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Pharmaceutica Acta Helvetiae 73 (1998) 3–10

PHARMACEUTICA ACTA HELVETIAE

Investigations on the influence of 2′-*O*-alkyl modifications on the base pairing properties of oligonucleotides

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Received 1 October 1997; accepted 8 October 1997

Abstract

The antisense strategy has gained wide acceptance as a promising drug development concept. Antisense drugs hybridize with selected complementary sequences in a highly specific manner. However, as a main prerequisite for extensive therapeutic use of this new class of drugs, selected structural modifications are required to adjust pharmacokinetic and pharmacodynamic behavior. In continuation of our earlier investigations on 2′-*O*-modified oligonucleotides (Cotten, M., Oberhauser, B., Brunar, H., Holzner, A., Issakides, G., Noe, C.R., Schaffer, G., Wagner, E., Birnstiel, M.L., 1991. 2′-*O*-methyl, 2′-*O*-ethyl oligoribonucleotides and phosphorothioate oligodeoxyribonucleotides as inhibitors of the in vitro U7 snRNP-dependent mRNA processing event. Nucleic Acids Res. 19, 2629–2635; Wagner, E., Oberhauser, B., Holzner, A., Brunar, H., Issakides, G., Schaffner, G., Cotten, M., Knollmüller, M., Noe, C.R., 1991. A simple procedure for the preparation of protected 2′-*O*-methyl or 2′-*O*-ethyl ribonucleoside-3′-*O*-phosphoramidites. Nucleic Acids Res. 19, 5965–5971) further series of modified oligonucleotides containing different modified 2′-*O*-adenosines have been synthesized. On the one hand linear alkyl moieties of increasing length, on the other hand oxyethylene moieties of corresponding length were introduced at the 2′-*O*-position of adenosine. Following another approach a cationic charge was introduced by insertion of an aminohexylmodification at the 2′-*O*-position of adenosine (Brunar, H., Haberhauer, G., Werner, D., Noe, C.R., 1994. 2′-*O*-Modified oligonucleotides: Synthesis and biophysical analysis. Eur. J. Pharm. Sci. 2, 150). Molecule dynamics simulations had shown that this cationic modification upon duplex formation leads to both intra- and interstrand interactions. To determine the influence of the different modifications, such as cationic charge, alkyl chainlength and introduction of oxygen into the chain, on duplex stability melting temperatures were measured by recording circular dichroism versus temperature.

Keywords: 2′-*O*-alkyl modifications; Pairing properties; Oligonucleotides

1. Introduction

As a result of the great progress in biological and biochemical research direct action upon genes to correct malfunctions or defects has become feasible in principle within the concept of gene therapy. Due to their sequence specific interaction with target sequences, even rather short antisense oligonucleotides may be used as drugs in this type of therapy. However, the application of these compounds is not only restricted to spectacular therapies of cancer or viral diseases, but might even lead to rather general alternatives in pharmacotherapy. Nowadays many new drugs result from a systematic search for antagonists for different receptors. Taking into account that also receptor proteins are encoded by genes, a certainly significant advance in receptor oriented pharmacotherapy might result from the development of antisense drugs which suppress receptor gene expression. Last but not least, short oligonucleotides interact not only with nucleic acids but also with nucleic acid binding proteins and other proteins, thus opening the new therapeutic approach of aptamers.

Abbreviations: CD: circular dichroism; DNA: deoxyribonucleic acid; ODN: oligodeoxynucleotide; RP-HPLC: reversed phase HPLC

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0031-6865/98/$19.00
PII S0031-6865(97)00041-1

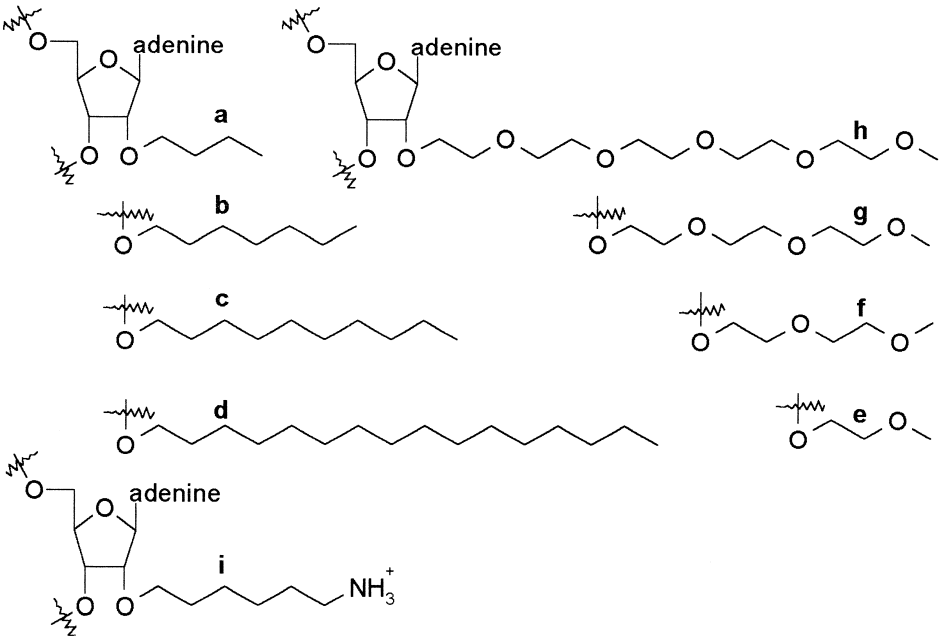


Fig. 1. Selected 2′-*O*-modifications.

The physicochemical properties of oligonucleotides, which differ greatly from the nature of traditionally applied drugs, have hitherto presented the greatest obstacle in their application as drugs. They are oligomeric compounds with a high molecular weight. Due to their polyanionic character, oligonucleotides do not penetrate lipophilic cell membranes easily. There is no previous experience on their pharmacokinetic behavior. The mechanisms of the distribution and the cellular uptake of ODNs are poorly understood. Data available indicate that the cellular uptake is a saturable receptor-mediated endocytosis and depends on temperature, concentration, length, specific sequences and modification [1]. In addition, the phosphodiester backbone is rapidly degraded by serum and cellular nucleases. Taking all these factors into account, it is not surprising that in the meantime various approaches have been reported to adjust the properties of ODNs to the therapeutical requirements.

Most of the reported ‘first generation’ modifications of ODNs were changes of the phosphodiester backbone for isosteric groups. In addition, replacement of the natural sugars ribose and deoxyribose, respectively by other sugars or rings has been described (for a review see Englisch and Gauss, 1991; Reddy, 1996). A third group of modifications comprises attachment of a variety of different moieties to the nucleobases or the hydroxy groups of the ribose. Among these, the modification of oligonucleotides at the 2′-position of the sugar has been shown to enhance nuclease stability.

In continuation of our earlier investigations on 2′-*O*-modified oligonucleotides (Cotten et al., 1991; Wagner et al., 1991) we were interested in conducting a systematic study on the effect of different modifications at the 2′-position. A decrease in duplex stability of long chain alkyl modifications has been reported by others (Sproat et al., 1991). Therefore the influence of a systematic increase in alkyl chainlength was studied first. Bearing in mind that long alkyl chains tend to form coils, which might be responsible for the loss of pairing properties due to steric hindrance, we decided to study bioisosteric oxyethylene chains with corresponding chainlengths (Fig. 1a–h). It is known that due to stereoelectronic effects (gauche-effect) such chains tend to adopt rather linear helical conformations.

Following a reaction sequence worked out earlier by our group (Birnstiel et al., 1991), the 2′-*O*-modifications were first attached to adenosine, followed by automated oligomerization using standard 2-cyanoethyl phosphoramidite chemistry with extended coupling times. A series of modified dodecamers were synthesized, incorporating up to six 2′-*O*-alkyl nucleotides within the sequence. Double strand geometry and hybridization thermodynamics of

[1] (Erickson and Izant, 1992; Crooke, 1995)

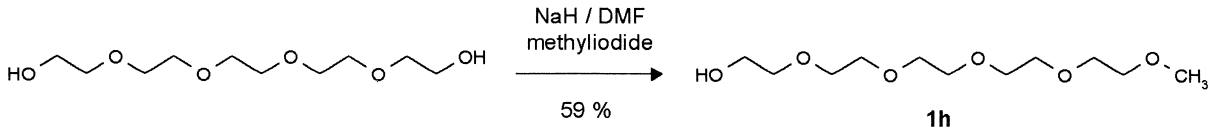


Fig. 2. Synthesis of pentaethylene glycol monomethylether.

these model compounds were studied systematically. The effects were compared with those of a zwitterionic modification (Fig. 1i), which seemed to support double strand formation.

Such modifications are not unknown in principle. Some 2′-*O*-alkylmodifications have been attached to adenosine (Lesnik et al., 1993), ODNs had been synthesized and their double strand geometry and hybridization thermodynamics were studied. At the time of the present study oxyethylene moieties were introduced into the 2′-position of thymidine (Martin, 1995). ODNs containing some substituted thymidines in the middle of the sequences have been synthesized and hybridization properties had been investigated.

2. Results and discussion

2.1. Synthesis of modified building blocks

According to the method developed earlier for the preparation of 2′-*O*-methyl and 2′-*O*-ethyl nucleotides (Wagner et al., 1991), all 2′-*O*-alkyladenosines were prepared via direct alkylation of unprotected adenosine (Fig. 1). To prepare the modifications a to d (Fig. 1), we used

Table 1
Synthesis of the 2′-*O*-alkyladenosines reported in Fig. 3

Compound	Formula (mw)	Mp (°C)	Yield (%)	^{1}H-NMR: δ (ppm); J (Hz); H-C(1′)	Elemental analysis (C, H, N) or MS (ESI)
1c	$C_{14}H_{21}N_5O_4$	156–158	48	5.99 (d, $J = 6$)[a]	52.28, 6.72, 21.45
2c	$C_{17}H_{27}N_5O_4$	127	12	5.84 (d, $J = 2.6$)[b]	54.58, 7.4, 18.73 (0.5 H_2O)
3c	$C_{20}H_{33}N_5O_4$	117	37	5.96 (d, $J = 6$)[a]	58.98, 8.22, 17.11
4c	$C_{26}H_{45}N_5O_4$	116	18	5.95 (d, $J = 4.2$)[a]	66.78, 8.33, 11.83
5c	$C_{13}H_{19}N_5O_5$	124	16.8	5.89 (d, $J = 5.9$)[c]	48.21, 6.18, 18.94(0.3 H_2O)
6c	$C_{15}H_{23}N_5O_6$	oil	22	6.0 (d, $J = 4.5$)[a]	367.9 [M^+]
7c	$C_{17}H_{27}N_5O_7$	oil	24	6.0 (d, $J = 4$)[d]	49.48, 6.5, 16.89
8c	$C_{21}H_{35}N_5O_9$	oil	10.7	5.88 (d, $J = 7.68$)[d]	49.73, 7.14, 13.49 (0.4 H_2O)

Solvents: [a]DMSO-d_6, [b]$CDCl_3$:CD_3OD 1:2, [c]$CDCl_3$:DMSO-d_6 2:1, [d]$CDCl_3$.

Table 2
Phosphitylation of protected 2′-*O*-alkyladenosines (Fig. 4)

Compound	Formula (mw)	Mp (°C)	Yield (%)	^{31}P – NMR: δ (ppm); $CDCl_3$	Elemental analysis (C, H, N) or MS (ESI+)
6a	$C_{51}H_{60}N_7O_8P$	45	99	151.35, 150.65	930.06 [M+H]$^+$
6b	$C_{54}H_{67}N_7O_8P$	58	85	151.26, 150.58	973.7 [M+H]$^+$
6c	$C_{57}H_{70}N_7O_8P$	54	88	151.49, 150.77	66.64, 6.85, 9.55 (0.8 H_2O)
6d	$C_{63}H_{84}N_7O_8P$	44	92	151.51, 150.76	67.95, 7.67, 8.71 (0.9 H_2O)
6e	$C_{51}H_{60}N_7O_{10}P$	34	92	152.44, 150.92	961.06 [M+H]$^+$
6f	$C_{52}H_{62}N_7O_{10}P$	37	85	151.12, 150.64	977.1 [M+H]$^+$
6g	$C_{61}H_{70}N_7O_7P$	45	99	151.35, 150.8	1125.0 [M+H]$^+$
6h	$C_{58}H_{74}N_7O_{13}P$	40	92	151.09, 150.64	1109.8 [M+H]$^+$

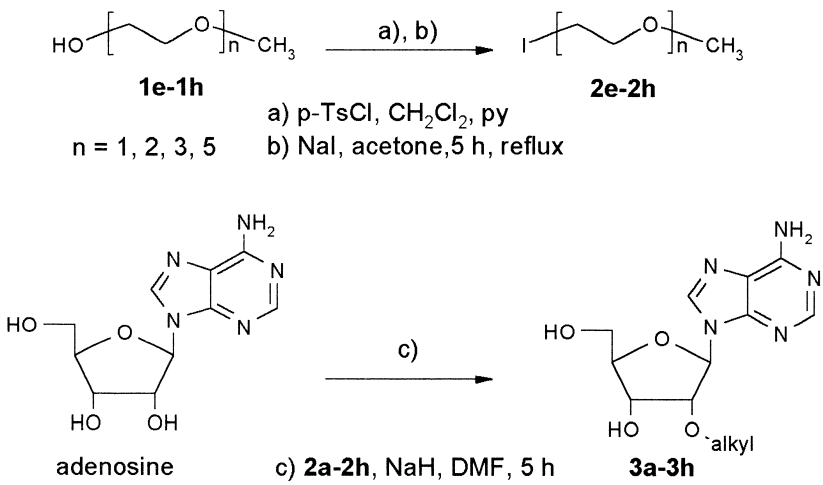
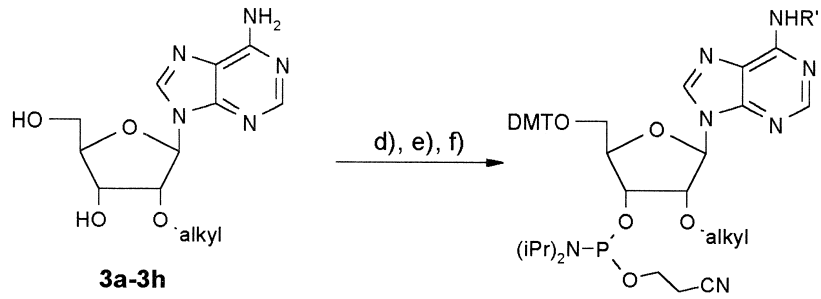


Fig. 3.

butyliodide (**2a**), heptyliodide (**2b**), decyliodide (**2c**) and hexadecyliodide (**2d**). The alkylation reagents for the oxyethylene modifications were prepared from mono-, di- or triethylene glycol monomethyl ethers (**1e**–**1g**). With the pentaethylene glycol modification only the diol was available. Therefore pentaethylene glycol was deprotonated with NaH/DMF and alkylated using 1.2 eq methyliodide to form the corresponding monomethyl ether (**1h**) in 59% yield. The dimethyl ether obtained as a side product in an extent of 5.5% was easily removed by vacuum-flash-chromatography (Fig. 2).

The monomethyl ethers were tosylated and converted to the iodides by a *Finkelstein* reaction. The synthesis of the 2′-*O*-alkyladenosines followed the same procedures as outlined earlier (Wagner et al., 1991). After selective deprotonation of the 2′-hydroxy group of adenosine by use of NaH/DMF and addition of the alkylation reagents the 2′-*O*-ether linkages were formed in the given yields (Table 1).

Synthesis of the 2′-*O*-modified adenosine building blocks was followed by standard protection procedures established for automated ODN synthesis. First the exocyclic amino function was protected in order to avoid side reactions. Both the phenoxyacetyl and the benzoyl group were used for this purpose applying the method of *transient protection* (Ti et al., 1982). For protection of the 5′-hydroxy function the di-*p*-methoxytrityl group was used (Wagner et al., 1991). Phosphitylation (Table 2) of the protected alkylated nucleosides was accomplished as described (Sinha et al., 1984). The reaction products were purified by chromatography. Control of purity of these nucleoside phosphoramidites included use of ^{31}P-NMR. The phosphoramidite (**6i**) bearing the trifluoracetamide protected 2′-*O*-aminohexyl modification (i) was synthe-



d) TMSCl, BzCl (to give **4a-4d**, **4f-4h**), $(PhOCH_2CO)_2O$ (to give **4e**), py
e) DMTCl, py, CH_2Cl_2
f) $Cl(N(iPr)_2)POCH_2CH_2CN$, $EtN(iPr)_2$, CH_2Cl_2

Fig. 4.

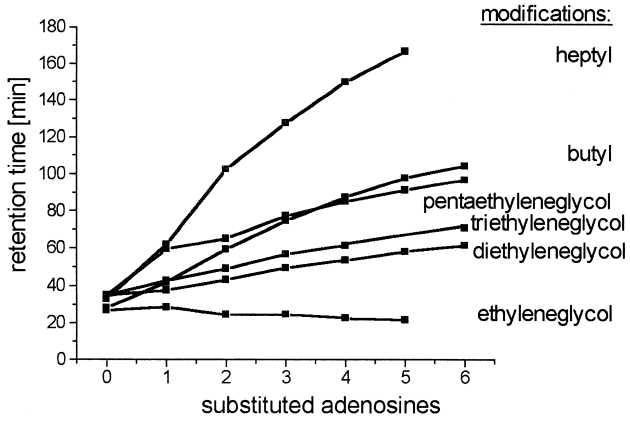


Fig. 5. Retention times of the test oligonucleotides **7a**–**b**,**e**–**i** to **12a**–**b**,**e**–**i** referring to an unmodified A12. **7c**–**d** to **12c**–**d** were not included, they were too lipophilic to be analyzed via RP-HPLC.

sized as described earlier (Brunar, 1993; Noe and Brunar, 1997).

2.2. Synthesis of modified oligonucleotides

The phosphoramidites **6a**–**6i** were oligomerized using an automated DNA synthesizer applying standard 2-cyanoethyl phosphoramidite chemistry with extended coupling times. To evaluate the influence of the modifications on the properties of oligonucleotides a series of test dodecamers was synthesized in a pattern designed earlier (Brunar et al., 1994). They bore modifications at the 5′-end in an increasing number up to six modified nucleotides. Position and extent of substitution of natural nucleotides in oligonucleotide dodecamers are shown in Table 3.

Coupling yields for incorporation of 2′-*O*-alkyladenosine varied between 70 and 99%. All oligonucleotides were purified by trityl off reversed-phase HPLC or anion exchange chromatography. ODNs containing 2′-*O*-decyl and hexadecyl adenosine (**7c,d**–**12c,d**) proved to be too lipophilic to be separated by reversed-phase HPLC. ODNs with these modifications were purified by anion exchange chromatography performed on a DEAE-material with a NaCl-gradient (Rockstroh et al., 1988). The integrity of ODNs was verified by MALDI-TOF mass spectra indicating that modified adenosines had been successfully incorporated into the oligomers.

Table 3
Modified dodecamers

(7a–i)	5′-AAA AAA AAA AAA-3′
(8a–i)	5′-AAA AAA AAA AAA-3′
(9a–i)	5′-AAA AAA AAA AAA-3′
(10a–i)	5′-AAA AAA AAA AAA-3′
(11a–i)	5′-AAA AAA AAA AAA-3′
(12a–i)	5′-AAA AAA AAA AAA-3′

2.3. HPLC-characterization

Before purification all modified ODNs were analyzed by reversed-phase HPLC using the same gradient program. Retention times were taken as indicators on the lipophilicity of the different sequences.

Retention times were consistent with full length oligomers. As expected, Fig. 5 shows prolonged retention times with increasing number of introduced modified 2′-*O*-adenosines. It also demonstrates the differences in lipophilicity between alkyl and oxyethylene modifications. The change from alkyl chain to the corresponding oxyethylene chain led to a dramatic increase in hydrophilicity.

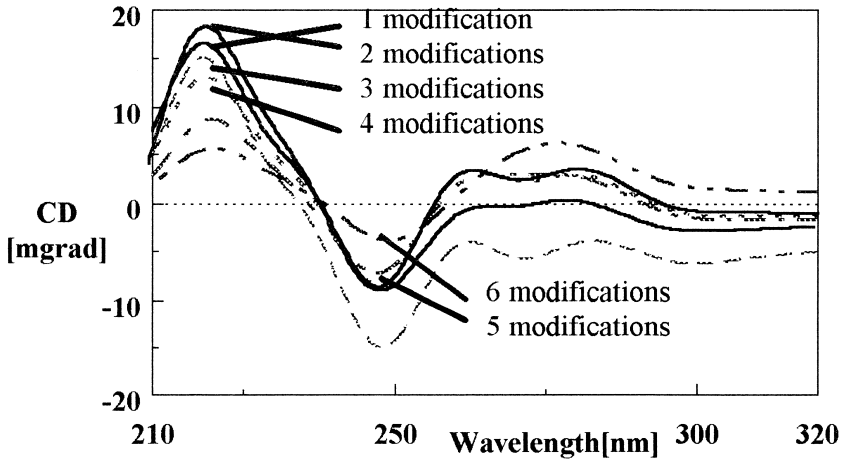


Fig. 6. CD-spectra of duplexes formed upon hybridization of T12 with pentaethyleneglycol modified dA12 **7h**–**12h** (0°C; 9 μM; 0.15 M NaCl, 0.01 M Tris–HCl; pH 7).

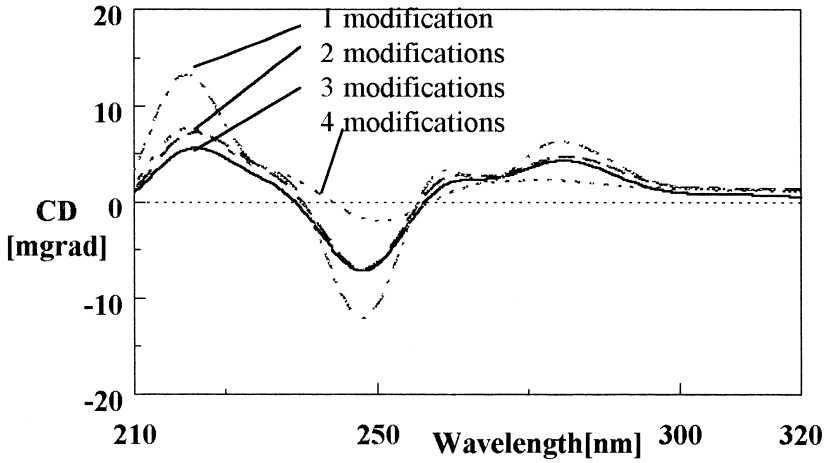


Fig. 7. CD-spectra of duplexes formed upon hybridization of T12 with hexadecyl modified dA12 **7d**–**10d** (0°C; 9 μM; 0.15 M NaCl, 0.01 M Tris–HCl; pH 7).

2.4. CD-spectra of RNA–DNA hybrids

A main point of interest was the change of duplex geometry and stability caused by different modifications and different extent of modification. Generally, a transition from B to A form occurs within a mixed hybrid, when a DNA–DNA hybrid segment (B-form) transforms to a RNA–DNA hybrid (A-form) (Wang et al., 1982). It is also known that a hybrid duplex containing purine-rich RNA segments adopts a conformation with more A-form similarities than B-form similarities (Ratmeyer et al., 1994). To investigate the duplex geometry of these dodecamers, they were hybridized to an unmodified T12 strand.

In Figs. 6 and 7 selected examples of CD-spectra are given. All spectra of the modified ODNs took characteristic B-form shapes. With increasing extent of modifications a flattening out of the curves was detectable. This effect was more distinct in the alkyl series than the oxyethylene series. However, a complete switch of double helix conformation of a 2′-*O*-modified RNA–DNA hybrid duplexes into an A form could not be found, regardless of the extent of modification. CD peaks of the hybrid duplexes both at 220 nm and 265 nm, bore in all cases resemblance to the B form shape of the dA12-T12 duplex.

2.5. Melting behavior

Measurement of the melting temperatures is a common method to determine duplex stability. In this investigation, duplex stabilities were measured by recording temperature dependences of the CD signal at 250 nm in a temperature range from 0–70°C. Oligonucleotide concentrations were 9

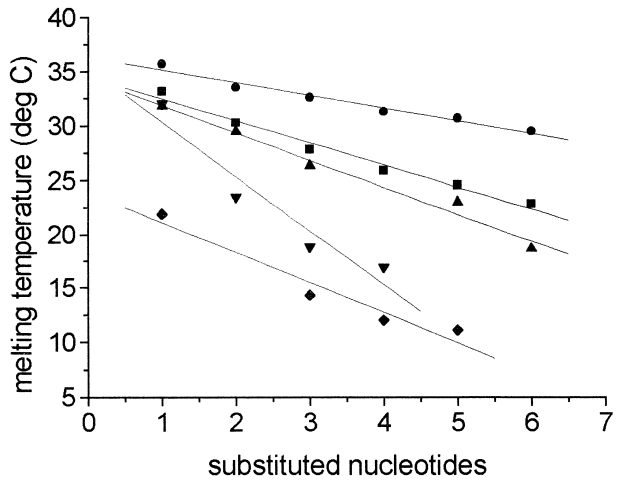


Fig. 8. Melting temperature versus strand substitution in 2′-*O*-alkyl series with **i** (circle), **a** (square), **b** (up triangle), **c** (down triangle), **d** (diamond) modified ODNs. Concentration (each strand) is 9 μM.

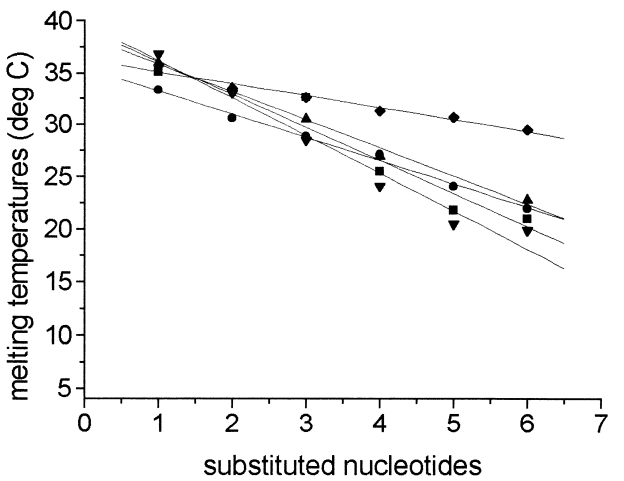


Fig. 9. Melting temperature versus strand substitution in 2′-*O*-oxyethylene series with **i** (circle), **e** (square), **f** (up triangle), **g** (down triangle), **h** (diamond) modified ODNs. Concentration (each strand) is 9 μM.

μM for each strand. All modified oligonucleotide dodecamers were hybridized to T12.

Sequences **7i**–**12i** exhibited melting temperatures which were higher than those of all other modifications. This supports the assumption of a stabilizing effect of aminohexyl modifications and this effect exceeded that of all other shown modifications. Taking into account that the repulsion of the negatively charged backbones of the complementary strands is a destabilization factor in double helix formation, melting temperatures of **7i**–**12i** support the concept of duplex stabilization through charge neutralization by introduction of zwitterionic sites. Whether an additional stabilizing effect is exerted by interstrand salt formations between phosphate anions and ammonium cations, as suggested by molecule dynamics simulations, can not be decided at present.

Not surprisingly, a comparison between the alkyl series (Fig. 8) and the oxyethylene series (Fig. 9) showed lower melting temperatures and thus lower duplex stabilities of the alkyl series. Unpredictable however, was the result that ODNs with only one modified oxyethylene nucleotide **7e**,**g**,**h** exhibited higher melting temperatures when compared to unmodified A12–T12 (34.8°C (9 μM)), while upon introduction of further modified units an expected slight decrease in duplex stability was observed.

2.6. Thermodynamic data of 2′-O-modified sequences

Duplex free energy values were determined from melting curves by plotting $1/T_m$ versus the natural logarithm of the total oligonucleotide concentration. Linear regression leads to determination of ΔH, ΔS and ΔG (Table 4) (Marky and Breslauer, 1987).

In the alkyl series **7a**–**c** the data exhibited a decrease with increasing chainlength Fig. 10. The ΔG of **7d** showed a value similar to **7a**. In the oxyethylene series, **7e**,**g**,**h** showed values higher than the unmodified A12–T12. This indicated stabilization forces of the oxyethylene moieties. This effect had a minimum at the **7f** modification. The ΔG value of **7i** was similar to the modifications of corresponding chainlength **7b** and **7f**.

Table 4
Thermodynamic data

Modification	ΔH (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})	ΔG (kJ mol^{-1})
a	−251.42	−713.74	−38.62
b	−162.80	−425.69	−35.88
c	−250.28	−740.96	−29.37
d	−276.5	−798.57	−38.41
e	−361.18	−1064.25	−43.87
f	−133.84	−328.73	−35.83
g	−308.06	−892.03	−42.1
h	−255.99	−717.36	−42.1
i	−328.89	−996.87	−31.68
Unmodified	−318.92	−929.45	−41.81

The calculations have been accomplished by choosing $T = 298.15$ K according to literature (Marky and Breslauer, 1987). Values are (kJ mol^{-1}).

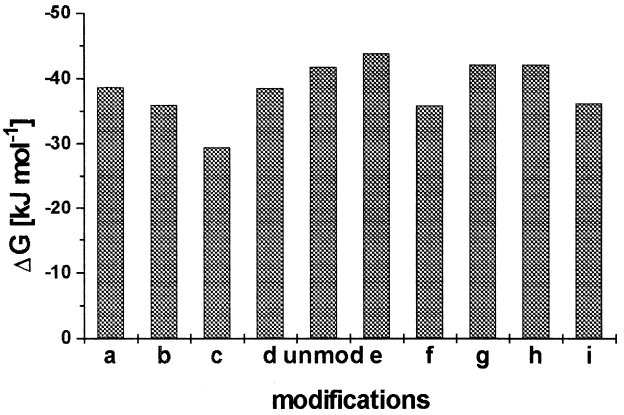


Fig. 10. The calculated ΔG of the modifications **7a**–**7i** including an unmodified A12–T12.

3. Conclusion

By its ability to reduce the net charge of the duplex, the aminohexyl modification lead to duplex stabilization. Also, oxyethylene modifications showed stabilization properties in case of introduction of only one moiety. However, in all cases an increase of extent of modification resulted in melting temperatures below the unmodified A12–T12 duplex. The decrease in melting temperatures took the following order:

aminohexyl > oxyethylene > alkyl modifications.

The steric hindrance exerted by a bulky alkyl 2′-*O*-moiety was compensated by the neutralizing effect generated by introduction of an amino group. Also, oxyethylene chains which might exert positive effects in therapeutic applications due to their amphiphilic character, exhibited in all cases increased duplex stability when compared to the corresponding alkyl modifications. These results indicate strongly that the introduction of zwitterionic modifications or oxyethylene chains into the 2′-*O*-position might provide useful tools for the modulation of properties of antisense oligonucleotides in their development as drugs.

4. Experimental protocols

4.1. Chemistry

For details of the chemical synthesis of the phosphoramidites **6a**–**6h** see Werner and Noe (1997). For details of the phosphoramidite **7i** see Brunar et al. (1997).

4.2. HPLC-analyses

Solutions of crude oligonucleotides were filtered on Schleicher and Schuell Spartan 13/20, 0.45 μm Braunrand H filters, concentrated via speed vac and redissolved in 300 μl H_2O (MilliQ) for determination of oligonucleotide concentrations by UV measurement (260 nm). For preparative HPLC the volume of oligonucleotide containing solution was reduced under 1000 μl and injected to reverse phase VarioPrep ET 250/10 Nucleosil® 100-7 C18 (Macherey Nagel). A solvent gradient of triethylammonium acetate (100 mM, pH 7.0) (A) and 80% CH_3CN in triethylammonium acetate (100 mM, pH 7.0) (B) was used as mobile phase (flow rate 16 ml/min; 30–50 psi). Purified oligonucleotides from preparative HPLC were lyophilized and redissolved in 500 μl H_2O (MilliQ), desalted on Waters SEP-PAK® columns, concentrated via speed vac and redissolved in 300 μl H_2O (MilliQ). Portions of 20 μl (0.2 mg/ml) were injected to a ET 250/4 Nucleosil® 100-5 C18 (Macherey Nagel) column for analytical HPLC (flow rate 1 ml/min; 150–160 psi).

4.3. CD-spectroscopy

Concentrations of purified and desalted oligonucleotides were determined by UV measurement at 260 nm using molar extinction coefficients of 15,400 cm^{-1} M^{-1} for the dA-, 2′-*O*-alkyl A residues and 8800 cm^{-1} M^{-1} for the T residues. Aliquots of purine and pyrimidine oligonucleotides were transferred into microcentrifuge tubes mixed with 30 μl of a 1.5 M NaCl, 0.1 M Tris–HCl buffer at pH 7.0 and enough H_2O (MilliQ) to a total volume of 300 μl. The oligonucleotide solutions were allowed to hybridize at 60°C for 3 min, then cooled to RT and transferred into a 0.1 cm pathlength cuvette. CD-spectra of the oligonucleotide duplexes were obtained at 10°C in a wavelength range of 320 to 210 nm. Temperature dependent measurement of ellipticity was performed from 0–60°C with a temperature slope of 50°C per hour. A range of oligomer strand concentrations and a 1:1 ratio of the two complementary strands were used to allow calculations of the thermodynamic parameter for duplex formation. Parameters were determined by the van't Hoff method (Marky and Breslauer, 1987).

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