Duplex-Stabilization Properties of Oligodeoxynucleotides Containing N^2-Substituted Guanine Derivatives

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Dedicated to Prof. Dr. *Frank Seela* on the occasion of his 60th birthday

Oligodeoxynucleotides **3**–**13** carrying different guanine derivatives with substituents at the N^2 position have been prepared from a common precursor. Duplexes containing these modified bases are more stable than unmodified duplexes. The highest stability is found in guanine derivatives carrying at N^2 an ethyl and propyl group substituted with a group that is protonated under physiological conditions, which is compatible with a possible interaction of the protonatable group with the phosphates.

1. Introduction. – The ability of oligonucleotides to form double-stranded structures with their complementary RNA and DNA sequences has been widely used for the isolation of cloned DNA sequences, the detection of specific genes, and the identification of specific mRNA sequences [1]. More recently, this ability is being used to inhibit gene expression (antisense and antigene strategy) [2] and to analyze the expression patterns of a large number of genes using oligonucleotide microarrays [3]. All these approaches rely on nucleic-acid hybridization, and they are expected to benefit from oligonucleotide analogs with better hybridization properties. A large number of nucleic-acid derivatives with enhanced binding properties have been developed [4]. Peptide nucleic acids (PNA) [5], hexitol nucleic acids (HNA) [6], 2′-fluoro-N3′-P5′ phosphoramidates [7], and locked nucleic acids (LNA) [8] are examples of backbone-modified derivatives with enhanced binding properties. However, simple substitutions in the sugar moiety such as 2′-*O*-alkyl-RNA derivatives [9] and substitutions in the nucleobases also lead to substantial increases in duplex stability.

N^2-Substituted derivatives of guanine (*Fig. 1*) are among these base-modified analogs with interesting binding properties [10–15]. First, the interest in the preparation of oligonucleotides carrying N^2-substituted derivatives of guanine was generated by the reaction of some carcinogenic compounds with guanine to yield adducts bound to the NH_2–C(2) group of guanine. These modified oligonucleotides were important intermediates in the carcinogenesis of polycyclic aromatic hydrocarbons (PAH) [16]. Later, the attachment of an imidazole moiety to the exocyclic amino group of guanine was described [11]. Oligonucleotide-imidazole conjugates were prepared to promote RNA hydrolysis [12]. Later, it was found that imidazolyl

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groups bound to the exocyclic amino group of G stabilized the duplex structure [12][13]. Molecular-modeling simulations suggested that the imidazole moiety stabilized the duplex by binding in the minor groove near the phosphate backbone [13]. More recently, it has been described that the incorporation of polyamine-derived groups, such as spermine- and spermidine-derived ones, and also the 3-aminopropyl group, at the N^2 position of guanine increase duplex stability [13] and strand replacement [10]. Finally, the introduction of the (naphthalen-1-ylmethyl) group at the N^2 position of guanine in G-tetrad-forming oligonucleotides increased the thrombin-inhibitory activity of these oligonucleotides [14].

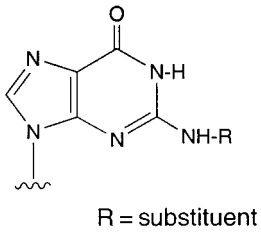
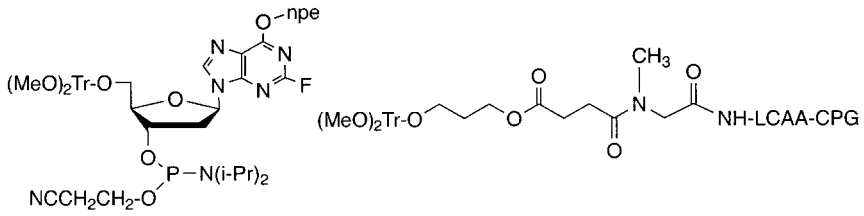


Fig. 1. *Structure of* N^2*-substituted guanine derivatives*

Two strategies have been described for the preparation of oligodeoxynucleotides carrying N^2-substituted G_d derivatives: 1) the use of a phosphoramidite carrying a protected N^2-modified G_d derivative and 2) the use of a precursor (usually a 2-fluoro-I_d derivative) [10][15] that is modified during deprotection of nucleobases by the appropriate amine (post-synthetic substitution). The first strategy is suitable for the introduction of several modifications in one oligonucleotide and the second approach is used for the introduction of fewer modifications [10b], especially when a complex amine is used, such as a PAH-substituted one [16]. A problem found when a polyamine is introduced into a guanine residue is the potential formation of different isomers [15][17]. In this communication, we describe the search for new, N^2-modified guanine derivatives with enhanced binding properties without the formation of isomers. Previous work indicated the importance of the imidazolyl group at the end of a propyl chain and the possibility that the imidazolyl group might interact with the phosphate [13]. Our results confirm this hypothesis, and we describe several new N^2-substituted derivatives of guanine with enhanced binding properties.

2. Results and Discussion. – We focused on the preparation of a dodecamer carrying a single N^2-substituted guanine. Our target oligonucleotide sequence was the dodecamer R5 (5′-d(CACC**G**ACGGCGC-p-propanediol)-3′), complementary to a mutated Ha-*ras* oncogene with antiproliferative activity [18]. The G_d residue in bold indicates the position of N^2-substituted G_d. A propane-1,3-diol group was added at the 3′-end to prevent oligonucleotide degradation by exonucleases [19]. We decided to follow the post-synthetic substitution strategy. For this purpose, the 2-fluoro-I_d derivative protected with the 2-(4-nitrophenyl)ethyl (npe) group (see **1** in *Fig. 2*) was prepared as described [15]. The sequence was assembled on an automatic DNA synthesizer from phosphoramidites protected with the (*tert*-butyl)phenoxyacetyl group [20]. A special support carrying the 4,4′-dimethoxytrityl($(MeO)_2Tr$)-protected

propanediol moiety connected by a succinyl linker to a sarcosyl-LCAA-CPG [21] (see **2** in *Fig. 2*) was prepared as described for similar supports [22]. Deprotection was performed in two steps: 1) 0.5M DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) in MeCN, 2 h, at room temperature (to remove the npe and the 2-cyanoethyl phosphate protecting groups) and 2) 1M aqueous amine solution (to perform deprotection of the natural bases, modification of the 2-fluoro-I_d derivative, and cleavage of the oligonucleotide-support linkage), overnight, 55°. The resulting products were desalted over *Sephadex G-25* (*NAP-10* columns) and purified by reversed-phase HPLC using the trityl-on and trityl-off protocols. In all cases, a major peak was obtained and collected. The purified oligonucleotides were analyzed by mass spectrometry (MALDI-TOF) and exhibited the expected mass (*Table*).



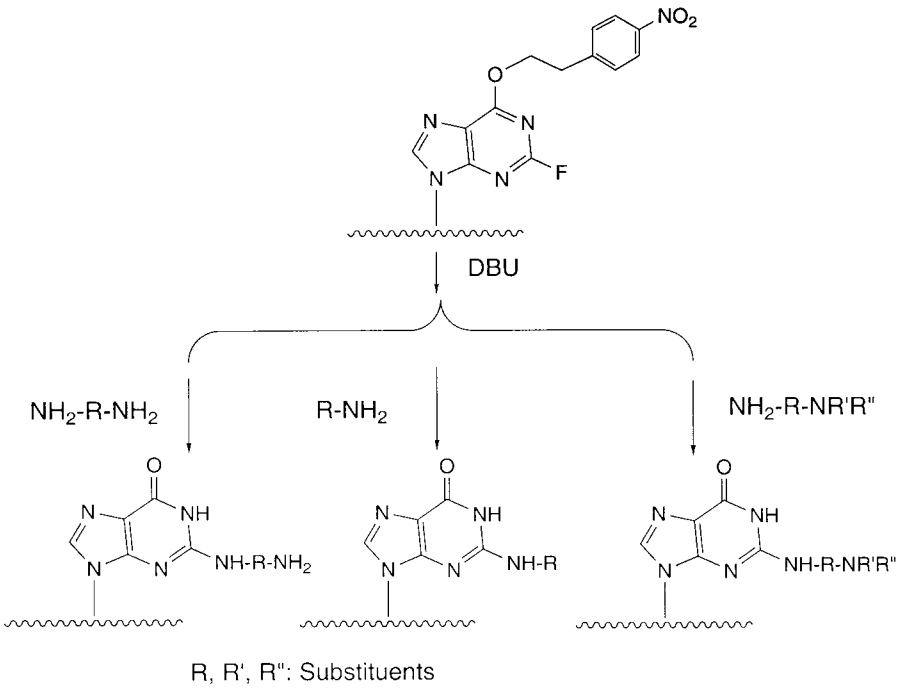
1 **2**

Fig. 2. *Phosphoramidite derivative* **1** *and solid support* **2** *used for the preparation of oligonucleotides* **3**–**13** (see *Table*). LCAA-CPG = (long-chain-alkyl)amine controlled-pore glass.

Thus, the dodecamers **3**–**13** were prepared by using appropriate amines during the deprotection-substitution step (see *Table* and *Scheme*). To avoid formation of different isomers, symmetric primary diamines (ethane-1,2-diamine (→**4**); propane-1,3-diamine (→**5**); hexane-1,6-diamine (→**8**); 2,2′-[ethane-1,2-diyl)bis(oxy)]bis(ethynamine) (→**10**)) and primary amines tethered with tertiary amines (1*H*-imidazole-1-propanamine (→**3**); *N*,*N*-dimethylethane-1,2-diamine (→**6**); pyridin-2-ethanamine (→**9**) were selected. Spermidine (= *N*-(3-aminopropyl)butane-1,4-diamine (→**7**) was chosen for comparison with previous data [10][15]. The reaction of 2-fluoro-I_d with spermidine may form three isomers. Butanamine (→**11**), and 3-aminopropanol (→**12**) were selected as controls, as well as the dodecamer R5 (**13**) lacking guanine modification.

Thermal denaturation of duplexes formed by modified dodecamers and their complementary DNA sequence were studied at medium salt concentration (0.15M NaCl, 50 mM *Tris*·HCl pH 7.5). Melting temperatures are shown in the *Table*. Thermal renaturation was also studied by running a decreasing-temperature gradient. No differences were observed, indicating that strand association and dissociation is fast, as observed in unmodified oligonucleotides. All the N^2-substituted guanine derivatives prepared here stabilized the double helix. The most stabilizing substituents were the 3-(1*H*-imidazol-1-yl)propyl, the 2-aminoethyl, the 3-aminopropyl, the 2-(dimethylamino)ethyl and the spermidine-derived groups (see **3**–**7**; 4–6° more stable than unmodified R5 (**13**), see *Table*). All these five substituents have a protonatable group (amino or imidazolyl) linked to a small chain of 2 or 3 C-atoms. A second group of substituents with moderate duplex stabilization properties was formed by the 6-

Scheme. *Preparation of Oligonucleotides Carrying* N^2*-Substituted Guanine Derivatives*



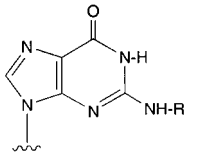
aminohexyl, 2-(pyridin-2-yl)ethyl, and the 2-[2-(2-aminoethoxy)ethoxy]ethyl groups (see **8**–**10**; 2–3° increase). Except for the piridinylethyl group, these derivatives have an amino group tethered by a long (> 3 residues) hydrocarbon or ethyleneglycol chain. Finally, the lowest melting temperatures were obtained with guanine derivatives substituted by a butyl or 3-hydroxypropyl group devoid of a protonatable group (see **11** and **12**). These data are in agreement with previous results for oligonucleotides containing G_d derivatives with a N^2-substituent derived from 1*H*-imidazole-1-propanamine, spermine, or spermidine [10–14]. In a previous paper [15], we reported that the melting temperature of the unmodified dodecamer duplex without the propanediol moiety at the 3′ end was 51°. On discovering that the sequence of the unmodified control oligonucleotide was incorrectly introduced in the synthesizer, we repeated the melting experiments described in [15], but using the correct sequence. The melting temperature of the duplex control was found to be 63°. Considering this correction, the stabilization properties found in [15] are in agreement with the present data.

These results indicate that the most beneficial substituents at the N^2 position of guanine for duplex stabilization are ethyl and propyl groups linked to an amino or imidazolyl group. Longer linkers between the exocyclic guanine NH_2–C(2) and the protonatable group reduce the duplex stability. A similar result was found on introduction of alkyl groups at the 2′-hydroxy function of RNA [23]. The $O^{2'}$ position as well as N^2 position of guanine point to the minor groove where the negative phosphate groups are located, suggesting a positive interaction between the phosphate group and

Table. *Melting Temperatures and Molecular Weights of Dodecamers* **3**–**13** *Carrying a* N^2*-Substituted Guanine Unit*[a])

	R	T_m [°C][b])	M^+ found	calc.
3	(im)$CH_2CH_2CH_2$	71.1	3863.8	3863.1
4	$NH_2CH_2CH_2$	71.0	3798.4	3798.0
5	$NH_2CH_2CH_2CH_2$	70.1	3812.3	3812.1
6	$Me_2NCH_2CH_2$	69.6	3827.9	3826.1
7	$NH_2(CH_2)_4NH(CH_2)_3$[c])	69.6	3883.6	3883.2
8	$NH_2(CH_2)_6$	68.0	3854.8	3854.1
9	(py)CH_2CH_2	68.0	3865.1	3860.1
10	$NH_2CH_2CH_2OCH_2CH_2OCH_2CH_2$	67.1	3885.5	3886.1
11	$MeCH_2CH_2CH_2$	66.1	3810.7	3811.1
12	$OHCH_2CH_2CH_2$	65.6	3810.2	3813.0
13	H	65.0	3756.2	3756.0

[a]) Modified R5 dodecamer 5′-d(CAC C**G**A CGG CGC-p-propanediol)-3′:



R = substituent

[b]) Complementary sequence: 3′-d(GTGGCTGCCGCG)-5′, 0.15M NaCl, 50 mM *Tris*·HCl pH 7.5. Error in T_m is ±0.4°. [c]) Only one isomer of the three possible is shown.

the protonated substituent [13][23]. The results presented here are consistent with this hypothesis.

In conclusion, oligonucleotides carrying a variety of N^2-substituted guanine residues have been prepared from a common precursor. This method can be used to generate series of oligonucleotides for the screening of duplex-stabilizing substituents. Some of the modified oligonucleotides have interesting binding properties and are easy to prepare either by post-synthetic substitution or by the use of a protected phosphoramidite. The study of the antiproliferative activity of the compounds prepared is in progress.

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Experimental Part

Oligonucleotide Synthesis. The oligonucleotide sequence 5′-d(CAC C**X**A CGG CGC-p-propanediol)-3′ (**X**$_d$ = 2′-deoxy-2-fluoro-O^6-[2-(4-nitrophenyl)ethyl]inosine) was synthesized on a DNA synthesizer, model *394* (*Applied Biosystems*, USA), from $(MeO)_2$Tr- and [(*tert*-butyl)phenoxyacetyl]-protected nucleoside 3′-(2-cyanoethyl phosphoramidites) (*PerSeptive Biosystems*, USA) and 2′-deoxy-5′-*O*-4,4′-dimethoxytrityl)-2-fluoro-O^6-[2-(4-nitrophenyl)ethyl]inosine 3′-(2-cyanoethyl phosphoramidite) (**1**; *Fig. 2*). The latter was prepared as described [15]. A special support carrying the $(MeO)_2$Tr-protected propane-1,3-diol moiety connected by a succinyl linkage to sarcosyl-controlled-pore glass [21] was prepared as described [22]. Standard 1-μmol-scale synthesis cycles were used. Coupling efficiencies were higher than 98%. The oligonucleotide support was treated with 1 ml of 0.5M DBU in MeCN for 30 min at r.t. The support was washed in MeCN, 1% Et_3N in MeCN and MeCN and dried. The resulting support was treated with 1 ml of 1M aq. soln. of the appropriate amine (*Table*) at

60° overnight, and the soln. was filtered and evaporated. The residue was dissolved in H_2O, and the soln. was desalted on a *Sephadex G-25* (*NAP-10*, *Pharmacia*, Sweden) column. The oligonucleotide-containing fractions were analyzed and purified by HPLC (*PRP-1*-10 μm column, 305 × 7 mm; *Hamilton*, USA, flow rate 3 ml/min). A 20-min linear gradient from 15 to 45% MeCN over 20 mM aq. $(Et_3NH)OAc$ was used for oligonucleotides carrying the $(MeO)_2Tr$ group. After removal of the $(MeO)_2Tr$ group with 80% $AcOH/H_2O$ (30 min), the resulting oligonucleotides were purified on the same column with a 20-min linear gradient from 5 to 25% MeCN over 20 mM aq. $(Et_3NH)OAc$. Purified oligonucleotides were analyzed by MALDI mass spectrometry (see *Table*). On average, we obtained 25 *OD* units at 260 nm of purified dodecamer per μmol.

Melting Experiments. Melting experiments of dodecamer duplexes were performed by mixing equimolar amounts of two dodecamer strands dissolved in a soln. that contained 0.15M NaCl and 0.05M *Tris*·HCl buffer (pH 7.4). Duplexes were annealed by slow cooling from 80 to 4°. UV Absorption spectra and melting curves (absorbance *vs.* temperature) were recorded in 1-cm path-length cells using a *Cary-1/3*-UV/VIS spectrophotometer (*Varian*, Australia) with a temp. controller with a programmed temp. increase of 0.5°/min. Melting curves were determined in triplicate on duplex concentrations of 4 μM at 260 nm. Results: see *Table*.

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