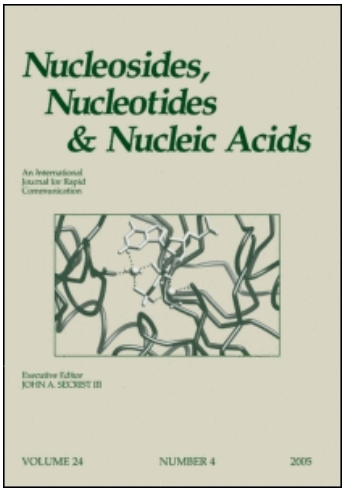

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Nucleotides LXIV[1]: Synthesis, Hybridization and Enzymatic Degradation Studies of 2'-O-Methyl-Oligoribonucleotides and 2'-O-Methyl/Deoxy Gapmers

Hagen Cramer[ab]; Wolfgang Pfleiderer[a]
[a] Fakultät für Chemie, Universität Konstanz, Konstanz, Germany [b] Gemini Technologies, Inc., Cleveland, OH, USA

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(10–12), 1765–1777 (2000)

NUCLEOTIDES LXIV[1] : SYNTHESIS, HYBRIDIZATION AND ENZYMATIC DEGRADATION STUDIES OF 2′-O-METHYL-OLIGORIBONUCLEOTIDES AND 2′-O-METHYL/DEOXY GAPMERS

HAGEN CRAMER[#] AND WOLFGANG PFLEIDERER*

Fakultät für Chemie, Universität Konstanz, Postfach 5560, 78434 Konstanz, Germany

In memoriam of Prof. Alexander Krayevsky

ABSTRACT. 2'-*O*-Methyloligoribonucleotides, deoxyoligonucleotides and 2'-*O*-methyl/deoxy gapmers were synthesized using solid phase phosphoramidite chemistry employing the 2-(4-nitrophenyl)ethyl (npe) protection strategy. Melting temperatures of the synthesized oligonucleotides as well as their stability against degradation by several different nucleases were determined. 2'-*O*-Methyloligoribonucleotides showed the highest melting temperatures (T_m's) whereas 2'-*O*-methyl/deoxy gapmers revealed either slightly higher or surprizingly no thermal stabilities compared with their deoxy analogs when using self-complementary sequences. Gapmers with four 2'-*O*-methyl nucleotides on both ends showed about the same stability as all 2'-*O*-methyloligoribonucleotides against micrococal nuclease, nuclease S_1, and snake venom phosphodiesterase.

INTRODUCTION. – 2'-Alkoxyoligoribonucleotides are a promising group of oligonucleotides as potential therapeutic agents [2-10] and as diagnostic probes [11]. These oligonucleotides have been shown to increase nuclease resistance [2,12-16] and exhibit good affinities for RNA targets [2,12,15,17]. By far the most often used 2'-modification is the 2'-*O*-methyl group due to its ease of preparation. The 2'-*O*-methyl phosphoramidites have been commercially available for several years now.

The 2'-*O*-methyl group is a naturally occurring modification found in RNA that enhances affinity for RNA targets due to the preference of 2'-*O*-methyl-modified sugars to adopt a C-3'-*endo* conformation [18,19]. Most antisense compounds rely on the action of RNase H for activity. RNase H degrades RNA only in a DNA/RNA hybrid but not in a 2'-*O*-methyl/RNA duplex. The only modifications tolerated by RNase H are 2'-deoxyoligonucletides with a negatively charged backbone [20], e. g. DNA with phosphorothioate or phosphorodithioate internucleotide linkages. Therefore, very often the gapmer approach is used, where a deoxy part in the middle of the oligonucleotide is flanked by a 2'-*O*-methyl

[#]Current address: Gemini Technologies, Inc., 11000 Cedar Ave., Cleveland, OH 44106, USA

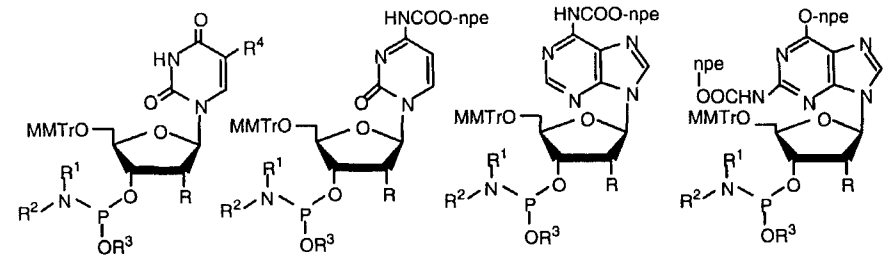
part. The deoxy part has to have a length of at least 5 residues to ensure good activation of human RNase H [8]. To further increase the antisense compound's stability against nucleases all phosphorothioate analogs of the deoxy/2'-*O*-methyl gapmers are often favored for *in vivo* experiments. But oligonucleotide phosphorothioates often show non-specific effects and therefore many controls are needed to verify that the obtained results rely on true antisense mechanisms [21-25].

Previously we described the detailed synthesis of homologous 2'-*O*-methyloligoribonucleotides using solid phase phosphoramidite chemistry employing the 2-(4-nitrophenyl)ethyl (npe) protection strategy [26]. Here we extend this approach towards the synthesis of 2'-*O*-methyloligoribonucleotides with up to 37 bases. In addition, we examined the hybridization properties of self-complementary and non self-complementary 2'-*O*-methyl, deoxy and mixed deoxy/2'-*O*-methyl sequences. We digested self-complementary and non self-complementary 2'-*O*-methyl, deoxy and mixed deoxy/2'-*O*-methyl sequences with nuclease S_1, micrococcal nuclease and snake venom phosphodieasterase (SVPD) in a time dependent manner using RP-HPLC for the analysis of the digested products.

SYNTHESES OF OLIGONUCLEOTIDES. – Oligonucleotides were synthesized on an automated DNA synthesizer using solid phase phosphoramidite chemi-stry and the 2-(4-nitrophenyl)ethyl (npe) protection strategy [26]. By using the 2-(4-nitrophenyl)ethyl and 2-(4-nitrophenyl)ethoxycarbonyl groups as base protecting groups, all protecting groups can be removed after oligonucleotide synthesis selectively by 1,8-diaza-bicyclo[5.4.0]-undec-7-ene (DBU) in aprotic solvents while the oligonucletide is still attached to the solid support. This offers the advantage of synthesizing very pure oligo-nucleotides in a direct manner. Therefore, oligonucleotides were collected as "trityl-off" products without being further purified. The quality of the oligonucleotides were confir-med by reversed-phase or anion exchange HPLC. The structures of the phosphoramidites em-ployed are listed in *scheme 1*. *Table 1* gives an overview of all oligonucleotides synthesized.

Any of the four differently substituted 2'-*O*-methyl phosphoramidites is suitable for making 2'-*O*-methyloligoribonucleotides using an automated DNA synthesizer [26]. Coupling efficiency usually exceeded 99%. Lower condensation yields were found for deoxy/2'-*O*-methyl gapmers due to poor quality of the deoxy phosphoramidites. For 2'-*O*-methyloligoribonucleotides containing cytidine and guanosine coupling times of 300 s were used. For the synthesis of homologous 2'-*O*-methyluridylates and 2'-*O*-methyladenylates coupling times of 2 min or less are sufficient [26]. All oligonucleotides were synthesized using a DBU-stable LCMAA-CPG support [27-29].

The synthesis of the 37-mer **39** required a 1000 Å support, while all other sequences were synthesized with glass beads having a pore size of 500 Å. The HPLC chromatogram of **39** (*figure 1B*) shows that the "npe strategy" is suitable for the synthesis of a 37 base long 2'-*O*-methyl oligoribonucleotides containing all four natural bases. *Figure 1A* shows



	R	R^1	R^2	R^3	R^4
1	OMe	Et	Et	npe	H
2	OMe	Et	iPr	npe	H
3	OMe	Me	iPr	npe	H
4	OMe	iPr	iPr	CE	H
5	H	iPr	iPr	CE	Me

	R	R^1	R^2	R^3
6	OMe	Et	Et	npe
7	OMe	Et	iPr	npe
8	OMe	Me	iPr	npe
9	OMe	iPr	iPr	CE
10	H	iPr	iPr	CE

	R	R^1	R^2	R^3
11	OMe	Et	Et	npe
12	OMe	Et	iPr	npe
13	OMe	Me	iPr	npe
14	OMe	iPr	iPr	CE
15	H	iPr	iPr	CE

	R	R^1	R^2	R^3
16	OMe	Et	Et	npe
17	OMe	Et	iPr	npe
18	OMe	Me	iPr	npe
19	OMe	iPr	iPr	CE
20	H	iPr	iPr	CE

MMTr = Monomethoxytrityl npe = 2-(4-nitrophenyl)ethyl- CE = 2-cyanoethyl-

Scheme 1

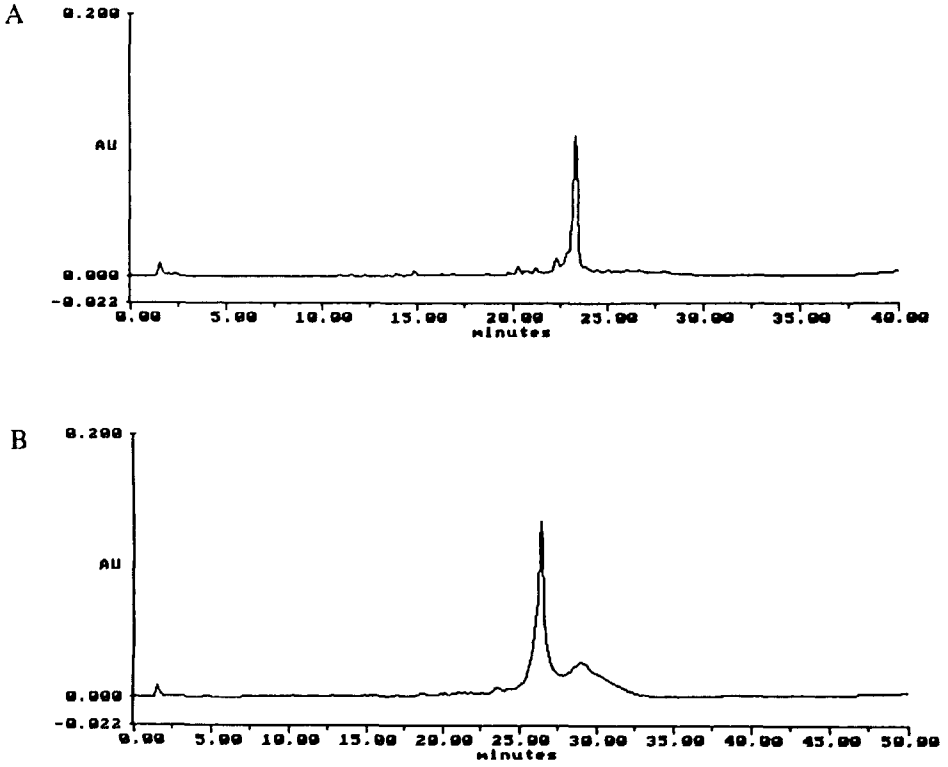


Figure 1: *Anion-exchange HPLC of the 18-mer* **37** (A) *and the 37-mer* **39** (B). Column: *NucleoPak PA-100*, 25 μm, 4 x 250 mm (*Dionex*); gradient: 0.02 M NaCl isocratic for 2 min, then 0.02-0.76 M NaCl in 30 min and 0.76-1.5 M NaCl in 5 min, 0.02 M NaOH, pH 12; flow: 1 ml /min.

Table 1. *Synthesized 2'-O-methyl, deoxy and mixed deoxy/2'-O-methyl sequences*

Sequence[a])	Amidites	Scale [μmol]	ASWY[b]) [%]	Yield [OD_{260}]
UUU UUU UUU U (**21**)	**1**	0.2	100	15
UUU UUU UUU UUU UUU UUU (**22**)	**4**	0.6	100	40
CCC CCC (**23**)	**9**	0.6	100	21
CCC CCC CCC C (**24**)	**8**	0.6	100	49
AAA AAA AAA A (**25**)	**13**	0.2	99.9	18
AAA AAA AAA AAA AAA AAA (**26**)	**12**	0.2	99.7	30
GGG GGG (**27**)	**19**	0.2	99.0	17
GGG GGG GGG G (**28**)	**16**	0.6	99.4	52
AUA UAU AUA UAU AUA UAU (**29**)	**3,13**	0.2	99.2	30
GCG CGC (**30**)	**6,16**	0.2	97.3	8
CGC GCG (**31**)	**6,16**	0.2	100	13
UGG AUC CA (**32**)	**1,9,14,16**	0.6	100	39
GGA CGC UAC U (**33**)	**2,7,12,17**	0.6	98.0	46
AGU AGC GUC C (**34**)	**1,9,14,16**	0.6	98.3	48
CCU GCG AUG A (**35**)	**1,6,11,16**	0.6	100	56
UCA UCG CAG G (**36**)	**1,9,14,16**	0.6	97.8	60
GGU UCC AUG CAU GGA ACC (**37**)	**1,6,11,16**	0.6	99.7	64
ACG UUC CUC CUG GGG GAA (**38**)	**1,6,11,16**	0.6	100	63
GGA GAG GUC UCC GGU UCG AUU CCG GAC UCG UCC ACC A (**39**)	**1,6,11,19**	0.6	100	116
GGt tcc atg cat gga aCC (**40**)	**6,18, 5,10,15,20**	0.6	96.7[c])	50
ggt tCC atg cau GGa acc (**41**)	**6,19, 5,10,15,20**	0.6	95.0[c])	38
ggt tcc atG CAU gga acc (**42**)	**4,9,14,19, 5,10,15,20**	0.6	95.7[c])	40
GGU Ucc atg cat ggA ACC (**43**)	**1,7,14,19, 5,10,15,20**	0.6	96.0[c])	68
ACg ttc ctc ctg cgg gAA (**44**)	**9,14, 5,10,15,20**	0.6	95.9[c])	64
ggt tcc atg cat gga acc (**45**)	**5,10,15,20**	n/a	n/a	n/a
acg ttc ctc ctg cgg gaa (**46**)	**5,10,15,20**	n/a	n/a	n/a

Coupling time 60 s for **21**, 120 s for **22**, **25**, **26**, **29** and 300 s for all other 2'-*O*-methyloligonucleotides

[a]): capital letters 2'-*O*-methyl, small letters deoxy, sequences listed in 5'-3' direction

[b]): average stepwise yield, taken from trityl values: solutions from the trityl port were diluted with 0.2 M p-TsOH in MeCN and their absorption determined photometrically

[c]): low coupling yields due to poor quality of deoxyphosphoramidites

the HPLC chromatogram of the 18-mer **37**. Obviously, the "npe strategy" yields sufficiently pure products for most applications without the need of further purification steps.

The 2'-*O*-methyloligonucleotide **38** and the self-complementary 2'-*O*-methyl sequence **37** were also synthesized as 2'-deoxy analogs **45** and **46**, respectively. In the sequences **40** and **41** four of the deoxynucleosides of **45** were replaced with 2'-*O*-methyl nucleosides at different locations, while **43** consisted of four 2'-*O*-methylnucleosides at the 3'- as well the 5'-end of the molecule. To apply the "npe strategy" to the synthesis of mixed deoxy/2'-*O*-methyloligonucleotides the deoxyribonucleotide phosphoramidites **5**, **10**, **15** and **20** were used [28,29].

MELTING TEMPERATURES. – The thermal stabilities of duplexes containing 2'-*O*-methyloligoribonucleotides were evaluated by recording UV-absorbance temperature profiles. Hereby, two complementary strands or a self-complementary strand were dissolved in concentrations of sodium phosphate buffer ranging from 0.003 M to 0.36 M (see *Table 2*). The melting temperature of $(A_m)_{10}$ (**25**)/$(U_m)_{10}$ (**21**) rose as expected from 25.8° to 27.9° when increasing the Na^+ concentration from 0.26 to 0.36 M. For the pair $(G_m)_{10}$ (**28**)/$(C_m)_{10}$ (**24**) the Na^+ concentration was reduced to 0.006 M and 0.003 M, respectively, to decrease the melting temperature to better detectable values. Corresponding oligodeoxyribonucleotides were found to have T_m values of 15.9° for the T/A pair and 52.6° for the G/C pair at Na^+ concentrations of 0.12 M [30].

The order of the nucleobases in an oligonucleoide plays an important factor on the T_m value as well. The homologous pair $(A_m)_{18}$ (**26**)/$(U_m)_{18}$ (**22**) shows a T_m value, which is almost 20° lower than the alternating self-complementary sequence $(A_mU_m)_9$ (**29**), whereas the homologous pair $(G_m)_6$ (**27**)/$(C_m)_6$ (**23**) has a melting temperature, which is on average about 10° higher than those of the self-complementary sequences $(G_mC_m)_3$ (**30**) and $(C_mG_m)_3$ (**31**). Sequence **30** and **31** demonstrate that, especially for shorter sequences, it is of importance which of the four bases is on the 5'- and 3'-end, respectively. The T_m value of $(G_mC_m)_3$ is about 6° higher than the one for $(C_mG_m)_3$. However, the decamer **33** shows about the same melting temperature with its complementary sequence **34** as the decamer **35** (which has a reversed sequence of **33**) with **36**.

The self-complementary sequence **37** showed a T_m value of 75.4°C at a Na^+ concentration of 0.03 M, 15°C higher than the one found for its deoxy analog **45**. However, the replacement of 4 deoxy bases at each end by 2'-*O*-methyl nucleotides (**43**) increased the melting temperature only by about 1°C. A surprising result was obtained when in the analogs **40**-**42** only 4 deoxyribonucleotides were replaced at different sites of the oligomer by the corresponding 2'-*O*-methylribonucleotides since no melting temperature at Na^+ concentrations of 0.03 - 0.3 M could be observed. This strange results may be attributed to relatively stable hairpin-structures in preference toduplex formation.

ENZYMATIC DIGESTION. – Sproat *et al.* [16] investigated enzymatic resistance of 2'-*O*-methyloligoribonucleotides in comparison to deoxy- and ribooligonucleotides

Table 2. *Melting Temperatures (Tm's)*

Sequence[a])	T_m [°C] (Na^+ conc. in M)
5'- AAA AAA AAA A -3' (**25**) 3'- UUU UUU UUU U -5' (**21**)	25.8 (0.26) 26.3 (0.30) 27.9 (0.36)
5'- AAA AAA AAA AAA AAA AAA -3' (**26**) 3'- UUU UUU UUU UUU UUU UUU -5' (**22**)	29.5 (0.03) 46.6 (0.30)
5'- AUA UAU AUA UAU AUA UAU -3' (**29**)[b])	47.5 (0.03)
5'- GGG GGG GGG G -3' (**28**) 3'- CCC CCC CCC C -5' (**24**)	75.4 (0.003) 79.5 (0.006)
5'- GGG GGG -3' (**27**) 3'- CCC CCC -5' (**23**)	62.5 (0.15)
5'- GCG CGC -3' (**30**)[b])	55.5 (0.15)
5'- CGC GCG -3' (**31**)[b])	49.7 (0.15)
5'- UGG AUC CA -3' (**32**)[b])	53.7 (0.15)
5'- GGA CGC UAC U -3' (**33**) 3'- CCU GCG AUG A -5' (**34**)	53.0 (0.03)
5'- CCU GCG AUG A -3' (**35**) 3'- GGA CGC UAC U -5' (**36**)	52.0 (0.03)
5'- ggt tcc atg cat gga acc -3' (**45**)[b])	60.4 (0.03)
5'- GGU UCC AUG CAU GGA ACC -3' (**37**)[b])	72.8 (0.01) 75.4 (0.03)
5'- GGU Ucc atg cat ggA ACC -3' (**43**)[b])	61.7 (0.03)
5'- GGt tcc atg cat gga aCC -3' (**40**)[b])	-[c]) (0.03)
5'- ggt tCC atg cau GGa acc -3' (**41**)[b])	-[c]) (0.03)
5'- ggt tcc atG CAU gga acc -3' (**42**)[b])	-[c]) (0.03) -[c]) (0.14)

[a]): capital letters 2'-*O*-methyl, small letters deoxy [b]): self-complementary sequence

[c]): no T_m found for Na^+ concentration indicated

against a variety of nucleases in a concentration-dependent manner. He and his co-workers studied the degradation of the oligonucleotides using polyacrylamide gel electrophoresis.

We used the endonucleases, micrococcal nuclease and nuclease S_1, and the 5'-exo-nuclease snake venom phosphodiesterase (SVP) for our investigations and followed the process of degradation of the oligonucletides in a time-dependent manner using reversed-phase HPLC. Since nuclease S_1 shows a preference for single stranded nucleic acids [31], only non self-complementary sequences like **38**, **44** and **46** were digested and showed with nuclease S_1 as well as with micrococcal nuclease (*figure 2*) many intermediate products which also on addition of phosphatase did not decrease the amount of products formed (data not shown). In contrast, however, degradation by SVP and addition of a large excess of phosphatase led to a conversion of the oligonucleotides directly to their nucleosides

Table 3. *Nuclease S_1 digestion (0.005 U/OD_{260})*

	percentage of degraded oligonucleotide after indicated time [a])							
Sequence [b])	5'	10'	15'	30'	2h	4h	24h	48h
acg ttc ctc ctg cgg gaa (**46**)	15	30	50	90	100			
ACg ttc ctc ctg cgg gAA (**44**)	20	40	70	100				
ACG UUC CUC CUG GGG GAA (**38**)	0	0	0	0	0	0	0	0

[a]): determined by comparing the integral of the oligonucleotide peak to the sum of the integrals of all digestion products

[b]): capital letters 2'-*O*-methyl, small letters deoxy, sequences listed in 5'-3' direction

Table 4. *Micrococcal nuclease digestion (0.005 U/OD_{260})*

	percentage of degraded oligonucleotide after indicated time [a])							
Sequence [b])	5'	10'	15'	20'	30'	2h	4h	24h
ggt tcc atg cat gga acc (**45**) [c])	100							
acg ttc ctc ctg cgg gaa (**46**)	100							
GGt tcc atg cat gga aCC (**40**) [c])	100							
ggt tCC atg cau GGa acc (**41**) [c])	100							
ggt tcc atG CAU gga acc (**42**) [c])	100							
GGU Ucc atg cat ggA ACC (**43**) [c])	20	30	40	50	60	70	75	85
GGU UCC AUG CAU GGA ACC (**37**) [c])	0	0	0	0	2	0	40	80
ACG UUC CUC CUG GGG GAA (**38**)	0	0	10	20	50	80	90	100

[a]), [b]): see *table 4* [c]): self-complementary sequence

Table 5. *Snake venom phosphodiesterase (SVP) digestion (0.0075 U/OD_{260})*

	percentage of degraded oligonucleotide after indicated time[a])						
Sequence [a])	5'	15'	30'	1h	4h	24h	48h
ggt tcc atg cat gga acc (**45**) [c])	95	100					
acg ttc ctc ctg cgg gaa (**46**)	98	100					
GGt tcc atg cat gga aCC (**40**) [c])	98	100					
ggt tCC atg cau GGa acc (**41**) [c])	98	100					
ggt tcc atG CAU gga acc (**42**) [c])	95	100					
GGU Ucc atg cat ggA ACC (**43**) [c])	15	25	40	55	65	80	90
GGU UCC AUG CAU GGA ACC (**37**) [c])	5	10	20	25	35	50	75
ACG UUC CUC CUG GGG GAA (**38**)	3	5	10	15	25	35	80

[a]), [b]): see *table 4* [c]): see *table 5*

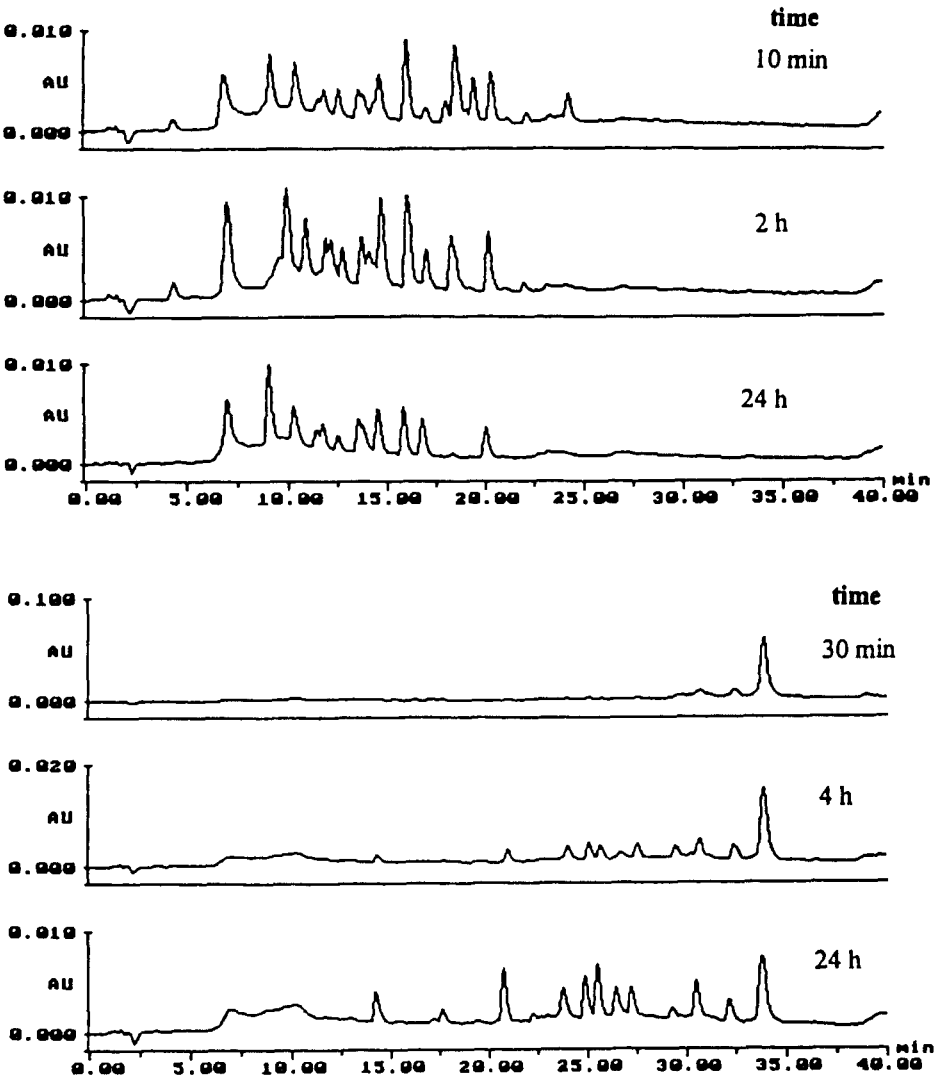


Figure 2. HPLC-chromatogram of the deoxy sequence **46** (top) and ist 2`-O-methyl analog **38** (bottom) when subjected to digestion with micrococcal nuclease at 45°C. Column: LiChrospher 100 RP-18, 5 µm, 4x125 mm (Merck); gradient: 0.1 M TEAAc pH 7 for 2 min and then 0-20% MeCN in 0.1 M TEAAc pH 7 within 33 min; flow 1 ml/min.

without any detectable formation of intermediates (see *figure 3*). Here, the composition of each degradation peak was confirmed by comparing the retention time with authentic samples, e. g. the deoxy and 2'-*O*-methyl nucleosides. Each enzyme was used in a concentration of about 0.005 U per OD_{260} of oligonucleotide.

The exchange of two deoxynucleotides by 2'-*O*-methyl nucleotides at both ends of the deoxy sequence **45** and **46** did not enhance its stability against any of the enzymes tested in our investigation (see *table 3* through *5*). There was also no difference in stability when a total of 4 deoxy nucleotides were replaced within the sequence (oligonucleotide **41** and **42**). However, sequence **43**, where 4 deoxy nucleotides of the oligodesoxyribonucleotide sequence **45** were replaced at its 5'- as well as 3'-end with 2'-*O*-methyl nucleotides, showed enhanced stability not only against the 5'-exonuclease snake venom phosphodiesterase but also against the endonuclease micrococcal nuclease as well. Incubating oligonucleotide **45** with micrococcal nuclease resulted in complete degradation within 5 min. However, sequence **43** was not completely digested even after 24 h under the same conditions. *Table 4* shows that the oligonucleotide stabilized by only four 2'-*O*-methyl nucleotides at both ends was almost as stable as its all 2'-*O*-methyl analog **37**. A similar result was found for the digestion with snake venom phosphodiesterase (see *table 5* and *figure 3*). In *figure 3* the undigested oligonucleotide **43** appears as two peaks. The self-complementary sequences **37** and **43** have similar melting temperatures slightly above 60 °C and both show two peaks in the reversed-phase HPLC chromatogram. Reinjection of one of the peaks resulted again in the formation of both peaks. Therefore, we concluded that the earlier eluting peak resembles the oligonucletide in its single stranded form while the later eluting peak resembles its duplex.

CONCLUSION. –The use of ß-eliminating groups as phosphate as well as aglycon protecting group ("npe strategy") proved to be very useful for the synthesis of 2'-*O*-methyl oligonucleotides as well as deoxy/2'-*O*-methyl gapmers up to a length of 37 bases. The advantage over standard approaches is that there is no need for purification steps after completion of the synthesis.

2'-*O*-Methyl oligonucletides showed an expected increase in their T_m's over deoxy sequences. We were able to show variations in the melting temperature when altering the sequence in 2'-*O*-methyl oligonucleotides. A 2'-*O*-methyl sequence has a greatly increased stability against the nucleases nuclease S_1, micrococcal nuclease and snake venom phosphodiesterase in comparison to its deoxy analog. The replacement of each of the four terminal bases of the oligodeoxyribonucleotide by 2'-*O*-methyl nucleotides led to a compound with almost the same stability against snake venom phosphodiesterase and micrococcal nuclease as the all 2'-*O*-methyl sequence itself.

EXPERIMENTAL SECTION

General: Synthesizer: *Applied Biosystems 380 B* and *392*. Reagents: DNA-grade

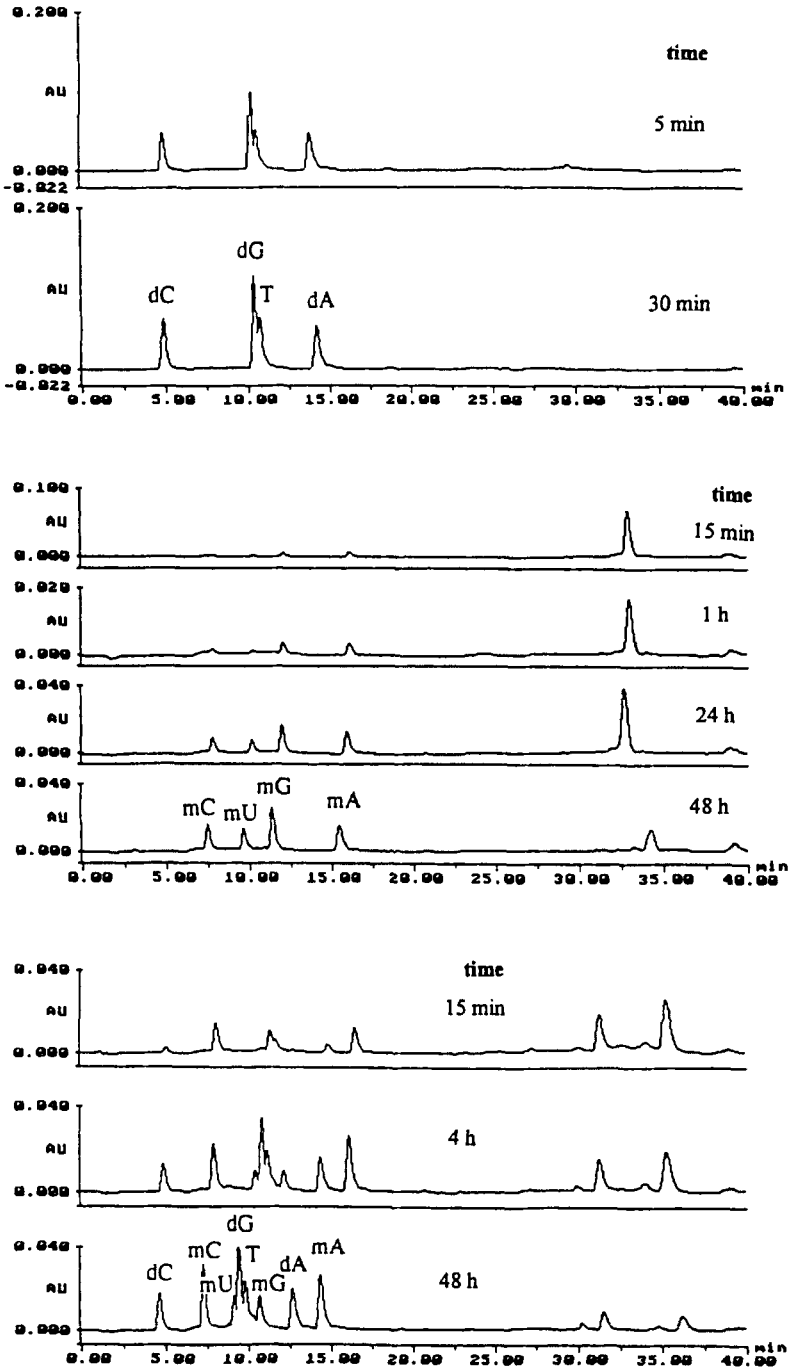


Figure 3: HPLC-chromatogram of the deoxy sequence **46** (top), the 2′-O-methyl sequence **38** (middle) and the mixed 2′-O-methyl/deoxy sequence **44** (bottom) when subjected to digestion with snake venom phosphodiesterase at 45°C. Column: see figure 2.

MeCN (<30 ppm H_2O); DBU *purum* was dried over molecular sieve; 1*H*-tetrazole was freshly sublimed; THF *p.a.* was freshly destilled from CaH_2; TCA, CH_2Cl_2, Ac_2O, 1-methyl-1*H*-imidazole, pyridine, I_2 and conc NH_3 soln. were used in *p.a.* grade, 2,6-dimethylpyridine in *purum* grade. Lyophilization: *Savant Speed-vac* concentrator in 1.5-ml *Eppendorf* tubes under high vacuum. HPLC of oligonucleotides: *Merck/Hitachi* system, gradient pump *L-6200*, interface *D-6000*, UV-detector *L-4000*, HPLC manager *D-6000* software. HPLC of enzymatic digestion studies: gradient pump *L-6200*, UV-detector *UVIKON 730 S LC*, intelligent auto sampler *AS 4000* with *Eppendorf* rack (parameter: X_1=32.5, Y_1=19.5, X_2=230.5, Y_2 =163.0, Z_1=47.5) and sharp edge needle: 20 µl sample loop, syringe capacity of 0.5 ml, lead and rear volume 0 µl, injection volume 20 µl. UV/Vis: *Perkin-Elmer, Lambda 15*. Melting temperatures: *Perkin-Elmer, Lambda 2* with computer controlled peltier thermostatted multi cell holder using the programs *PETEMP* and *PECSS*. Enzymes: Micrococcal nuclease (116 units/mg solid) and nuclease S_1 (400 U/µl, 75% in glycerol) were from *Sigma*, snake venom phosphodiesterase from *Crotalus durissus* (3 U/ml in 50% glycerol) and alkaline phosphatase (1000 U/ml) from *Boehringer Mannheim*.

Assembly of Oligonucleotides [26]. Syntheses were carried out using an *Applied Biosystems 380B* or *392* DNA synthesizer. Nucleoside-functionalized CPG material (**69-76** ; 0.6 or 0.2 µmol) was packed into a 1 µmol *ABI* crimp column. Cycles of nucleotide addition were carried out by a programmed series of reagent and solvent washes based on recommended procedures. For the npe/npeoc base deprotection the support was treated consecutively with a 1 M DBU solution in MeCN for a total time of 10.5 h before final cleavage with conc. ammonia.
The NH_3 solution was collected and lyophilized in a *Speed-vac* concentrator under high vacuum. The isolated amount of oligonucleotide was determined by measuring the absorbance at 260 nm.

Melting Temperatures. For measuring melting temperatures, 1000 µl of a self complementary solution or 500 µl of each of the two complementary oligonucleotide solutions having a concentration of 1 OD_{260}/ml were added to a 3 ml quarz cuvette. The concentration of sodium ions in the solution was adjusted by adding the appropriate amount of phosphate buffer (pH 7.4, 1.84 g NaH_2PO_4 x H_2O and 9.49 g Na_2HPO_4 x $2H_2O$ dissolved in H_2O and filled to 100 ml; containing 0.66 M PO_4^{3-} and 1.2 M Na^+ ions) and filling the cuvette with H_2O to a volume of 2 ml. To avoid evaporation, the oligonucleo-tide containing solution was topped with 200 µl of pentadecan. The UV measurements were made at a wavelength of 260 nm while stirring. The temperature was increased and decreased at a rate of 0.2°C/min. The T_m curve was constructed and the T_m value was determined by using the algorithms of the *Perkin-Elmer* software (see general). Each melting temperature determination was repeated three times.

Enzymatic Digestion. Nuclease S_1: A soln. of 2 OD_{260} of oligonucleotide **37**, **44**, or **46** in 350 µl of sodium acetate buffer (pH 4.6, 30 mM, 50 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol) was treated with 50 µl of nuclease S_1 stock (4 U; 1 µl in glycerol) in 20 ml of sodium acetate buffer (pH 4.6, 30 mM, 50 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol)) to give an enzyme concentration of 0.005 U/OD_{260}. The solution was incubated at 45° and aliquots of 50 µl were taken after 5, 10, 15 and 30 min and 2, 4, 24 and 48 h. Aliquots were heated immediately at 100° for 5 min to denature the enzyme.

Micrococcal Nuclease: A soln. of 2 OD_{260} of oligonucleotide **37**, **38**, **40**, **41**, **42**, **43**, **45**, or **46** in 350 µl of Tris HCl buffer (pH 9, 100 mM, 10 mM $CaCl_2$) was treated with 50 µl of 1:100 diluted micrococcal nuclease stock (23 U; 0.2 mg solid) in 1.15 ml of Tris HCl buffer (pH 9, 100 mM, 10 mM $CaCl_2$)) to give an enzyme concentration of 0.005 U/OD_{260}. The solution was incubated at 45° and aliquots of 50 µl were taken after 5, 10, 15, 20 and 30 min and 2, 4 and 24 h. To denature the enzyme 5 µl of 10% perchloric acid was added immediately after taking the aliquots. The aliquots were neutralized *prior* analysis with 3.5 µl of 10% NaOH.

Snake venom phosphodiesterase (SVPD): 2 OD_{260} of oligonucleotide **37**, **38**, **40**, **41**, **42**, **43**, **45**, or **46** in 375 µl of Tris HCl buffer (pH 8, 50 mM, 10 mM $MgCl_2$) was treated with 5 µl (0.015 U) of SVPD solution (stored for 1 year at 4 ° in 50% glycerol, therefore actual concentration might be lower) and 20 µl (20 U) of alkaline phosphatase. The solution was incubated at 45° and aliquots of 50 µl were taken after 5, 15 and 30 min and 2, 4, 24 and 48 h. Aliquots were heated immediately at 100° for 5 min to denature the enzyme.
Samples were centrifuged and supernatant was analyzed with reversed-phase HPLC: *LiChrospher 100 RP-18* column (5 µm, 4 x 125 mm; *Merck*); flow rate 1 ml/min; gradient: isocratic for 2 min at 100% A, 0-40% B in A within 33 min, 40-100% B in A within 3 min followed by isocratic conditions for another 2 min, where A was 0.1 M $AcO(NEt_3)$ pH 7 and B 50% MeCN in A.

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