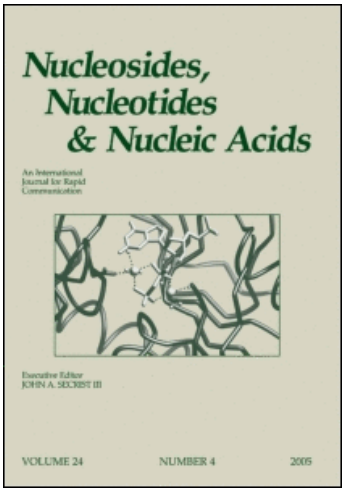

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

2'-O-alkyloligoribonucleotides Synthesis and Applications in Studying RNA Splicing

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To cite this Article Sproat, Brian S. , Iribarren, Adolfo , Beijer, Barbro , Pieles, Uwe and Lamond, Angus I.(1991) '2'-O-alkyloligoribonucleotides Synthesis and Applications in Studying RNA Splicing', Nucleosides, Nucleotides and Nucleic Acids, 10: 1, 25 — 36
To link to this Article: DOI: 10.1080/07328319108046433
URL: http://dx.doi.org/10.1080/07328319108046433

NUCLEOSIDES & NUCLEOTIDES, 10(1-3), 25-36 (1991)

2′-O-ALKYLOLIGORIBONUCLEOTIDES: SYNTHESIS AND APPLICATIONS IN STUDYING RNA SPLICING

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Abstract: Improved synthetic routes have been established for the preparation of appropriately protected 2′-O-allylribonucleoside-3′-O-phosphoramidites. 2′-O-Allyloligoribonucleotides prove to be superior antisense compounds for investigating RNA processing.

The use of 2′-O-methyloligoribonucleotides as novel nucleic acid probes has been demonstrated by several groups[1-7]. We have recently shown that these modified oligoribonucleotides have important applications in studying RNA processing, in particular, pre-mRNA splicing[8-10]. In particular, biotinylated 2′-O-methyloligoribonucleotides used in conjunction with streptavidin-agarose or anti-biotin antibodies can be used to isolate and/or purify RNA-protein complexes from crude nuclear extracts[9-10]. The reason that this procedure is successful is that a 2′-O-methyloligoribonucleotide-RNA duplex is not a substrate for RNase H[3], a nuclease abundant in nuclear extracts.

In order to identify RNA analogues with improved properties for antisense analysis we have synthesized and investigated 2′-O-alkyloligoribonucleotides in which the alkyl residue was the allyl or 3,3-dimethylallyl moiety[11]. It was shown that the 2′-O-allyl polymers are superior to the 2′-O-methyl analogues.

We recently developed new synthetic routes to prepare purine 2′-O-methylriboside-3′-O-phosphoramidites using a novel alkylation procedure, utilizing methyl iodide in acetonitrile in the presence of

the sterically hindered strong organic base 2-tert.-butylimino-2-diethylamino-1,3-dimethylperhydro 1,3,2-diazaphosphorin[12] (BDDDP). The utilization of highly versatile intermediates was a key feature of the synthetic routes developed. The 2′-O-methyluridine and 2′-O-methylcytidine building blocks were prepared via a common intermediate largely according to the procedure of Chattopadhyaya[13]. Initially the 2′-O-allylribonucleotide monomers were prepared in identical fashion to the 2′-O-methyl compounds using allyl bromide instead of methyl iodide for the alkylation reaction. We reckoned, however, that the recently published one step conversion of alcohols into allyl ethers under neutral conditions using allyl ethyl carbonate and a palladium (0) catalyst[14] might prove to be better with respect to cost and overall yield of the reaction for the 2′-O-allylation of appropriately protected ribonucleosides.

The present report describes the application of this allylation procedure in the improved synthesis of 2′-O-allylribonucleotide building blocks. In our hands the allylation reaction was extremely fast, a 15-30 min reflux in tetrahydrofuran was sufficient, giving isolated yields of protected 2′-O-allylribonucleosides in the range 79-89%. Our alternative procedure for allylation using allyl bromide and BDDDP gave yields around 60% with a reaction time in dry acetonitrile at room temperature of about 5 h.

In order to increase the binding stability of short 2′-O-alkyloligoribonucleotide probes to complementary RNA target sequences in RNA-protein complexes we decided to incorporate 2-aminoadenosine (2,6-diaminopurine riboside) in place of adenosine, thereby gaining an increase in stability of the Watson-Crick base pair with uridine by an additional hydrogen bond between the purine 2-amino group and the 2-oxo group of uracil. Similar experiments had already been performed with oligodeoxyribonucleotides[15, 16], however, incorporation of several 2-amino-2′-deoxyadenosines is complicated by the facile depurination of the acid labile di-N-acyl derivatives. Our published route to the protected 2′-O-methylguanosine-3′-O-phosphoramidite[12] gave us direct access already to 2′-O-methyl-2,6-diaminopurine riboside. Since depurination in the ribo series is more or less negligible we

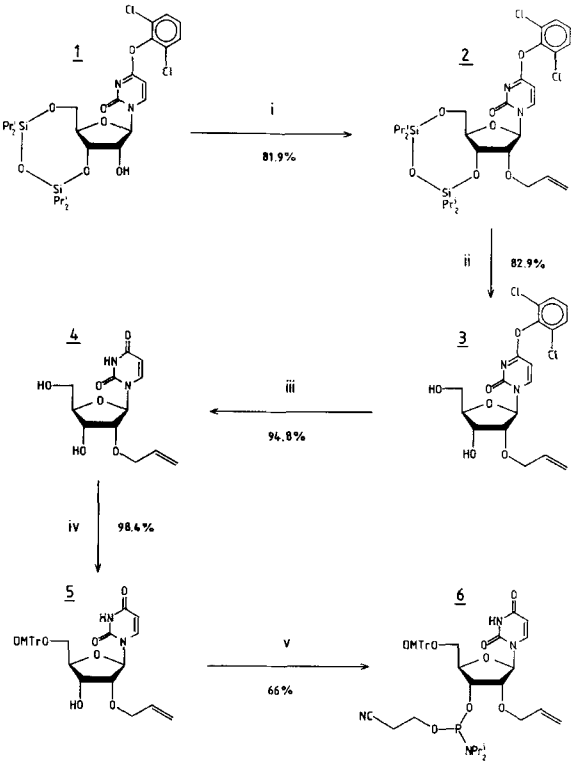

FIG.1

expected to observe no problems in the incorporation of several 2'-O-alkyl-di-N-acyl derivatives of 2-aminoadenosine in a solid phase modified oligoribonucleotide synthesis. Accurate T_m measurements in the DNA series have shown that an amino A/T base pair is stronger than an A/T one but weaker than a G/C one[16].

The synthetic routes to the protected 2'-O-allylribonucleoside-3'-O-phosphoramidites are illustrated in FIGS. 1-5 respectively for the nucleosides uridine, cytidine, adenosine, inosine and guanosine. Full descriptions of most of the reaction steps have already been published for the 2'-O-methyl analogues[12]. Examples of the new reaction steps will be given here.

FIG. 1 shows the synthetic route to the 2'-O-allyluridine monomer 6. Compound 1 was prepared analogously to the 4-O-(2-

nitrophenyl) derivative as described by Nyilas and Chattopadhyaya[13]. The allylation reaction was performed as follows: Tris (dibenzylideneacetone)dipalladium(0) (1.83 g, 2 mmol) and 1,4-bis (diphenylphosphino)butane (3.41 g, 8 mmol) were suspended in dry THF (400 ml) under argon. A solution of compound 1 (126.3 g, 200 mmol) and allyl ethyl carbonate (52 g, 400 mmol) in dry THF (600 ml) was added and the mixture refluxed for 30 min. Silica gel t.l.c. in petrol/ethyl acetate (2:1 v/v) showed complete reaction with a new spot of R_f 0.48 (starting material has R_f 0.23). After removal of solvent and purification by preparative liquid chromatography compound 2 was obtained as a cream coloured foam (110 g, 81.9%), with ^{13}C n.m.r. spectrum ($CDCl_3$) δ: 169.61 (C-4), 154.49 (C-2), 144.64 (phenyl C-1), 144.37 (C-6), 134.29 (allyl CH), 128.75 (phenyl C-2 and C-6), 128.51 (phenyl C-3 and C-5), 126.93 (phenyl C-4), 116.85 (allyl = CH_2), 93.50 (C-5), 89.94 (C-1′), 81.64 (C-2′), 80.40 (C-4′), 70.90 (O-CH_2 of allyl), 67.49 (C-3′), 59.34 (C-5′), 17.24, 17.10, 16.79 and 16.63 (isopropyl CH_3s), 13.21, 12.83, 12.70 and 12.30 p.p.m. (isopropyl CHs).

Desilylation of compound 2 gave compound 3 in good yield. Conversion of compound 3 to 2′-O-allyluridine, compound 4, a white crystalline solid (from methanol) required 18 h treatment with 2-nitrobenzaldoximate in acetonitrile. Two further steps gave the desired 2′-O-allyluridine building block 6. The overall yield from uridine was 28%.

FIG. 2 depicts the route to a suitably protected 2′-O-allylcytidine monomer, compound 10. The versatile intermediate 2 was treated with ammonia/THF in a bomb for 72 h at 70°C to displace the 2,6-dichlorophenoxide moiety, and after removal of solvent, benzoylation yielded the N^4-benzoylcytidine derivative 7 in excellent yield. Three more steps yielded the desired 2′-O-allylcytidine monomer, 10. The overall yield of compound 10 starting from uridine was 41.5%.

The route to the protected 2′-O-allyladenosine monomer, 16 is illustrated in FIG. 3, starting with the previously prepared 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-6-(2,6-dichlorophenoxy) purine riboside[12], compound 11. Allylation as described above gave

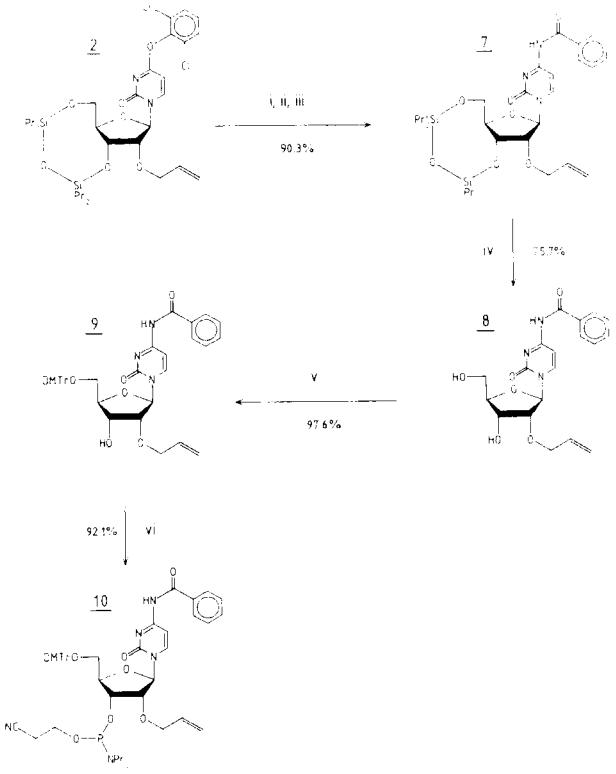

FIG.2

compound 12 in 79% yield. Subsequent reaction steps largely as described for the 2'-O-methyl analogue[12] yielded the 2'-O-allyladenosine monomer, 16. Overall yield of 16 starting from 6-chloropurine riboside was 32%.

The versatile intermediate 12 was also converted into a protected 2'-O-allylinosine monomer 20 as illustrated in FIG. 4. The desilylation was performed prior to the oximate reaction as partial cleavage of the 3'-end of the silyl bridge was observed when compound 12 was exposed to oximate in acetonitrile at reflux for 24 h. The moderate yield of 69% for the conversion of compound 17 to compound 18 was due to isolation problems caused by low solubility of 2'-O-allylinosine in organic solvents. It should be noted that dimethoxytritylation of 2'-O-allylinosine yields at first a compound containing two DMTr groups, one of them being on the

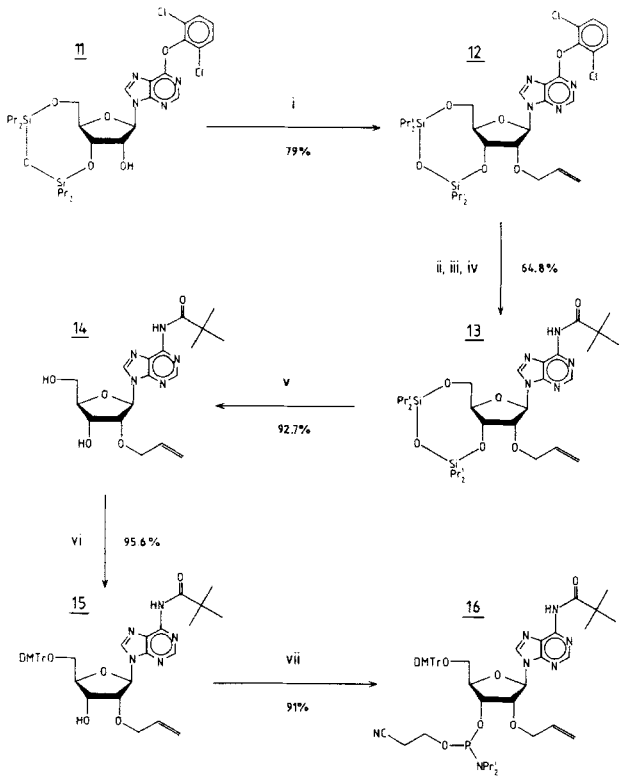

FIG.3

heterocycle (this compound has R_f 0.68 on silica gel t.l.c. in 10% EtOH/$CHCl_3$ containing 1% triethylamine, whereas compound 19 has R_f 0.37). Removal of the second trityl group was achieved by stirring a solution of the material in Et_3N/EtOH/$CHCl_3$ (1:3:6 v/v/v) with silica gel prior to chromatography. The overall yield of the monomer 20 was 20% starting from 6-chloropurine riboside.

FIG. 5 depicts the synthetic route to the 2'-O-allylguanosine monomer 28. The starting material for the synthesis was our previously described highly versatile intermediate 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-2-chloro-6-(2,6-dichlorophenoxy)purine riboside[12], compound 21. Allylation as described above gave an excellent yield of compound 22. Subsequent reaction with sodium azide followed by a careful reduction with

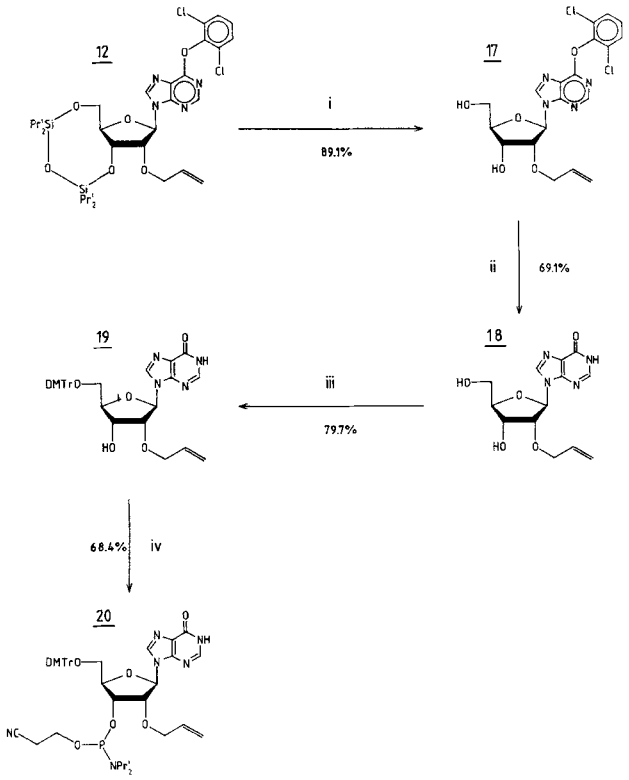

FIG.4

Lindlar catalyst further poisoned with quinoline gave the 2,6-diaminopurine riboside derivative 24. The overall success of this route was due to the acceptance of 2'-O-allyl-2,6-diaminopurine riboside, compound 25 as a substrate for adenosine deaminase, giving a quantitative yield of 2'-O-allylguanosine. Adenosine deaminase handles a range of substrates[17], and it was already known that 2'-O-methyl-2,6-diaminopurine riboside was also a substrate[18]. The conversion to the N^2-protected 2'-O-allylguanosine 26 was performed without intermediate purification. The protected 2'-O-allylguanosine monomer, compound 28 was obtained in an overall yield of 27% starting from 2-amino-6-chloropurine riboside.

Our route to a suitably protected 2'-O-propyl-2,6-diaminopurine riboside monomer, compound 33 is illustrated in FIG.

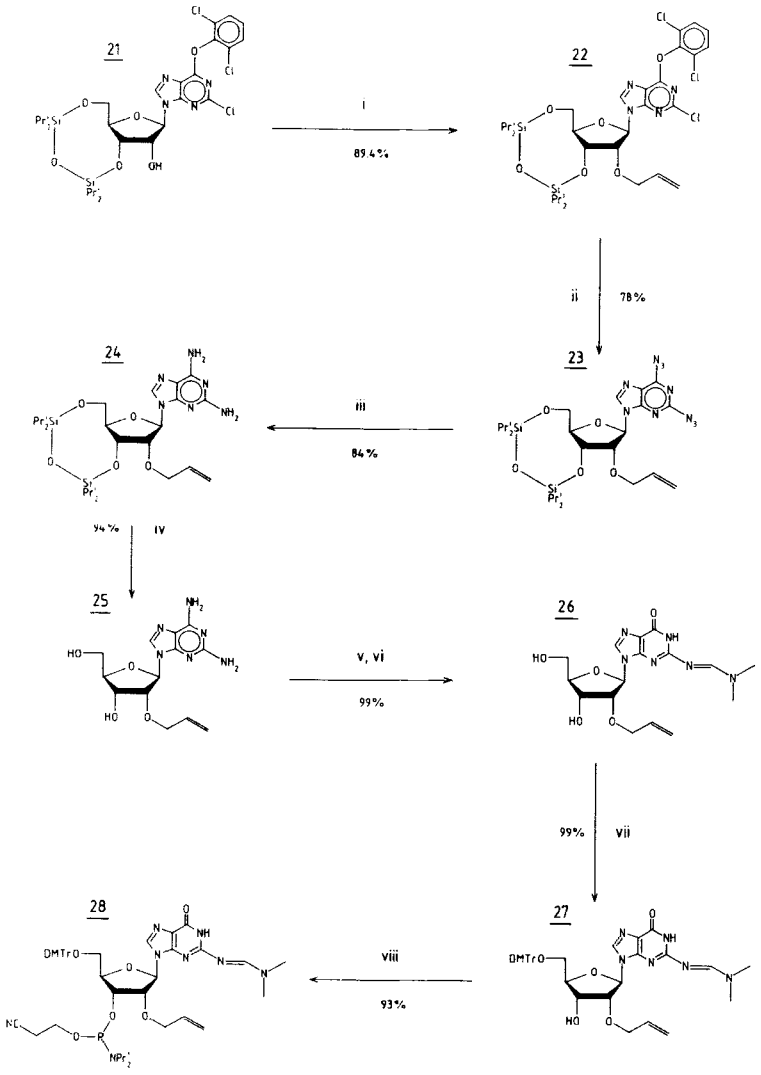

FIG.5

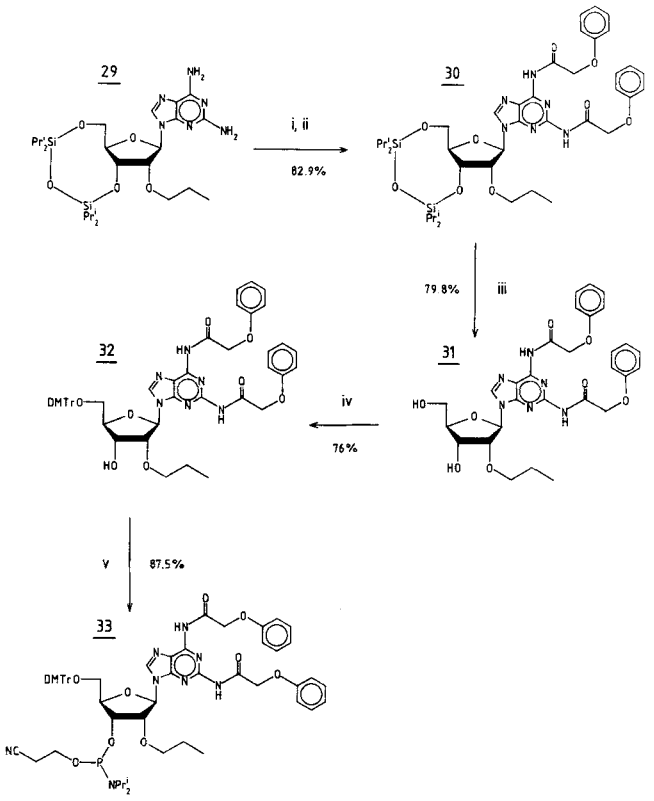

FIG.6

6. The starting material, compound 29, was readily obtained from compound 23 by reduction over Pd/C. Our choice of N-acyl protecting group was influenced by problems of deacylation of bis N-acyl protected 2'-deoxy-2,6-diaminopurine riboside previously reported[15, 16]. The phenoxyacetyl group, reported as a highly labile protecting group for adenine and guanine[19, 20] proved to be suitable for N-protection of the 2,6-diaminopurine moiety. Compound 30 was quantitatively converted back to compound 29 by overnight treatment with methanolic ammonia at 60°C. Compound 29 (R_f 0.26 on silica gel t.l.c. in ethyl acetate) was acylated with a five fold excess of phenoxyacetic anhydride in pyridine at room temperature for 4 h. Excess reagent was quenched with water during 3 h and compound 30 was isolated by chromatography as a foam (R_f 0.59 on

silica gel t.l.c. in 1:1 petrol/ethyl acetate). Subsequent treatment with tetrabutylammonium fluoride for 4 min yielded N^2,N^6-diphenoxyacetyl-2′-O-propyl-2,6-diaminopurine riboside, compound 31 with ^{13}C n.m.r. spectrum ($CDCl_3$) δ: 167.98 and 166.59 (carbonyl of phenoxyacetyls), 157.19 and 156.97 (C-1 of phenyls), 151.44 and 150.93 (C-2 and C-6), 148.80 (C-4), 143.14 (C-8), 129.50 and 129.50 (C-3 and C-5 of phenyls), 122.04 and 121.84 (C-4 of phenyls), 119.93 (C-5), 114.76 (C-2 and C-6 of phenyls), 88.41 (C-1′), 86.83 (C-4′), 81.08 (C-2′), 72.83 (OCH_2 of propyl), 69.99 (C-3′), 62.16 (C-5′), 22.68 (CH_2 of propyl), and 10.08 p.p.m. (CH_3 of propyl). Conversion of compound 31 to the desired monomer 33 was straightforward.

All monomers were obtained as solid white foams after purification by short column chromatography. Silica gel t.l.c. analysis and ^{31}P n.m.r. spectroscopy (referenced to trimethyl phosphate) yielded the following data: Compound 6, R_f 0.42 and 0.39 in EtOAc/CH_2Cl_2 (4:1 v/v) containing 5% Et_3N, δ_p: + 146.84 and 146.33 p.p.m. Compound 10, R_f 0.48 and 0.43 in above solvent system, δ_p: + 146.86 and 146.04 p.p.m. Compound 16, R_f 0.53 and 0.49 in above solvent system, δ_p: + 147.01 and 146.73 p.p.m. Compound 20, R_f 0.64 in Et_3N/EtOH/CH_2Cl_2 (1:2:17 v/v/v), δ_p: + 146.95 and 146.70 p.p.m. Compound 28, R_f 0.66 in Et_3N/EtOH/CH_2Cl_2 (1:2:17 v/v/v), δ_p: + 147.19 and 147.02 p.p.m. Compound 33, R_f 0.60 and 0.52 in petrol/CH_2Cl_2 (2:1 v/v) containing 10% Et_3N, δ_p: 147.46 and 146.83 p.p.m. Monomer 33 was insoluble in pure acetonitrile and was dissolved in THF/CH_3CN (1:1 v/v) for synthesis. Functionalised aminopropyl controlled pore glass was loaded with the 3′-O-succinates of the compounds 5, 9, 15, 19, 27 and 32 in the usual way giving loadings of 36-38 μmol of nucleoside g^{-1} of carrier.

Polymer assembly was carried out as described for the 2′-O-methyloligoribonucleotides[7] but using a condensation time of 8 min. It is essential that the capping reaction for oligomers containing a protected 2,6-diaminopurine ribonucleotide moiety is carried out with phenoxyacetic anhydride/N-methylimidazole instead of with acetic anhydride in order to avoid a transamidation reaction. This problem was first encountered by Chaix *et al.*, with N^2-phenoxyacetylguanine[20]. Single or multiple biotinylation when

required was performed during the solid phase synthesis[21]. A short biotinylated 2'-O-allyloligoribonucleotide containing five 2,6-diaminopurine ribonucleotides proved successful for depletion of human U5 snRNP from crude nuclear extracts. The RNA in this particle is largely covered with proteins and only a very short region is available for hybridization.

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