZYLOXYMETHYL)-PROTECTED MONOMERS

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Abstract
A new, easily introduced protecting group for ribonucleoside 2'-hydroxyls, *o*-nitrobenzyloxymethyl, permits fast, effective solid phase synthesis of RNA.

Automated chemical synthesis of DNA is now a well-established and fruitful technique. In principle, the chemistry and general methodology evolved for construction of oligodeoxyribonucleotides should be applicable to oligoribonucleotides as well, but the ability to make RNA on a routine basis has been hampered by lack of a wholly satisfactory protection mode for the 2'-hydroxyl functions of ribonucleoside synthetic units. Even the commercially available system, that which uses 2'-*O*-(*t*-butyldimethylsilyl) ribonucleoside phosphoramidite monomers[1,2], still requires a lengthy coupling time (> 10 min) in each nucleotide addition cycle[3,4] compared with synthesis of an equivalent sequence of DNA. Our own past syntheses of RNA[5,6] have employed *o*-nitrobenzyl[7,8] for 2'-hydroxyl protection; however, phosphoramidite monomers derived from 2'-*O*-(*o*-nitrobenzyl) ribonucleosides likewise need extended coupling times[9], evidently due to steric hindrance by the bulky 2'-protecting group. Such steric crowding in the vicinity of the phosphoramidite function might be relieved by incorporating a flexible arm into the structure at the 2'-hydroxyl. Accordingly, we have developed a novel 2'-protecting group, *o*-nitrobenzyloxymethyl[10], that is designed to be compatible with currently employed DNA synthesis protocols[11] and to be quantitatively removable by UV photolysis[12].

Regioselective introduction of *o*-nitrobenzyloxymethyl onto the 2'(3')-hydroxyl of uridine was accomplished by using a new alkylating agent, *o*-nitrobenzyl chloromethyl ether[13]. This material (16 mmol in 20 mL of dry DMF) was added dropwise over 5 min to a stirred suspension of 2',3'-*O*-(dibutylstannylene)uridine[14] (5.1 g, 10.7 mmol) and a catalyst[15], tetra-*n*-butylammonium bromide (1.725 g, 5.3 mmol), in anhydrous DMF (40 mL). Stirring was continued for 2 h, then pyridine (5 mL) and water (2 mL) were added. After a further 20 min, the solution was concentrated under high vacuum to remove most of the DMF. The syrupy residue was dissolved in the minimum volume of pyridine : water (2:1, v/v) and mixed with silica gel (Merck 60, 10 g). The resulting slurry was dried, at first in a fume hood, then under vacuum desiccation. The dry powder thus obtained was layered on a 2.5 X 20 cm column of silica gel packed in chloroform, and the product was eluted with chloroform : methanol (95:5, v/v); the yield of mixed 2'- and 3'-*O*-(*o*-nitrobenzyloxymethyl)uridines was 4.24 g. The crude mixture of isomers was dried by coevaporation with pyridine, then treated with dimethoxytrityl chloride (3.86 g, 1.1 equiv.) and triethylamine (1.60 mL, 1.1 equiv.) in anhydrous pyridine (114 mL). After 3 h at 25°, water (5 mL) was added and the solution was concentrated to an oil, which was dissolved in ethyl acetate (150 mL). The organic layer was extracted with 1M $NaHCO_3$ (3 X 100 mL) and 10% NaCl (3 X 100 mL), dried (Na_2SO_4), and evaporated to a foam. This material was chromatographed on a 2.5 X 30 cm column of silica gel with chloroform : methanol (99:1, v/v)

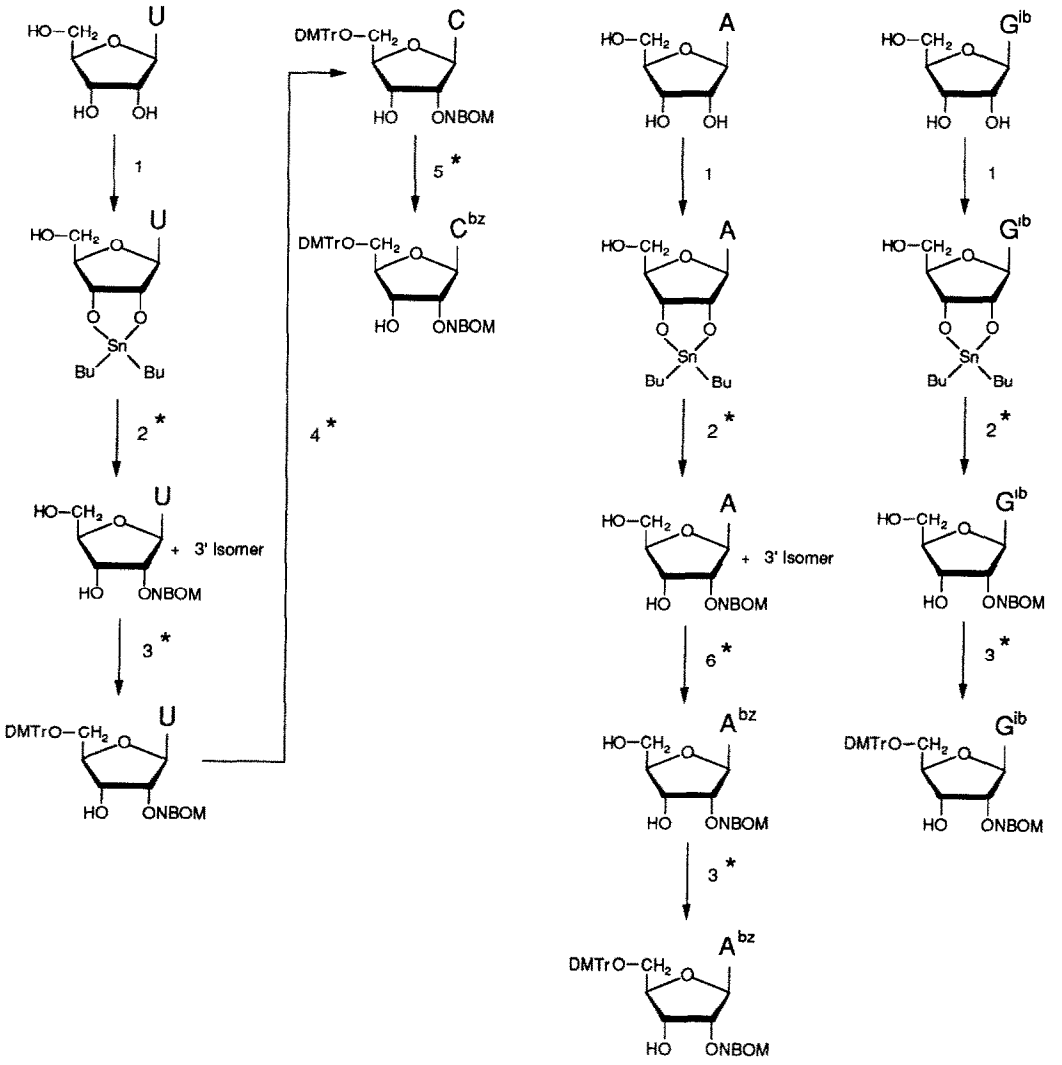

Figure 1. Synthetic scheme for the preparation of *o*-nitrobenzyloxymethyl (NBOM) protected ribonucleosides. The reagents represented by numbers are (1) dibutyltin oxide, (2) *o*-nitrobenzyl chloromethyl ether, (3) 4,4'-dimethoxytrityl chloride, (4) see text, (5) pentafluorophenyl benzoate, (6) benzoyl chloride and trimethylsilyl chloride. The symbol (*) denotes purification of product by silica gel chromatography.

containing 0.25% pyridine, to give the 2'-*O*-(*o*-nitrobenzyloxymethyl) derivative contaminated with a small amount of the slower-running 3'-isomer. Final purification was carried out by high-resolution centrifugal chromatography on silica gel (Chromatotron, Harrison Research, Palo Alto, CA) using the same solvent; the yield of 5'-*O*-dimethoxytrityl-2'-*O*-(*o*-nitrobenzyloxymethyl)uridine[16] was 2.95 g (39% from uridine).

The method outlined above was then applied, with some modifications, to other ribonucleosides[17] (Figure 1). For example, alkylation of the dibutylstannylene derivative of adenosine gave a crude mixture of 2'- and 3'-*O*-(*o*-nitrobenzyloxymethyl)adenosines, from which the pure 2'-isomer was isolated in 24% yield by silica gel column chromatography. This material was benzoylated using the transient protection method of Ti *et al.*[18], then tritylated to produce 5'-*O*-dimethoxytrityl-2'-*O*-(*o*-nitrobenzyloxymethyl)-6-*N*-benzoyl-adenosine.

The base-protected nucleoside 2-*N*-isobutyrylguanosine[19], as its dibutylstannylene derivative, also gave a mixture of 2'- and 3'-isomers upon alkylation with the chloromethyl ether. However, in this case alone, omission of the tetrabutylammonium bromide catalyst resulted in predominant formation of the 2'-*O*-(*o*-nitro-benzyloxymethyl) nucleoside. The yield, after purification on silica gel, was 25%; subsequent tritylation gave 5'-*O*-dimethoxytrityl-2'-*O*-(*o*-nitrobenzyloxymethyl)-2-*N*-isobutyrylguanosine.

As a simple way of obtaining the cytidine monomer without having to separate 2'- and 3'-isomers, 5'-*O*-dimethoxytrityl-2'-*O*-(*o*-nitrobenzyloxymethyl)uridine was acetylated to provide temporary protection for

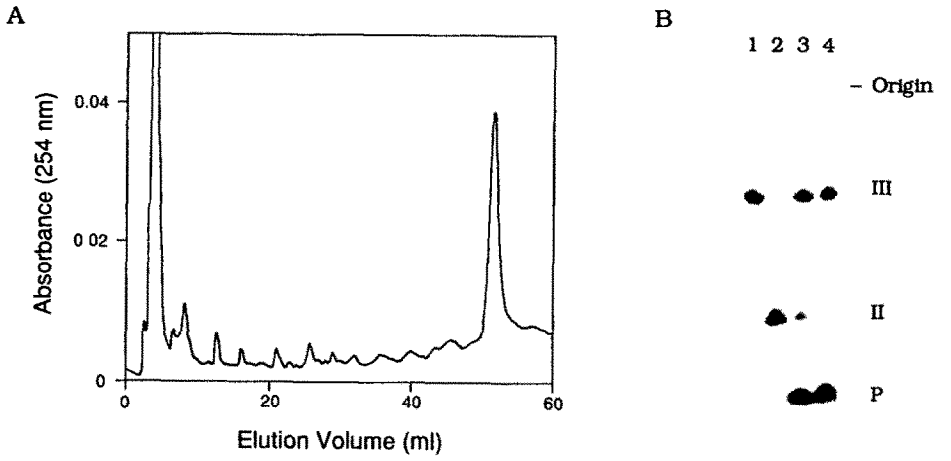

Figure 2. (A) Anion exchange HPLC profile of the crude deprotection mixture from the synthesis of the 16-mer I. Column: crosslinked polyethyleneimine-silica[20] (0.4 X 25 cm). Solvent: 150 mL of aqueous 0.05 M KH_2PO_4 containing 30% (v/v) MeOH and a linear gradient of 0 - 0.5 M $(NH_4)_2SO_4$ at pH 6; flow rate: 1 mL/min. (B) Autoradiogram from gel (20% acrylamide, 7 M urea) electrophoretic analysis of 5'-^{32}P labeled oligomers II and III, and a demonstration of their ribozyme function. Lanes 1 and 2 show electrophoretically purified III and HPLC purified II, respectively, after incubation for 2 h at 50°C in the presence of 50 mM Tris-HCl (pH 7.6 at 23°C) – 0.5 mM spermine – 20 mM $MgCl_2$. Lanes 3 and 4 show the labeled hexamer (P) produced by a mixture of II and III incubated under the same conditions for 2 and 6 h.

the 3'-hydroxyl, then converted into 5'-*O*-dimethoxytrityl-2'-*O*-(*o*-nitrobenzyloxymethyl)cytidine in 71% yield using the base-transformation procedure of Sung[21]. Selective benzoylation of the 4-*N* amino group with pentafluorophenyl benzoate[22] led to the desired protected nucleoside, 5'-*O*-dimethoxytrityl-2'-*O*-(*o*-nitrobenzyloxymethyl)-4-*N*-benzoylcytidine.

The 3'-(2-cyanoethyl *N,N*-diisopropylphosphoramidites) of the four 5'-*O*-dimethoxytrityl-protected ribonucleosides were prepared by the method of Sinha *et al.*[23], and purified by flash column chromatography using ethyl acetate : hexane (80:20, v/v) as solvent. The amidites were dissolved in acetonitrile at 0.15 M concentration and used in a Gene Assembler Plus (Pharmacia) to synthesize RNA[24]. The manufacturer's standard software protocols for DNA synthesis, which prescribe a two minute condensation time, were adopted without modification. A variety of RNA molecules have been made under these conditions, including 3 components of ribozyme structures: GAAAUAGCAAUAUUUA (I), GCUGUCACCGCG (II), and CGCGGUCUGAUGAGUCCGUGAGGACGAAACAGC (III). The average condensation yield in each synthesis was greater than 98%. Machine-synthesized oligoribonucleotides were released from their support cassettes by extruding the resin into pyridine : conc. NH_4OH (1:4, v/v) and heating the resulting suspension in a pressure tube at 50°C for 24 h, thereby removing all protecting groups except the nitrobenzyloxymethyls. These were subsequently taken off by exposure of solutions of the 2'-protected oligonucleotides (*ca.* 1 AU_{260}/mL in 50% aqueous *t*-butanol, pH 3.7) to long-wave UV light for 4.5 h. These conditions, the low pH in particular[7], cause efficient removal of *o*-nitrobenzyl moieties. The 16-mer I (Figure 2A) and the 12-mer II were purified by HPLC; the 33-mer III was purified by gel electrophoresis in 20% polyacrylamide. Oligomer I exists in the form of a hairpin (ΔH = −40 kcal/mol, ΔS = −125 eu, Tm = 44.0°C, in 100 mM NaCl – 3.7 mM spermidine – 5 mM $MgCl_2$), and undergoes self-cleavage[25] between G1 and A2 in the presence of manganous ion. The sequence for this 16-mer was taken from the central region of a 31-mer recently shown by Dange *et al.*[26] to undergo Mn^{++}-dependent cleavage at the equivalent G–A position. The sequences of oligomers II and III were designed to correspond to the general pattern of the substrate and catalytic regions, respectively, of hammerhead ribozymes. Incubation of a mixture of II and III results in specific cleavage of II between C6 and A7 (Figure 2B).

The foregoing indicates that it is now possible, for the first time, to machine synthesize oligoribonucleotides effectively with the same speed as their deoxy counterparts.

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10. The parent group benzyloxymethyl, although widely used for hydroxyl protection[27], is unsuitable for nucleic acids because of the conditions necessary for its removal.
11. For example, the 2'-protecting group must be capable of surviving the repeated acid treatments that constitute the detritylation cycles in automated synthesis. The remarkable acid stability of formaldehyde acetals is evident from the kinetic data of Kreevoy and Taft, Jr.[28].
12. Cleavage of the *o*-nitrobenzyloxymethyl group is expected to occur in two stages: removal of the photolabile *o*-nitrobenzyl moiety on irradiation with UV[29], followed by rapid breakdown of the resulting formaldehyde hemiacetal in mild acid. There is enough ultraviolet emission from overhead fluorescent lighting to cause slow loss of *o*-nitrobenzyls, so it is advisable to replace the lamps with yellow-tinted equivalents when conducting the syntheses described here.
13. The alkylating agent *o*-nitrobenzyl chloromethyl ether was generated immediately before use by the method of Benneche *et al.*[30] for conversion of benzylic *O,S*-acetals into the corresponding chloromethyl benzyl ethers. The necessary *O,S*-acetal starting material in our case was *o*-nitrobenzyl methylthiomethyl ether, synthesized by a modification of the general procedure of Pojer and Angyal[31] as follows: a solution of *o*-nitrobenzyl alcohol (23 g, 150 mmol) in DMSO (150 mL) was treated with acetic anhydride (108 mL) and acetic acid (77 mL) and allowed to stand for 24 h at room temperature. It was then added dropwise to a stirred suspension of $NaHCO_3$ (330 g) in water (1500 mL). After 24 h stirring, the aqueous supernatant was decanted and the oily product was dissolved in ethyl acetate (200 mL), to which hexane (200 mL) was added. The organic phase was extracted with saturated aqueous $NaHCO_3$ (200 mL) and 10% NaCl (200 mL), dried (Na_2SO_4), and evaporated under reduced pressure. The residue was purified on a column (5 X 56 cm) of silica gel, using a stepwise gradient as follows: 500 mL hexane, then 1 L each of 5%, 10%, and 15% ether in hexane (v/v). Pure *o*-nitrobenzyl methylthiomethyl ether was obtained in 52% yield. Bp 119° C (0.017 kPa). ^{1}H NMR spectrum (200 MHz, $CDCl_3$) δ : 2.40 (-CH_3, s, 3H), 4.80 (-O-CH_2-S, s, 2H), 5.00 (Ar-CH_2, s, 2H), 7.46 (Ar C5-H, ddd, J = 1.4, 7.4, 8.0 Hz, 1H), 7.65 (Ar C4-H, ddd, J = 1.3, 7.4, 8.0 Hz, 1H), 7.78 (Ar C6-H, d, J = 7.6 Hz, 1H), 8.08 (Ar C3-H, dd, J = 1.2, 8.2, 1H). ^{13}C NMR spectrum (50 MHz, $CDCl_3$) δ : 13.22 (-CH_3), 65.65 (-O-CH_2-S-), 74.60 (Ar-CH_2-), 123.73 (Ar C-5), 127.24 (Ar C-4), 127.92 (Ar C-6) , 132.77 (Ar C-3), 133.53 (Ar C-1), 146.41 (Ar C-2). Anal. calcd for $C_9H_{11}NO_3S$: C, 50.69; H, 5.19; N, 6.57; S, 15.04. Found: C, 50.95; H, 5.39; N, 6.86; S, 14.78.
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16. ^{1}H NMR analyses have shown that the 2'-*O*- and 3'-*O*-(*o*-nitrobenzyloxymethyl)uridine derivatives are correctly identified. The isomers are distinguishable according to the rules established by Fromageot *et al.*[32] for protected ribonucleosides: the H1' signal of the 2'-isomer is more downfield than that of the 3'-

isomer. Also, the 2'-isomer has a smaller coupling constant for H1' and H2' than the 3'-isomer. For 5'-*O*-dimethoxytrityl-2'-*O*-(*o*-nitrobenzyloxymethyl)uridine, ^{1}H NMR (200 MHz, $CDCl_3$) δ:3.56 (H5', bs, 2H, becomes a multiplet when D_2O is added), 4.11 (H4', bs, 1H), 4.40 (H2', dd, $J_{H2'-H1'}$= 2.6 Hz, $J_{H2'-H3'}$= 2.8 Hz, 1H), 4.51 (H3', m, 1H), 6.06 (H1', d, $J_{H1'-H2'}$= 2.6 Hz, 1H), 5.21 (H5, d, J_{H5-H6}= 7.0 Hz, 1H), 7.96 (H6, d, J_{H6-H5}= 7.0 Hz, 1H), 10.0 (N3-H, s, 1H), 5.00-5.15 (-CH_2O-CH_2-phenyl, bs*, 4H), 7.40-8.41 (NBOM-phenyl, m, 4H), 3.82 (-OCH_3, s, 6H), 6.80-7.40 (DMTr- phenyl, m, 13H). For 5'-*O*-dimethoxytrityl-3'-*O*-(*o*-nitrobenzyloxymethyl)uridine, ^{1}H NMR (200 MHz, $CDCl_3$) δ: 3.50 (H5', m, 2H), 4.26 (H4', bs, 1H), 4.34 (H2', dd, $J_{H2'-H1'}$= 3.6 Hz, $J_{H2'-H3'}$= 3.8 Hz, 1H), 4.40 (H3', m, 1H), 5.94 (H1', d, $J_{H1'-H2'}$= 3.6 Hz, 1H), 5.37 (H5, d, J_{H5-H6}= 8.2 Hz , 1H), 7.86 (H6, d, J_{H6-H5}= 8.2 Hz, 1H), 10.25 (N3-H, s, 1H), 4.90-5.00 (-CH_2O-CH_2-phenyl, bs*, 4H), 7.40-8.20 (NBOM-phenyl, m, 4H), 3.78 (-OCH_3, s, 6H), 6.79-7.40 (DMTr- phenyl, m, 13H). * not resolved.

17. Full details concerning the synthesis, isolation, and characterization of all the ribonucleoside and ribonucleotide derivatives reported in this communication are included in a forthcoming manuscript (M.E. Schwartz and G. R. Gough, in preparation).
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24. In order to construct oligoribonucleotides on the DNA synthesizer, the manufacturer's pre-packed solid support cassettes were detritylated on the machine and derivatized by reaction with 2'(3')-*O*-dimethoxytrityl-3'(2')-*O*-benzoyluridine 5'-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite)[17]. Attachment of this "reversed" uridine linker gives us the capability of synthesizing either DNA or RNA on a single type of support. The concept of the universal support is described in ref. 9.
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