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Highly efficient chemical synthesis of 2'-O-methyloligoribonucleotides and tetrabiotinylated derivatives; novel probes that are resistant to degradation by RNA or DNA specific nucleases

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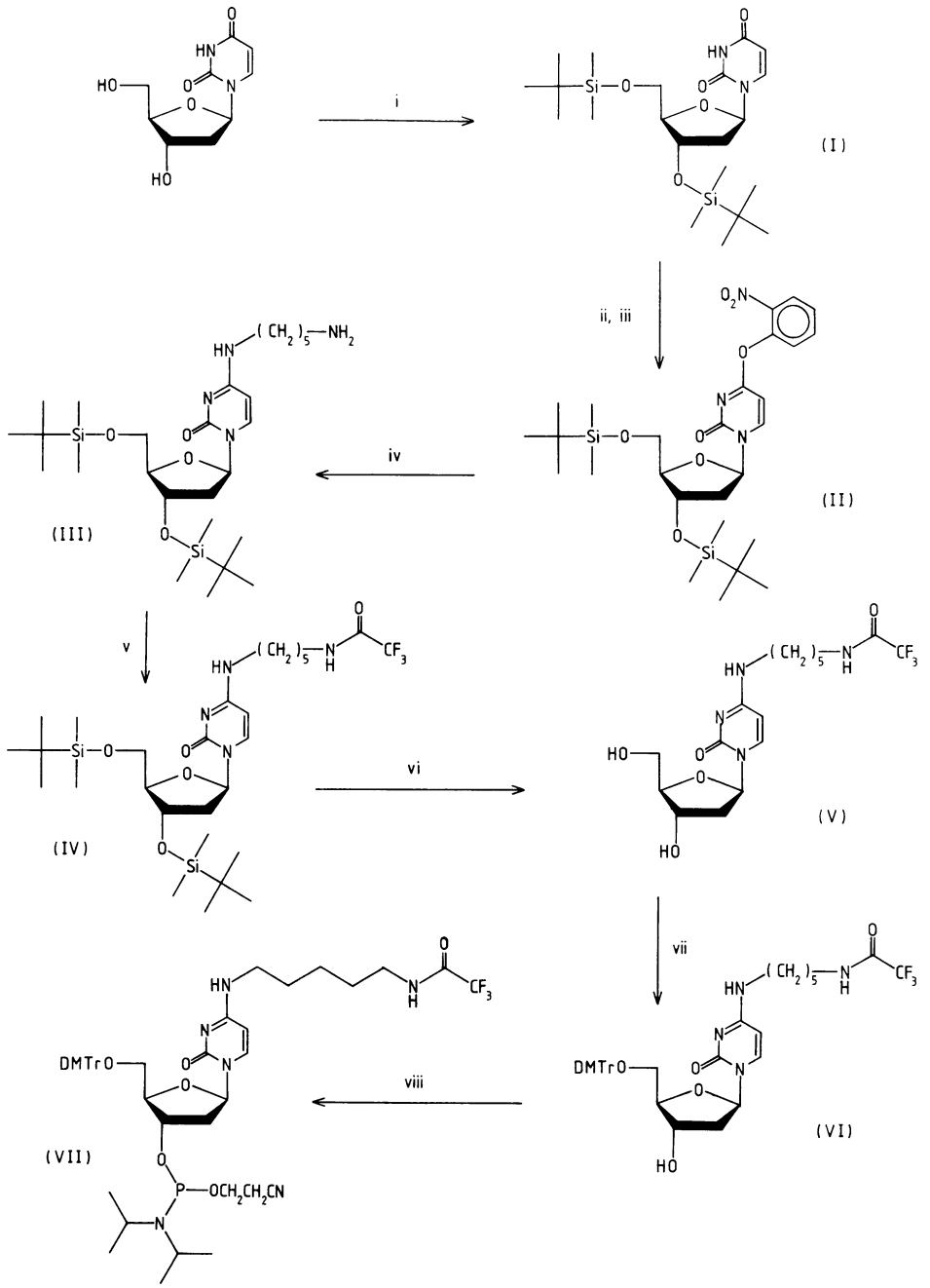
ABSTRACT

2'-O-Methyloligoribonucleotides have been synthesised on solid phase from base protected 5'-O-dimethoxytrityl-2'-O-methylribonucleoside-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidites) using 5-(4-nitrophenyl)-1H-tetrazole as activator. Coupling yields greater than 99% were achieved, as judged by trityl cation release. The preparation of a modified 2'-deoxycytidine building block bearing an N^4-(5-trifluoroacetylaminopentyl) spacer is also described. The latter compound enabled the chemical synthesis of 2'-O-methyloligoribonucleotide probes carrying several 5'- terminal biotinylation sites (in general four modified residues were used), which can be conveniently ^{32}P end-labelled enzymatically using polynucleotide kinase. Used in conjunction with streptavidin-containing derivatives, such biotinylated probes have important applications in biochemical purification and electron microscopy of RNA-protein complexes. The 2'-O-methyloligoribonucleotides are completely resistant to degradation by either RNA or DNA specific nucleases. In contrast, nucleases with dual RNA/DNA specificity show a complete spectrum of cleavage rates.

INTRODUCTION

The use of 2'-O-methyloligoribonucleotides as novel nucleic acid probes has recently been described in the literature (1–6). Of particular interest were the findings that i) a 2'-O-methyloligoribonucleotide–RNA duplex is much more thermally stable than the corresponding oligodeoxyribonucleotide–RNA duplex (4), and ii) that the former duplex is not a substrate for RNase H (3). RNase H specifically cleaves RNA when it is in an RNA–DNA heteroduplex (7). The previously described syntheses used phosphotriester chemistry on polystyrene (4), which gave rather low condensation yields (*ca.* 85%). Recently, brief mention was made of syntheses from phosphoramidite building blocks (2) utilising both the methyl and β-cyanoethyl procedures (8–10) that were developed originally for DNA synthesis.

We describe here a highly efficient synthesis of 2'-O-methyloligoribonucleotides by the β-cyanoethyl phosphoramidite method (10), using a urethane linked (11) aminopropyl controlled pore glass (CPG) support and 5-(4-nitrophenyl)-1H-tetrazole as activator (12) to achieve fast, high yield condensations. To enable biotinylation of probes we have previously used a 5'-aminolinker moiety, introduced chemically as 6-(N-monomethoxytrityl) aminohexyloxy, 2-cyanoethoxy N,N-diisopropylaminophosphine (13,14). This yields monobiotinylated probes that cannot be ^{32}P-end labelled with polynucleotide kinase. We describe here the preparation of a modified 2'-deoxycytidine building block bearing an N^4-(5-trifluoroacetylaminopentyl) spacer to expand the flexibility of the probes. This compound can be chemically incorporated, either singly or as multiple residues, at the 5'- terminus of chemically synthesised 2'-O-methyloligoribonucleotides thus permitting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both single or multiple biotinylation and conventional enzymatic ^{32}P end labelling with T4 polynucleotide kinase. Alternatively, such probes could be ^{32}P labelled and fluorescence tagged, e.g. for microinjection work. As streptavidin has four biotin binding sites, we routinely incorporated four of the base modified cytidine residues at the 5'- terminus of the 2'-O-methyloligoribonucleotide probes to optimise the binding interaction.

The properties of the 2'-O-methyloligoribonucleotide probes have been studied with respect to chemical and enzymatic stability, and compared with corresponding oligodeoxyribonucleotide and oligoribonucleotide probes of identical sequence.

RESULTS AND DISCUSSION

Synthesis of the base modified 2'-deoxycytidine building block

The synthetic route used for preparation of the base modified 2'-deoxycytidine building block VII is illustrated in Figure 1. Thus, 2'-deoxyuridine was silylated to give the bis(silyl) compound I in 99% yield. Reaction of this compound with mesitylenesulphonyl chloride followed by displacement of mesitylenesulphonate from the intermediate with 2-nitrophenoxide, using a procedure described for modified ribonucleosides by Nyilas and Chattopadhyaya (15), gave a 78% isolated yield of the 4-O-(2-nitrophenyl)ether II. Subsequent displacement of 2-nitrophenol using 1,5-diaminopentane generated the modified 2'-deoxycytidine derivative III in 87% yield. Protection of the primary amino group with S-ethyl trifluorothioacetate giving compound IV was quantitative. Desilylation of this compound yielded N^4-[5-(N-trifluoroacetyl)aminopentyl]-2'-deoxycytidine (V) in 66% yield. Conversion to the 5'-O-dimethoxytrityl compound VI was achieved in 94% yield, and subsequent phosphitylation generated the desired building block VII in 57% isolated yield.

Assembly of 2'-O-methyloligoribonucleotides

The principal advantages of using urethane linked supports as compared to succinate linked supports have been described previously (11). In our opinion the ease of preparation of nucleoside functionalised supports (which requires only small quantities of the relatively valuable base protected 5'-O-dimethoxytrityl-2'-O-methylribonucleosides) and the high stability of the urethane bond to trace impurities in solvents and reagents far outweighs the only real disadvantage, which is the relatively long deprotection step with ammonia. The latter has no detrimental effects as the 2'-O-methyloligoribonucleotides are alkali stable, just like DNA (and unlike RNA). The presence of completely free 3'- terminal hydroxyl group was demonstrated by digestion of ^{32}P end labelled oligonucleotides with snake venom phosphodiesterase, which went to completion.

High yield coupling, >99%, of our 2'-O-methylribonucleoside building blocks (manuscript in preparation) was achieved using a 0.1M solution of 5-(4-nitrophenyl)-1H-tetrazole in acetonitrile as activator (12). A coupling time of 6 min was necessary to achieve

Figure 1. Reaction scheme for the preparation of the modified 2'-deoxycytidine building block. Reagents: i, t-butyldimethylsilylchloride and imidazole in DMF; ii, 2-mesitylenesulphonylchloride, triethylamine and 4-dimethylaminopyridine in 1,2-dichloroethane; iii, 2-nitrophenol, triethylamine and 1,4-diazabicyclo[2.2.2.]octane in dichloroethane; iv, 1,5-diaminopentane in acetonitrile; v, S-ethyl trifluorothioacetate in dichloromethane; vi, tetrabutylammonium fluoride in tetrahydrofuran; vii, 4,4'-dimethoxytrityl chloride and triethylamine in pyridine; viii, 2-cyanoethoxy N,N-diisopropylaminochlorophosphine and N,N-diisopropylethylamine in dichloroethane.

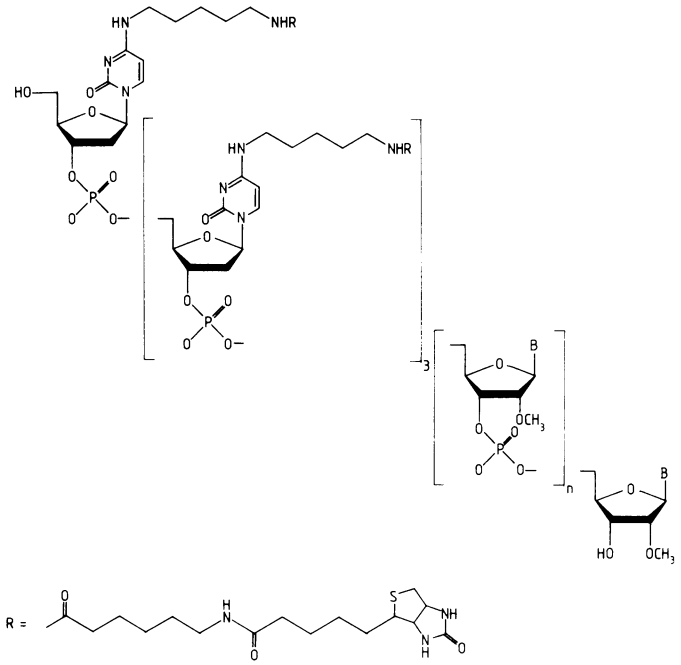

Figure 2. Structure of the tetrabiotinylated 2'-O-methyloligoribonucleotides.

complete reaction, due to the steric hindrance caused by the neighbouring 2'-O-methyl group. Under these conditions no over condensation was observed (n+1 and n+2 peaks are often observed in DNA synthesis using 0.5M tetrazole as activator). A coupling time of 5 min using 0.5 M tetrazole as activator was not effective, and moreover a 0.4 M solution of N-methylanilinium trichloroacetate (16) gave slightly poorer results than those obtained with 0.1 M 5-(4-nitrophenyl)-1H-tetrazole. We have now synthesised more than fifty 2'-O-methyloligoribonucleotides with chain lengths of 16 to 30 residues, and have obtained isolated yields in the range from 14−24%, using the method described here. These high yields are in part due to the special support and in part due to the virtual absence of depurination during the polymer chain assembly. This is readily observed from h.p.l.c. analyses of DMTr protected 2'-O-methyloligoribonucleotides. As a whole the actual chain assembly works better than for oligodeoxyribonucleotide synthesis.

Biotinylated probes

The basic structure of the tetrabiotinylated probes that were synthesised is illustrated in Figure 2. Since the biotin moities are located at the end of long flexible spacer arms, all four should bind to a single streptavidin molecule. As would be expected, the presence of four terminal biotins instead of one, accelerates the kinetics of binding of the probe to streptavidin-gold (manuscript in preparation). We have used 2'-O-methylinosine in place of 2'-O-methylguanosine, and have shown that the probes bind to complementary RNA target sequences in the correct place (manuscript in preparation). It has been shown that inosine behaves approximately as a guanosine residue but can 'pair' with any of the four natural bases with or without forming hydrogen bonds (17,18). In our case inosine can

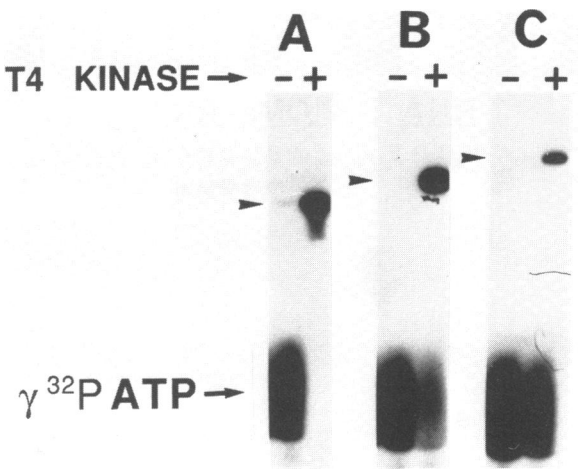

Figure 3. Enzymatic phosphorylation of an oligonucleotide made of A/ DNA, B/ 2′-OMe RNA or C/ 2′-OMe RNA plus 5′- tetrabiotin. The sequences of the oligonucleotides were; A/ 5′-d[TACTGCCACTGCGCAAAGCU], B/ 5′-(2′-OMe) r[UACUICCACUICICAAAICU], C/ As in B/ with four 5′-terminal biotinylated deoxycytidine residues. The kinased oligonucleotide band is indicated by an arrowhead at the left side of each panel. Oligonucleotides were analysed on a 20% polyacrylamide/8M urea gel run in 1×TBE buffer, pH 8.3.

form two properly oriented hydrogen bonds with the C in the opposite strand, so the energy of one hydrogen bond is lost relative to the normal GC base pair. Work describing the use of 2′-O-methylguanosine and including a novel synthesis of the building block will be reported later.

Mono- or tetrabiotinylation of the amino functionality of the modified probes proceeded in very high yield and the products were readily purified by reversed phase h.p.l.c. The isolated yields of four monobiotinylated probes were in the range from 7 to 20% based on the amount of CPG carrier used for the synthesis, and the isolated yields of twelve different tetrabiotinylated probes, of length 20 to 29 residues including the 4 base modified dC residues, were in the range from 4−10%.

In Figure 3 we compare the enzymatic phosphorylation of an oligodeoxyribonucleotide with an oligonucleotide of equivalent sequence made of 2′-OMe RNA, either with or without four biotin residues at the 5′-terminus. This shows clearly that the 2′-OMe RNA is a good substrate for T4 polynucleotide kinase, labelling with an efficiency comparable to single stranded DNA. In the presence of 5′-terminal biotin residues the efficiency of the phosphorylation reaction is somewhat reduced, presumably through steric hindrance of the kinase. However, even in the presence of biotin residues, we have always found it possible, (with a sample of sixteen separate tetrabiotinylated oligonucleotides), to satisfactorily 5′ end label the 2′-OMe RNA using γ^{32}P ATP and T4 polynucleotide kinase. Figure 3 also shows that the h.p.l.c. purified products of oligonucleotide assembly made using 2′-OMe RNA nucleotides are essentially free of the shorter chains, resulting from depurination, that are commonly observed with DNA oligonucleotides. Gel purification of the modified RNA oligonucleotides is therefore seldom necessary to isolate homogeneous, full-length probes for subsequent use in biological experiments.

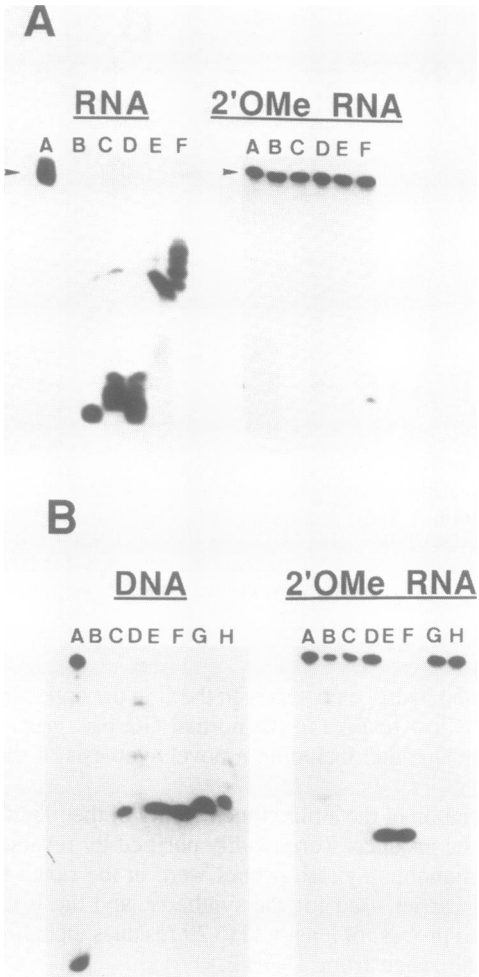

Figure 4. Nuclease sensitivity of oligonucleotides made of either RNA, DNA or 2'-OMe RNA. The sequences of the DNA and 2'-OMe RNA oligonucleotides are the same as those used in Figure 3. The 2'-OMe RNA oligonucleotide was not biotinylated. The RNA oligonucleotide is identical in sequence to the 2'-OMe RNA but with G residues instead of I residues. Digestion products were analysed on a 20% polyacrylamide/8M urea gel run in 1×TBE buffer, pH8.3. The nucleases tested were; PANEL A: A/ no enzyme control, B/ RNase A, C/ RNase T1, D/ RNase T2, E/ RNase CL3 and F/RNase U2. PANEL B: A/ no enzyme control, B/ micrococcal nuclease, C/ S1 nuclease, D/ mung bean nuclease, E/ P1 nuclease, F/ snake venom phosphodiesterase, G/ DNase1 and H/ exonucleaseIII.

Nuclease sensitivity of 2'-OMe RNA

The presence of a 2'- OMe group on the ribose would be expected to significantly alter the sensitivity of the resulting polymer to degradation by nucleases. In principle, both steric hindrance of the binding of a nuclease's active site and the lack of the nucleophilic 2'-OH could contribute to lowering the rate of nuclease cleavage of 2'-OMe RNA. The latter

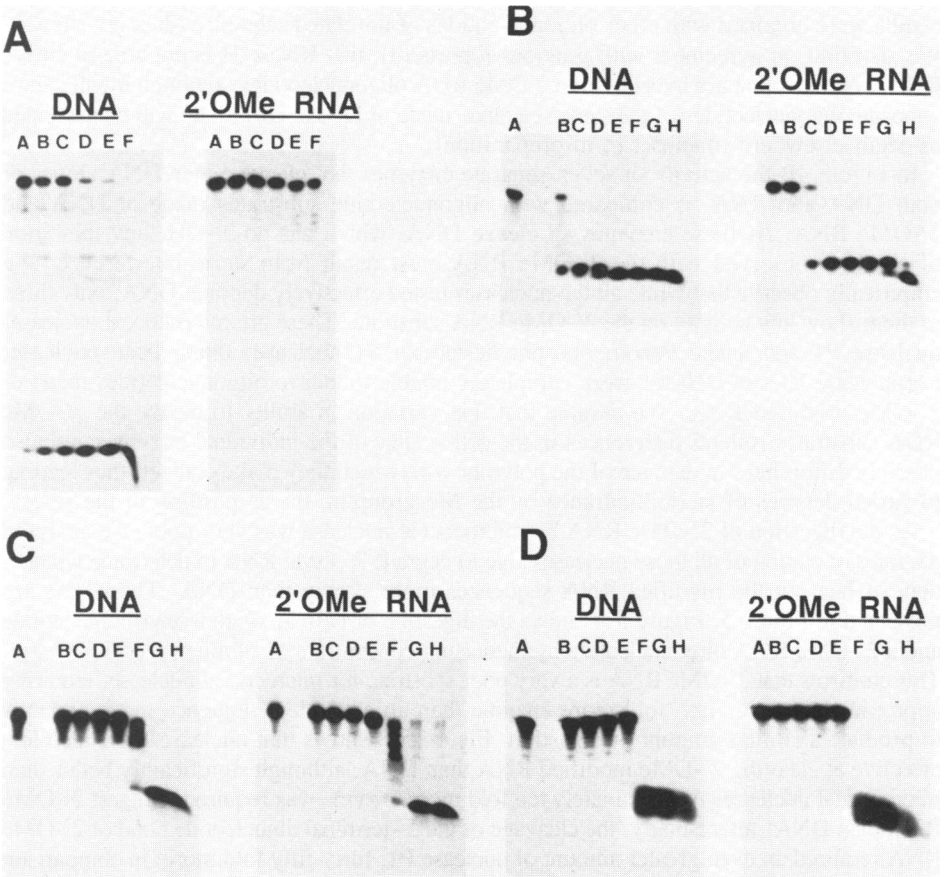

Figure 5. Comparison of nuclease cleavage rates with either DNA or 2'-OMe RNA oligonucleotide substrates. The oligonucleotides have the same sequence as described in Figure 3 and were not biotinylated. Oligonucleotides were analysed on a 20% polyacrylamide/8M urea gel run in 1×TBE buffer, pH 8.3. PANEL A: Digestion with micrococcal nuclease. Incubations were done for 1 hour at 50°C using A/ no enzyme, B/ 0.12, C/ 0.6, D/ 2.4, E/ 12, F/ 30 and G/ 120 units of micrococcal nuclease. PANEL B: Digestion with P1 nuclease. Digestions were done for 1 hour at 30°C using A/ no enzyme, B/ 0.001, C/ 0.01, D/ 0.05, E/ 0.2, F/ 1 and G/ 5 units of P1 nuclease. PANEL C: Digestion with snake venom phosphodiesterase. Digestions were done at 30°C using A/ no enzyme, B/ 0.001, C/ 0.01, D/ 0.05, E/ 0.2, F/ 1 and G/ 5 units of snake venom phosphodiesterase. PANEL D: Digestion with Bal31 nuclease. Digestions were done at 30°C using A/ no enzyme, B/ 0.00004, C/ 0.0002, D/ 0.002, E/ 0.01, F/ 0.04, G/ 0.2 and H/ 1 unit of Bal31 nuclease.

is relevant to those RNases which have a reaction mechanism that utilises the 2'-OH group of the ribose as a nucleophile to attack the adjacent 3',5'-phosphodiester bond.

In Figure 4A the ability of five separate RNA-specific nucleases to digest oligonucleotides made of either RNA or 2'-OMe RNA is compared. These RNases exhibit different base specificities in their cleavage patterns and are commonly used for enzymatic sequencing of RNA. In each case, equivalent concentrations of nuclease that produced total digestion of RNA were unable to cleave the same base sequence made of 2'-OMe RNA. Identical

results were obtained with other oligonucleotides of unrelated sequence (data not shown). We also find, in agreement with previous reports (3), that RNase H is not able to cleave RNA sequences that are hybridised to 2'OMe RNA oligonucleotides, although it will cleave opposite oligonucleotides of the same sequence made of DNA. These data will be presented in detail elsewhere (manuscript in preparation).

In Figure 4B the activity of seven separate enzymes that cleave either DNA alone, or both DNA and RNA, is compared with oligonucleotide substrates made of DNA and 2'-OMe RNA. As these enzymes all cleave DNA, which has no 2'-OH, any inhibition of cleavage observed with the 2'-OMe RNA must result from steric interference. We empirically observe that while all the nucleases tested effectively degrade DNA, only three of them show any activity on the 2'-OMe RNA substrate. These are micrococcal nuclease, nuclease P1 and snake venom phosphodiesterase. S1 nuclease, mung bean nuclease, exonuclease III and DNase1 were completely unable to cleave oligonucleotides made of 2'-OMe modified RNA. We assume that this variation in ability to cleave the 2'-OMe RNA substrates reflects differences in the active sites of the individual enzymes and also possibly differences in the face of the polymer with which they make contact, thus leading to varied degrees of steric hindrance by the Me group on the 2' position of the ribose.

As the digestion of 2'-OMe RNA by micrococcal nuclease was very poor, we analysed the rates of cutting of all those nucleases able to degrade 2'-OMe RNA to determine whether they in fact cut the modified RNA sequences more slowly than DNA. These data are presented in Figure 5. Figure 5 A shows the digestion of both an oligodeoxyribonucleotide and a 2'-OMe RNA oligonucleotide by increasing concentrations of micrococcal nuclease. This confirms that 2'-OMe RNA is a very poor substrate for micrococcal nuclease, requiring approximately a hundred fold more enzyme than an equivalent sequence made of DNA to produce a similar amount of digestion. Figure 5 B shows that nuclease P1 is also less effective at cleaving 2'-OMe modified RNA than DNA, although significantly better than micrococcal nuclease. Approximately ten fold more enzyme was required to digest 2'-OMe RNA than DNA. Interestingly, the cleavage of the 5'-terminal dinucleotide bond of 2'-OMe RNA required an even greater amount of nuclease P1, forty-fifty fold more, in comparison to DNA. In contrast with both micrococcal and P1 nucleases, which cut 2'-OMe modified RNA more slowly than they cut DNA, snake venom phosphodiesterase is able to cleave both polymers at approximately the same rate (figure 5 C). Thus the collection of nucleases tested, which have dual RNA/DNA cleavage specificities, show a spectrum of abilities to cleave 2'-OMe RNA ranging from inactivity through weak activity to efficient digestion.

Another enzyme with dual RNA/DNA specificity is Bal 31, a nuclease that is widely used in molecular biology for the generation of deletion mutations. A recent report has been made that Bal 31 is unable to cleave sequences made of double stranded 2'-OMe RNA (5). As Bal 31 nuclease is known to have two separate activities, a double strand specific exonuclease activity and a single strand specific endonuclease activity, we therefore tested the oligonucleotides reported in this study for sensitivity to the single stranded endonuclease activity of Bal 31 (figure 5 D). It is clear from these data that Bal 31 is able to cleave the 2'-OMe RNA very efficiently, indeed at a rate which is essentially indistinguishable from that for cleaving DNA. We have found a similar, high rate of cleavage by Bal 31 with other 2'-OMe RNA oligonucleotides with different sequences and have also observed efficient cleavage when the 2'-OMe RNA is hybridised to a complementary DNA oligonucleotide (data not shown). We have also performed h.p.l.c. analysis of the digestion products of Bal 31 cleavage as an additional control against the

possibility that Bal 31 was not in fact efficiently degrading the majority of the 2'-OMe RNA but rather dephosphorylating the oligonucleotides. These data confirm beyond doubt that the 2'-OMe RNA is completely hydrolysed by Bal 31 when it is single stranded. In this study we did not assay the double strand exonuclease activity on double stranded 2'-OMe RNA substrates. However, comparing our data with that of Mukai *et al.* (5), it is remarkable that such a profound difference should exist in the ability of the enzyme to cleave single but not double stranded 2'-OMe RNA.

Applications of 2'-OMe RNA

We have described an efficient route for the synthesis of 2'-OMe modified RNA oligonucleotides that can additionally be tagged with cytidine derivatives carrying biotin. This system allows for highly efficient affinity binding of the oligonucleotides to streptavidin derivatives (e.g. streptavidin agarose) while retaining the option of allowing the oligonucleotides to be conveniently kinased at the 5'-terminus. The synthesis of 2'-OMe RNA can be performed using a standard DNA synthesiser and in our experience gives consistently better results than DNA in terms of per cycle condensation yields, overall stability and extent of depurination during synthesis. Additionally, oligonucleotides made of 2'-OMe RNA are resistant to degradation by most nucleases, making them attractive probes to use in biological experiments where it is not possible to exclude or inhibit nuclease activity. Their high stability also makes them good candidates for use in 'antisense' experiments where previous experience has shown that conventional DNA or RNA probes can be subject to rapid turnover *in vivo*. Our initial results support this view (unpublished observations). Additionally, we foresee particularly important applications for these probes in the study of RNA biochemistry where the possibility of forming stable hybrids between RNA molecules and biotinylated 2'-OMe RNA oligonucleotides allows the design of novel strategies for the purification and functional analysis of ribonucleoprotein complexes.

EXPERIMENTAL

General Materials and Procedures

2-Cyanoethoxy N,N-diisopropylaminochlorophosphine was purchased from BioSyntech (Hamburg, FRG). 2'-Deoxyuridine was obtained from Pharma-Waldhof GmbH (Düsseldorf, FRG) and sulphosuccinimidyl 6-(biotinamido)hexanoate [NHS-LC-biotin] was obtained from Pierce (Oud-Beijerland, Holland). Tolylene-2,6-diisocyanate was obtained from Janssen Chimica (Beerse, Belgium). Controlled pore glass (CPG) of 80/120 mesh size and 500Å mean pore diameter was obtained from Electro-Nucleonics, Inc. (Fairfield, New Jersey, USA). Enzymes were obtained from Boehringer Mannheim (Mannheim, FRG) except for Bal 31 nuclease, mung bean nuclease and T4 polynucleotide kinase which were purchased from New England Biolabs. Reagents for oligodeoxyribonucleotide synthesis were purchased from Applied Biosystems (Foster City, California) or BioSyntech. Reagents for oligoribonucleotide synthesis according to the procedure of Ogilvie (19) were purchased from Peninsula Laboratories, Inc. (Belmont, California). All other reagents used were of the highest purity available.

6-(N-Monomethoxytrityl)aminohexyloxy 2-cyanoethoxy N,N-diisopropylamino-chlorophosphine was synthesised according to the general procedure of Connolly (13) and purified by short column chromatography. 5-(4-Nitrophenyl)-1H-tetrazole was synthesised in 93% yield on a 0.5 mol scale using the procedure described for 5 phenyltetrazole (20); the crystalline material (from absolute ethanol) was finely powdered and dried thoroughly *in vacuo* over phosphorus pentoxide. Base protected 5'-O-dimethoxytrityl-2'-O-

methylribonucleoside-3'-O-(2-cyanoethyl N,N-diisopropylphos-phoramidites) were synthesised using a novel route (manuscript in preparation). Aminopropyl CPG 500Å was prepared from CPG 500Å using the procedure described by Atkinson and Smith (21) for functionalising Fractosil 500.

Column chromatography was performed on Kieselgel 60 (Fluka, Neu-Ulm, FRG) and ascending mode t.l.c. was performed on aluminium foil supported silica containing a 254 nm fluor.

^{13}C and ^{31}P n.m.r. spectra were recorded on a Bruker AM250 spectrometer using tetramethylsilane and external trimethyl phosphate as the respective references. ^{31}P spectra were recorded using broad band proton noise decoupling. ^{13}C data below are reported with broad band proton noise decoupling, however signal assignments were made with the aid of the off-resonance spectra.

Oligodeoxyribonucleotides were synthesised on an Applied Biosystems synthesiser model 380B-02 (Foster City, California) or on a Milligen 7500 DNA synthesiser (Bedford, Massachusetts, USA) using β-cyanoethyl phosphoramidite chemistry (10). Oligoribonucleotides were synthesised by the methyl phosphoramidite procedure according to Ogilvie *et al.* (19) on the Applied Biosystems synthesiser.

Synthesis of the base modified 2'-deoxycytidine monomer

3',5'-O-Bis(t-butyldimethylsilyl)-2'-deoxyuridine (I) : 2'-Deoxyuridine (4.56 g, 20 mmol) was dried by addition and evaporation of dry N,N-dimethylformamide (80 ml; DMF) *in vacuo*, and redissolved in dry DMF (40 ml). Imidazole (6 g, 88 mmol) and t-butyldimethylsilylchloride (7.53 g, 50 mmol) were added with stirring and exclusion of moisture under dry nitrogen. After 20 h at room temperature t.l.c. in ethanol/dichloromethane (5 : 95 v/v) showed a single spot of R_f 0.41. Solvent was evaporated *in vacuo*, the residue was dissolved in chloroform (200 ml), and the solution was washed with 1 M sodium bicarbonate solution (2×200 ml) followed by saturated brine (200 ml). The organic layer was dried (Na_2SO_4), filtered and evaporated *in vacuo*. Evaporation of dry toluene left the desired product I as a white foam (9.05 g, 99%). ^{13}C n.m.r. spectrum ($CDCl_3$) δ : 163.33 (C-4), 150.24 (C-2), 140.06 (C-6), 102.12 (C-5), 87.70 (C-1'), 85.15 (C-4'), 71.13 (C-3'), 62.38 (C-5'), 41.81 (C-2'), 25.84 and 25.70 (CH_3s of t-Bu), 18.32 and 17.95 (quaternary Cs of t-Bu), −4.63, −4.86, −5.53 and −5.59 p.p.m. (CH_3-Si).

3',5'-O-Bis(t-butyldimethylsilyl)-4-O-(2-nitrophenyl)-2'-deoxyuridine (II) : Added triethylamine (14 ml, 100 mmol), 4-dimethylaminopyridine (600 mg, 5 mmol) and mesitylenesulphonylchloride (6.31 g, 28 mmol) to a stirred solution of I (9.05 g, 19.8 mmol) in dry 1,2-dichloroethane (100 ml). A precipitate immediately formed and after 30 min under anhydrous conditions t.l.c. in ethanol/dichloromethane (5 : 95 v/v) showed complete reaction with a new spot of R_f 0.85. The reaction mixture was diluted with dichloroethane (100 ml) and poured into vigorously stirred 1 M sodium bicarbonate solution (400 ml). The organic layer was separated, dried (Na_2SO_4), filtered and evaporated *in vacuo*. Evaporation of dry toluene from the residue left the intermediate mesitylenesulphonyl derivative as a foam. This material was dissolved in 1,2-dichloroethane (125 ml) and triethylamine (4.4 ml, 31.4 mmol), 1,4-diazabicyclo [2.2.2]octane (450 mg, 4 mmol) and 2-nitrophenol (4.4 g, 31 mmol) were added with stirring under dry nitrogen. After 1 h at room temperature t.l.c. in ethanol/dichloromethane (5 : 95 v/v) showed complete reaction with a new spot of R_f 0.76 due to the desired product. The reaction mixture was diluted with dichloromethane (200 ml) and washed with 1 M sodium bicarbonate solution (2×500

ml). The organic layer was dried (Na_2SO_4), filtered and evaporated *in vacuo* leaving a yellow oil. The crude product was purified by chromatography on silica gel (120 g), using dichloromethane as eluant followed by a gradient up to 3% ethanol in dichloromethane. Pure fractions were pooled and evaporated *in vacuo* to give the title compound II as an almost colourless oil (8.9 g, 77%). ^{13}C n.m.r. spectrum ($CDCl_3$) δ : 170.50 (C-4), 154.72 (C-2), 145.01 (phenyl C-1), 144.91 (C-6), 141.65 (phenyl C-2), 134.73 (phenyl C-5), 126.52, 125.90 and 125.54 (phenyl C-3,4, and 6), 94.20 (C-5), 87.60 (C-1'), 86.74 (C-4'), 69.71 (C-3'), 61.62 (C-5'), 42.14 (C-2'), 25.86 and 25.64 (CH_3s of t-Bu), 18.32 and 17.87 (quaternary Cs of t-Bu), −4.59, −4.81 and −4.95 p.p.m. (Si-CH_3s).

3',5'-O-Bis (t-butyldimethylsilyl)-N^4-(5-aminopentyl)-2'-deoxycytidine (III): Compound II (8.9 g, 15.4 mmol) was dissolved in dry acetonitrile (100 ml) to which were added triethylamine (2.2 ml, 16 mmol) and dry 1,5-diaminopentane (15.7 g, 154 mmol). The solution was stirred overnight at room temperature under dry nitrogen. The now deep yellow solution was evaporated to dryness; t.l.c. in ethanol/dichloromethane (2 : 8 v/v) showed no starting material left. A new spot of R_f 0.17 due to product and a very high R_f yellow spot due to 2-nitrophenol were observed. The crude product was purified on a column of silica gel (100 g), eluting first with dichloromethane and then with a gradient from 10 to 20% ethanol in dichloromethane. Pure product fractions were pooled and evaporated *in vacuo* to give the desired product III as a yellow oil (7.24 g, 87%). ^{13}C n.m.r. spectrum ($CDCl_3$) δ : 163.23 (C-4), 156.06 (C-2), 137.85 (C-6), 95.31 (C-5), 86.43 (C-1'), 84.77 (C-4'), 70.07 (C-3'), 61.54 (C-5'), 41.29, 40.87 and 39.81 (-NH-CH_2, CH_2-NH_2, and C-2'), 31.87, 28.03 and 23.42 (C-2,3 and 4 of pentyl), 25.20 and 25.06 (CH_3s of t-Bu), 17.68 and 17.24 (quaternary Cs of t-Bu), −5.27, −5.56, −6.16 and −6.22 p.p.m. (Si-CH_3s).

3',5'-O-Bis(t-butyldimethylsilyl)-N^4-[5-(N-trifluoroacetyl)aminopentyl]-2'-deoxycytidine (IV) : Compound III (7.24 g, 13.4 mmol) was dissolved in dry dichloromethane (50 ml) and S-ethyl trifluorothioacetate (2.37 g, 15 mmol) was added with stirring. After 15 min t.l.c. in ethanol/dichloromethane (5 : 95 v/v) showed complete reaction with a product of R_f 0.4. Solvent was removed *in vacuo* and the residual oil was dried by evaporation of dry toluene. The title compound IV was obtained as a colourless viscous oil (8.54 g, 100%) which was pure by t.l.c. and n.m.r. spectroscopy. ^{13}C n.m.r. spectrum ($CDCl_3$) δ : 163.63 (C-4), 157.78 (carbonyl of trifluoroacetyl), 156.64 (C-2), 138.71 (C-6), 115.90 (CF_3), 95.75 (C-5), 87.06 (C-1'), 85.32 (C-4'), 70.62 (C-3'), 62.05 (C-5'), 41.76, 40.16 and 39.35 (-NH-CH_2, C-2' and CH_2-NH-CO), 27.98, 27.38 and 23.53 (C-4, 2 and 3 of pentyl), 25.64 and 25.48 (CH_3s of t-Bu), 18.04 and 17.71 (quaternary Cs of t-Bu), −4.86, −5.12, −5.74 and −5.80 p.p.m. (Si-CH_3s).

N^4-[5-(N-Trifluoroacetyl)aminopentyl]-2'-deoxycytidine (V) : The bis (silyl) compound IV (8.54 g, 13.4 mmol) in dry dioxan (100 ml) was treated with 1 M tetrabutylammonium fluoride in tetrahydrofuran (40 ml). T.l.c. in ethanol/dichloromethane (5 : 95 v/v) after 1 h showed complete conversion of starting material to a new spot of R_f 0.09. Water (10 ml) was added and the reaction mixture was evaporated to dryness *in vacuo*. The residue was dissolved in pyridine/methanol/water (200 ml, 3 : 1 : 1 v/v/v) and stirred for 30 min with 100 ml of pyridinium form Dowex 50. The resin was filtered off and washed with pyridine/methanol/water (2×100 ml, 3 : 1 : 1 v/v/v). The filtrate and washings were combined and evaporated *in vacuo*. After drying by evaporation of toluene, the crude product was purified by column chromatography on silica gel (100 g), eluting with dichloromethane followed by a gradient of 0−10% ethanol in dichloromethane. Pure

product fractions were pooled and evaporated *in vacuo* yielding the title compound V as a solid white foam (3.36 g, 66%). ^{13}C n.m.r. spectrum (DMSO -d_6) δ : 163.20 (C-4), 156.00 (carbonyl of trifluoroacetyl), 155.03 (C-2), 139.60 (C-6), 115.70 (quartet, CF_3), 94.57 (C-5), 87.08 (C-1'), 84.76 (C-4'), 70.41 (C-3'), 61.38 (C-5'), [-NH-CH_2, CH_2-NH-CO and C-2' under DMSO peak], 28.08, 27.90 and 23.62 p.p.m. (C-4, 2 and 3 of pentyl).

5'-O-Dimethoxytrityl-N^4-[5-(N-trifluoroacetyl)aminopentyl]-2'-deoxycytidine (VI) : The 2'-deoxycytidine derivative V (3.63 g, 8.9 mmol) was 5'-O-dimethoxytritylated in the usual way in dry pyridine (100 ml). Silica gel t.l.c. in ethanol/dichloromethane (1 : 9 v/v) showed complete reaction after 2 h with a product spot of R_f 0.33. After the usual work up the crude product was purified by column chromatography on silica gel (100 g), eluting with a gradient of 0 to 10% ethanol in dichloromethane containing 1% triethylamine. Pure product fractions were evaporated *in vacuo* yielding the title compound VI as a solid yellow foam (5.95 g, 94%). ^{13}C n.m.r. spectrum (DMSO-d_6) δ : 163.19 (C-4), 158.05 (C-4 of methoxyphenyls), 156.00 (carbonyl of trifluoroacetyl), 154.95 (C-2), 144.65 (C-1 of phenyl), 139.21 (C-6), 135.40 and 135.30 (C-1 of methoxyphenyls), 129.67 (C-2 and C-6 of methoxyphenyls), 127.77 and 127.68 (o-and m- Cs of phenyl), 126.66 (C-4 of phenyl), 115.70 (quartet, CF_3), 113.15 (C-3 and C-5 of p-methoxyphenyls), 94.55 (C-5), 85.70 (quaternary C between phenyls), 85.11 (C-1'), 84.57 (C-4'), 70.03 (C-3'), 63.40 (C-5'), 54.98 (CH_3-O), [-NH-CH_2, CH_2-NH-CO and C-2' under DMSO peak], 28.07, 27.91 and 23.62 p.p.m. (C-4, 2 and 3 of pentyl).

5'-O-Dimethoxytrityl-N^4-[5-(N-trifluoroacetyl) aminopentyl]-2'-deoxycytidine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) (VII) : To a solution of compound VI (5.95 g, 8.38 mmol) in dry dichloromethane (60 ml) under argon was added diisopropylethylamine (3.48 ml, 20 mmol) and 2-cyanoethoxy-N,N-diisopropylaminochlorophosphine (2.37 ml, 10 mmol). T.l.c. in ethanol/dichloromethane (1 : 9 v/v) containing 1% triethylamine showed complete reaction after 30 min, with a product spot of R_f 0.75. After the usual work up the crude product was purified by column chromatography on silica gel (100 g), eluting with triethylamine/dichloromethane (5 : 95 v/v). Pure product fractions were pooled and evaporated *in vacuo* leaving a white foam. This foam was dissolved in dichloromethane (10 ml) and the solution was added dropwise to vigorously stirred petroleum ether (200 ml) at −30°C. The precipitate was filtered cold and immediately dried *in vacuo*. The desired building block VII was obtained as a white powder (4.4 g, 57%). ^{31}P n.m.r. spectrum (CH_2Cl_2, concentric external D_2O lock) δ : 145.52 and 145.29 p.p.m.

Synthesis of urethane linked CPG supports

A base protected 5'-O-dimethoxytrityl-2'-O-methylribonucleoside (0.2 mmol) was first reacted with tolylene-2,6-diisocyanate (28.4 μl, 0.2 mmol; *CARE : SEVERE POISON*), and the resultant monoisocyanate was further reacted with 2 g of 500Å pore diameter aminopropyl CPG, using the previously described procedure for preparation of long chain alkylamine CPG-DNA supports (11). The capping procedure and estimation of the support loading were as described, using ϵ_{500nm} = 71.7 cm^2 μmol^{-1} for the released dimethoxytrityl cation. In typical experiments the following loadings were obtained:

2'-O-Methyluridine support ------ 32.3 μmol nucleoside g^{-1}; 2'-O-methylcytidine support ------ 42.2 μmol nucleoside g^{-1}; 2'-O-methyladenosine support ------ 40.8 μmol

nucleoside g^{-1}; 2'-O-methylinosine support ------ 35.6 μmol nucleoside g^{-1};

Synthesis of 2'-O-methyloligoribonucleotides

2'-O-Methyloligoribonucleotides were normally synthesised on a 0.5 μmol scale on the Applied Biosystems synthesiser, using the trityl on manual ending method. The 2'-O-methylribonucleoside building blocks and the activator, 5-(4-nitrophenyl)-1H-tetrazole were made up as 0.1 M solutions in anhydrous acetonitrile. The small scale β-cyanoethyl phosphoramidite DNA synthesis cycle was used with the condensation wait time increased to 6 min.

In order to synthesise 2'-O-methyloligoribonucleotides functionalised with primary amino groups, either 6-(N-monomethoxytrityl) aminohexyloxy, 2-cyanoethoxy, N,N-diisopropylaminophosphine was added as a chemical linker molecule to the 5'-terminus, or the base modified 2'-deoxycytidine building block VII was used for the last four base additions.

Deprotection and purification of oligonucleotides

The carrier bound fully protected 2'-O-methyloligoribonucleotide was treated with 25% ammonia solution for 72 h at 60°C in a sealed vial, to remove all protecting groups and free the terminal 3'-hydroxyl group from the carrier. The cooled solution was evaporated *in vacuo* and the residue was taken up in 0.5 ml of 0.1 M triethylammonium acetate solution pH 7. Brief centrifugation removed insoluble material generated from the CPG support. The DMTr or MMTr protected 2'-O-methyloligoribonucleotide was then purified by reversed phase h.p.l.c. After evaporation of solvent from the product peak the residue was treated with 80% acetic acid (30 min for cleavage of DMTr-O and 2 h for cleavage of MMTr-NH). The solution was diluted with an equal volume of water, washed 5 times with 2 volumes of diethyl ether, and the aqueous phase plus a few drops of triethylamine was lyophilised.

Biotinylation procedure

The lyophilised 2'-O-methyloligoribonucleotide (generally 5 to 20 A_{260} units) functionalised with one or four primary amino groups was dissolved in 0.3 M sodium acetate solution (0.5 ml, pH 5.5) and precipitated with cold absolute ethanol (1.5 ml) in an Eppendorf tube. The sample was kept for 1 h at −20°C and then centrifuged; after careful removal of supernatant the pelleted oligonucleotide was dissolved in 400 μl of 250 mM sodium carbonate/sodium bicarbonate buffer pH 9. NHS-LC-biotin (4 mg, 7.2 μmol) was added to the solution in two portions over a period of 1 h with thorough mixing. Analysis by reversed phase h.p.l.c. showed >95% biotinylation after a 3 h reaction, with the mono- or tetra- biotinylated oligonucleotide eluting at a slightly higher (few per cent) acetonitrile concentration than the unlabelled material. Using a reasonably shallow acetonitrile gradient pure tetrabiotinylated material was easily obtained in quantities up to $10A_{260}$ units. After removal of solvent *in vacuo* the purified biotinylated probes were stored at −20°C.

Kinasing and nuclease digestions

Kinase reactions were done, in a total volume of 25 ml, with 20 mM Tris acetate pH 7.9, 10 mM Mg acetate, 50 mg/ml glycogen, 25 mCi $g^{32}P$ ATP (5,000 Ci/mmol), 10 units T4 polynucleotide kinase and 100 pmol oligonucleotide, incubated at 37°C for 45 minutes. All nuclease digestions were done in a total volume of 20 ml, using buffer

conditions recommended by the supplier, glycogen at 50 mg/ml and 4 pmol of kinased oligonucleotide. Incubations were for 1hour at 30°C, except for nucleases CL3, U2 and micrococcal, which were done at 50°C.

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