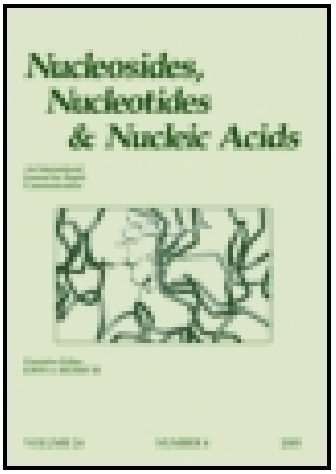

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OLIGONUCLEOTIDE CHARGE REVERSAL: 2′-O-LYSYLAMINOHEXYL MODIFIED OLIGONUCLEOTIDES

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□ *A novel cationic building nucleoside building block designed for antisense and siRNA oligonucleotides is presented. Protected L-lysine was coupled to 2′-O-aminohexyluridine and the resulting nucleoside was phosphitylated for automated oligonucleotide synthesis. An increasing number of these 2′-O-lysylaminohexyl nucleosides lowered the melting temperature of desoxy-thymidine homododecamers, but the decrease was lower than that for DNA/RNA hybrids. Incubation with an exonuclease showed the exceptionally high resistance against enzymatic degradation. CD spectrometry revealed a gradual transition towards an A-type oligonucleotide structure. Based on these data, the cationic building block is particularly suited for gapmer antisense as well as siRNA oligonucleotides.*

Keywords Antisense oligonucleotides; siRNA; cellular uptake; CD spectrometry

Gene silencing mediated by antisense oligonucleotides (ASO) or short interfering RNA (siRNA) has become an important technique in scientific research in the last decade. Attempts to exploit it for therapeutic purposes have so far been hampered by poor pharmacokinetic and pharmacodynamic properties as well as off-target effects caused by the chemical structures of oligonucleotides used in clinical trials.

In particular, the polyanionic nature of oligonucleotides all but prevents their uptake into living cells. Charge reduction or neutralization by zwitterionic nucleotide building blocks can give positive effects not only on cellular uptake, but also on target affinity.[1,2] Conjugating cationic peptides to oligonucleotides has been shown to improve cellular uptake.[3,4] We present a cationic building block that results in a charge reversal, as its incorporation in an oligonucleotide adds a positive charge instead of the negative one of a wild-type nucleoside.

The synthesis of the cationic uridine building block **5** proceeds in two steps from protected 2′-O-aminohexyl uridine[1] (Figure 1). A trifluoro

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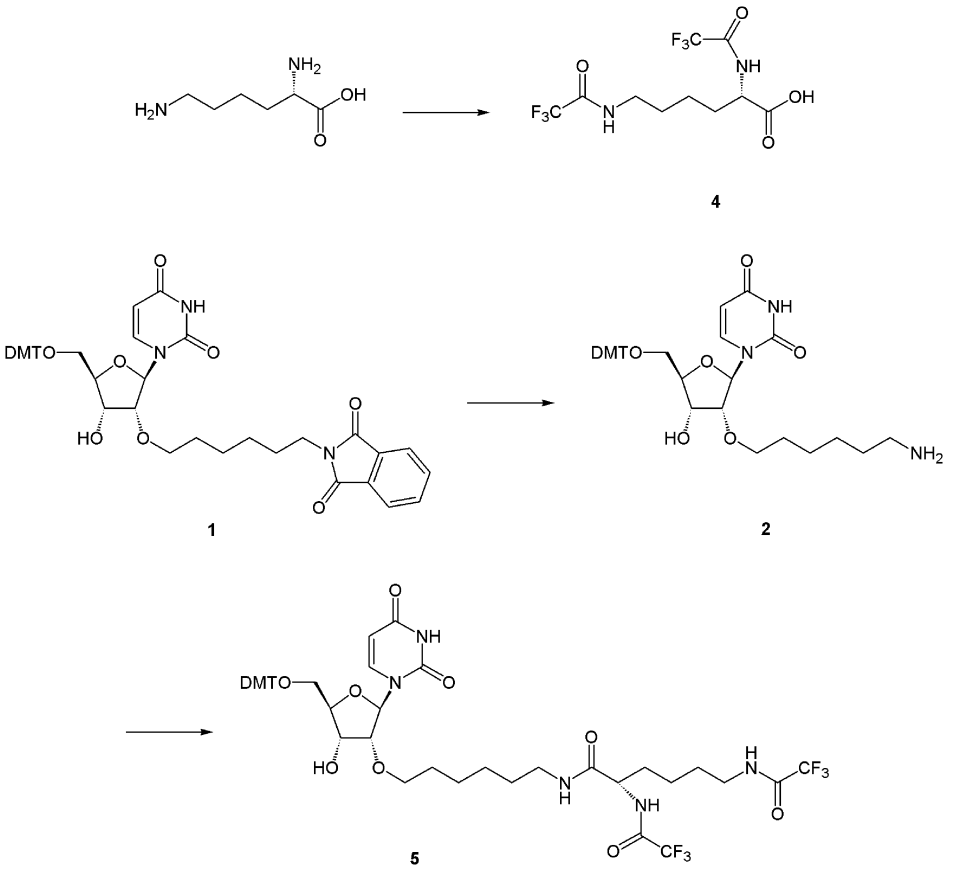

FIGURE 1 Synthesis scheme of 2′-O-lysylaminohexyl building block **5**.

acetate protected L-lysine building block, prepared from L-lysine using trifluoro acetate, is coupled to the free amino group in excellent yield using DIC as catalyst. After phosphitylation, the building block can be used in standard oligonucleotide synthesis when extended coupling times are employed.

For an evaluation of the biophysical parameters of charge reversed oligonucleotides, desoxy-thymidine homododecamers incorporating an increasing number of building block **5** were used. The decrease in target affinity as determined by transition temperatures (Tm) and free energy values (ΔG) was lower than expected for dT-rU hybrids, indicating an increase in target affinity caused by the cationic lysine chains (Table 1). The calculated values were determined by a nearest neighbor model and take into account the poorer hybridization affinity of rU compared to dT. Data show that a few terminal cationic nucleotides are tolerated very well, whereas a pronounced influence on duplex stability was observed with several modifications spread over the entire oligonucleotide strand.

TABLE 1 Sequences and properties of oligonucleotides **7–13**. U*: 2′-*O*-Lysylaminohexyl-uridine

	Sequence	Tm meas.	Tm calc.	ΔΔG meas.	ΔΔG calc.	MW calc.	MW found
	5′-TTT TTT TTT TTT-3′	34,7	31,0	0,0	0,0	3577,4	3576,3
7	5′-U*TT TTT TTT TTT-3′	33,6	25,1	0,0	1,7	3817,7	3816,0
8	5′-U*U*T TTT TTT TTT-3′	32,4	22,7	0,0	5,0	4047,0	4048,0
9	5′-U*U*U* TTT TTT TTT-3′	30,4	20,6	1,4	8,4	4276,3	4276,0
10	5′-U*U*U* U*TT TTT TTT-3′	28,3	18,6	3,8	11,8	4505,6	4505,6
11	5′-U*U*U* U*U*T TTT TTT-3′	26,2	16,8	3,9	15,1	4735,0	4734,7
12	5′-TTU* TTT U*TT TU*T-3′	17,6	19,6			4276,3	4282,0
13	5′-TU*T TU*T TU*T TU*T-3′	9,5	17,7			4505,6	4507,0

MALDI analyses confirmed the successful incorporation of lysine aminohexyl uridine into oligonucleotides. Neither transacetylation of lysine amino groups during synthesis nor cleavage of the amide bond during standard ammonia deprotection occurred.

Incubation of **7** with 5′-exonuclease revealed the excellent stability of charge reversed oligonucleotides (Figure 2). Only one modified nucleotide leads to practically complete resistance against enzymatic degradation. Lysylaminohexyl oligonucleotides are clearly superior to aminohexyl

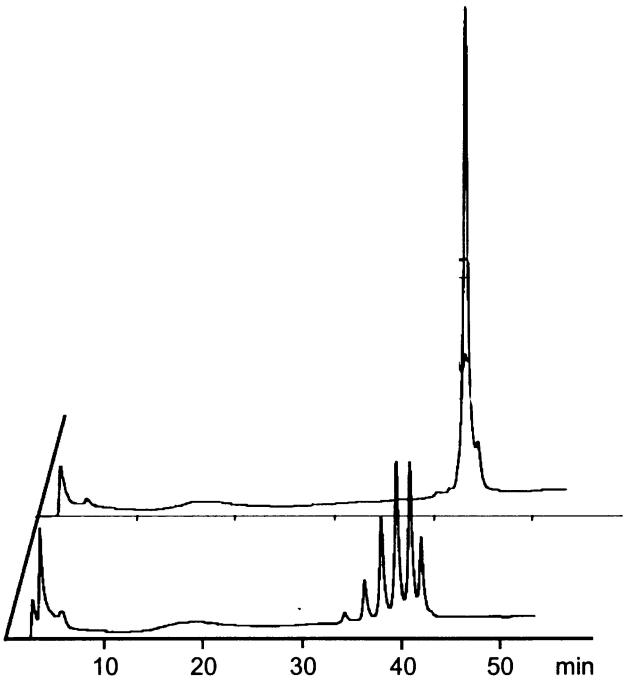

FIGURE 2 HPLC of **7** (top) and T_{12} (bottom) after 30 minutes of incubation with phosphodiesterase.

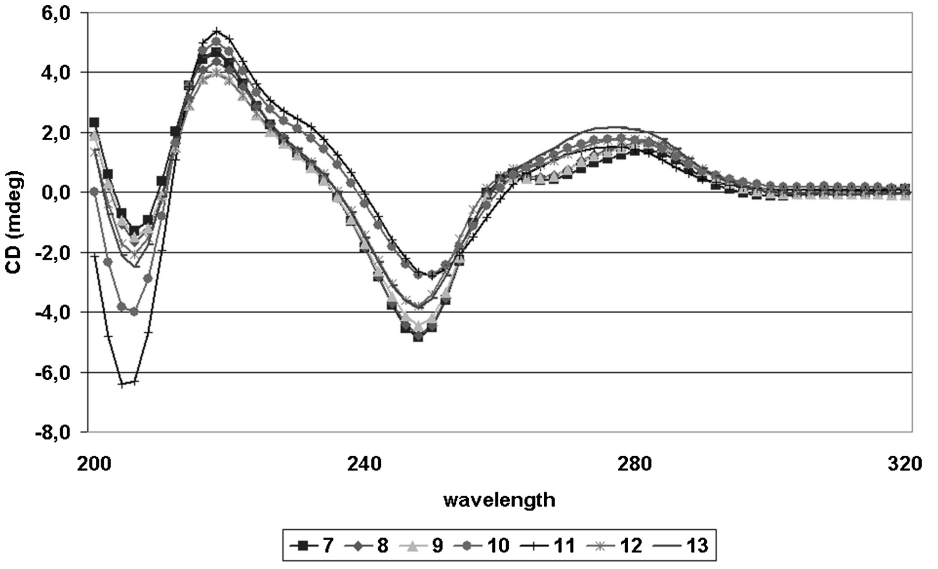

FIGURE 3 CD spectra of duplexes of **7–13** with dA_{12}.

modified ones, where three terminal modifications are required for a similar effect.[1]

CD spectra of duplexes with wild-type DNA sense showed a conversion towards an A-type oligonucleotide secondary structure with increasing number of modified building blocks (Figure 3). The characteristic double peak of dA_{12}-dT_{12} gradually disappears. In addition, the oligonucleotides with the highest number of cationic nucleotides exhibit a strong negative band in the far UV region, a characteristic of an A-type structure, often associated with RNA.

In conclusion, 2′-O-lysylaminohexyl modified oligonucleotides exhibit auspicious biophysical characteristics. In particular, the gapmer approach promises a positive effect on target affinity and sufficient stability against degrading enzymes at the same time.

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