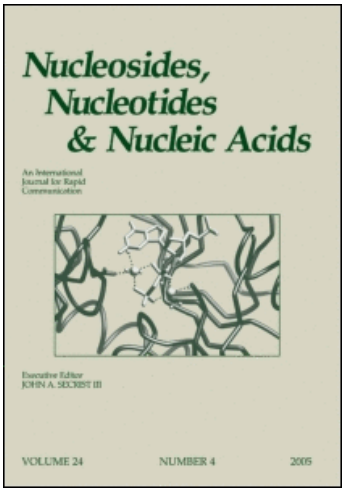

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The N^1-(3'-Deoxythymidin-3'-yl)-N^2-cyano-N^3-(5'-deoxythymidin-5'-yl) Guanidine Dimeric Building Block in Automated DNA Synthesis and Mass Spectrometric Analysis of Its Integrity

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THE N^1-(3'-DEOXYTHYMIDIN-3'-YL)-N^2-CYANO-N^3-(5'-DEOXYTHYMIDIN-5'-YL) GUANIDINE DIMERIC BUILDING BLOCK IN AUTOMATED DNA SYNTHESIS AND MASS SPECTROMETRIC ANALYSIS OF ITS INTEGRITY.

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Abstract: Nucleoside dimers with an *N*-cyanoguanidine linkage were synthesized and incorporated in oligonucleotides on an automated DNA synthesizer. The integrity of the dimer was investigated using mass spectrometry.

Natural oligonucleotides suffer from two serious limitations: the instability of the construct against enzymatic degradation and the poor cellular uptake due to their polyanionic character[1].
In order to investigate, whether or not the cyanoguanidine is a good isoster for the phosphodiester linkage in natural B-DNA, a molecular modelling study[2] was undertaken. The energy difference between the fully minimized dimer and the fit is about 4.3 kcal/mol. The overlap is shown in Figure 1.

The synthesis and properties of *N*-cyanoguanidine modified oligonucleotides have already been described.[3] Until now the proof of the absence of side reactions or chemical degradations came from the enzymatic stability test. An oligomer $d(T)_6$ with as sequence TT(TT)TT, with (TT) representing the dimer with the modified linkage, was enzymatically broken down with snake venom phosphodiesterase and treated with calf intestinal alkaline phosphatase. HPLC analysis of the the reaction mixture revealed the presence of only thymidine and the intact dimer.[3b] This indicates that the N^1-(3'-deoxythymidin-3'-yl)-N^2-cyano-N^3-(5'-deoxythymidin-5'-yl)guanidine dimer is not recognized by the enzymes used. It likewise proves the dimeric building block to be incorporated without any change in the oligonucleotide. A degradation of the cyano group could have occurred, as upon treatement of the dimer with an aqueous solution of trifluoroacetic acid, a quantitative conversion to the N^2-carboxamido analogue was observed[4].

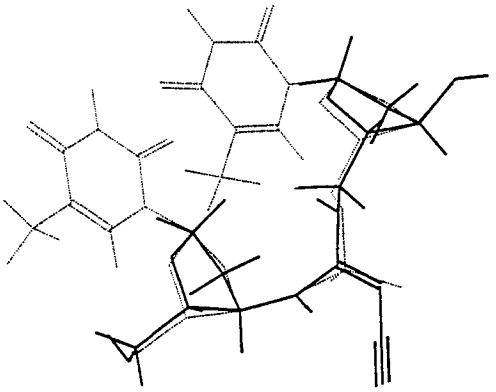

FIG.1: Overlay of a natural TT dimer and a cyanoguanidine linked dimer.

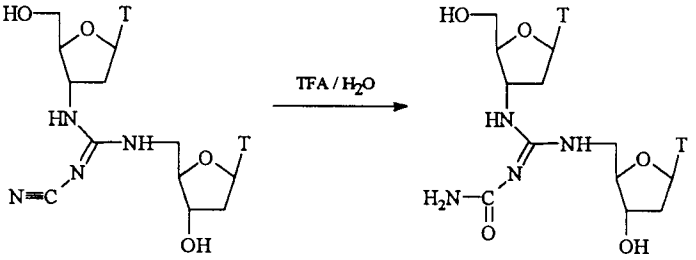

FIG. 2: Acidic hydrolysis of the *N*-cyanoguanidine.

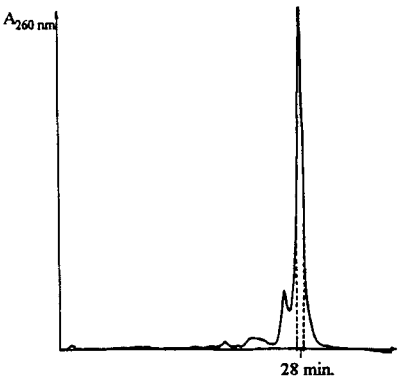

FIG. 3: HPLC profile of a crude eicosamer (anion exchange chromatography Mono Q HR 10/10, Pharmacia). Eluens buffer A=10 mM NaOH + 0.1 M NaCl; buffer B 10 mM NaOH + 0.9 M NaCl. pH=12.0

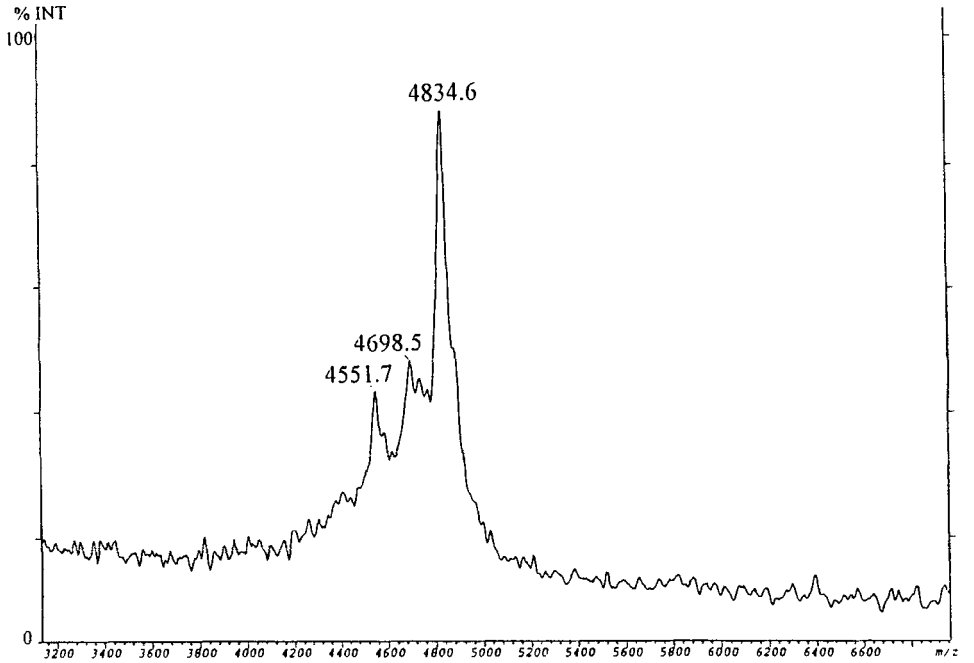

FIG. 4: Mass spectrum of a modified 16-mer.

As the guanidine function in this molecule has much more basic properties, it is protonated at physiological pH [5]. A charged molecule is less attractive as it permeates with more difficulty through the lipophilic cell membrane. A (partial) hydrolysis of the cyano functions certainly has to be avoided during synthesis of longer sequences.

The synthesis of mixed sequences on an automated DNA synthesizer, using the TT dimeric building block, gave good results. As an example, the profile of a crude 20-mer (c-*fos* sense sequence 277-297) is shown in figure 3.

An interesting analysis of the integrity of modified oligonucleotides is mass spectrometry. Mixed sequences, which need longer treatment with ammonia to deprotect the heterocyclic bases (18 hours at 55°C) were examined by mass spectrometry. A 16-mer, CCCTG(TT)CGGGCGCCA (an antisense sequence directed against the primer binding site (PBS) in HIV) was synthesized. The integrity of this sequence was proven by matrix-assisted laser desorption negative ion mass spectrometry using the method described by Pieles *et al.*[6]. The calculated value for this oligonucleotide was 4834.1 and the observed value for $[MH]^-$ was 4834.6. This method is very attractive as it requires only 8 pmol of the sample. Figure 4 shows the mass spectrum of the oligonucleotide.

All these results prove that a dimeric building block can be incorporated into oligonucleotides without any side reactions, using the standard procedures of oligonucleotide synthesis.

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4. ^{13}C(DMSO-d_6): δ 11.4 (CH_3); 11.5 (CH_3); 37.5 (C-2'); 38.5 (C-2'); 42.9 (N-C-5'); 51.8 (N-C-3'); 60.5 (C-5'); 70.9 (C-3'); 84.2, 84.4, 84.8 and 85.2 (2 x C-1' and 2 x C-4'); 110.7 and 110.9 (2 x C-5); 137.2 (2 x C-6); 151.3 (2 x C-2); 154.9 ($CONH_2$); 165.3 (2 x C-4) ppm.
LSIMS (thioglycerol): m/z $[M+H]^+$ 551
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7. An aliquot, 1μL of a solution containing 80 pmol oligonucleotide per μL in distilled water, was mixed with 10 μL of an 0.5 mM solution of 2,4,6-trihydroxyacetophenone in ethanol and 5 μL of an 0.1 M solution of dibasic ammonium citrate in water. From this mixture, 1 μL was spotted on the metal target and allowed to dry by air. The mass spectra were taken on a VG Tof Spec (VG Analytical, Wytenshawe, UK) instrument which is equipped with a nitrogen laser (337 nm). The instrument was run in linear mode. Spectra were recorded in the negative ion mode using an acceleration voltage of 20 kV and were obtained by overlaying 20-120 single laser pulses. The spectrometer was calibrated externally with the $[MH]^-$ mass peak of the matrix (2,4,6-trihydroxyacetophenone) and of dT_{10} as reference.