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Oligomer Synthesis and DNA / RNA Recognition Properties of a Novel Oligonucleotide Backbone Analog: Glucopyranosyl Nucleic Amide (GNA)

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Abstract: A general solid phase synthesis is described for the construction of novel amide linked oligonucleotide analogs having the glucopyranosyl configuration via solid phase N-Fmoc-type peptide chemistry. The selective binding to DNA and RNA sequences by these oligomers is characterized by measurement of melting temperatures and related thermodynamic constants. © 1997 Elsevier Science Ltd.

In the search for more efficacious, less toxic antisense agents, there has been much research to understand the factors which influence the affinity and selectivity of binding to RNA and DNA by novel oligonucleotide structures. Numerous reports describe the synthesis of modified linkages of oligonucleotide dimer units and their subsequent incorporation into natural, phosphodiester linked strands of DNA and RNA.[1] The binding affinities of these singly modified hybrid strands to natural DNA and RNA targets are often less than that of analogous, unmodified oligomers. It has been our goal to replace the phosphodiester linkages with novel nucleoside units throughout the strand. The possibility of success in this direction has been admirably demonstrated by the example of PNA (peptide nucleic acids);[2] however, PNA is not without problems related to self-aggregation, solubility, and cellular penetration. We aim to improve upon the idea of PNA by creating a dramatically different oligonucleotide backbone which is chirally pure, somewhat more rigid, amide linked and hydroxylated. Here we report the solid phase synthesis of two oligomers and the binding properties of this novel type of oligonucleotide derived from D-glucosamine: GNA (glucopyranosyl nucleic amide).

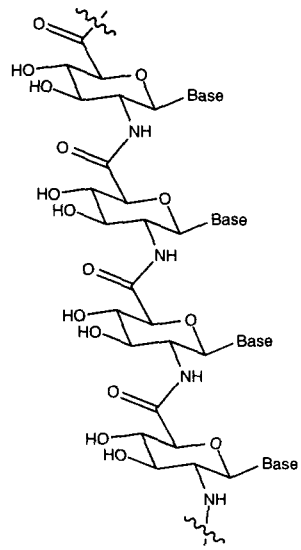
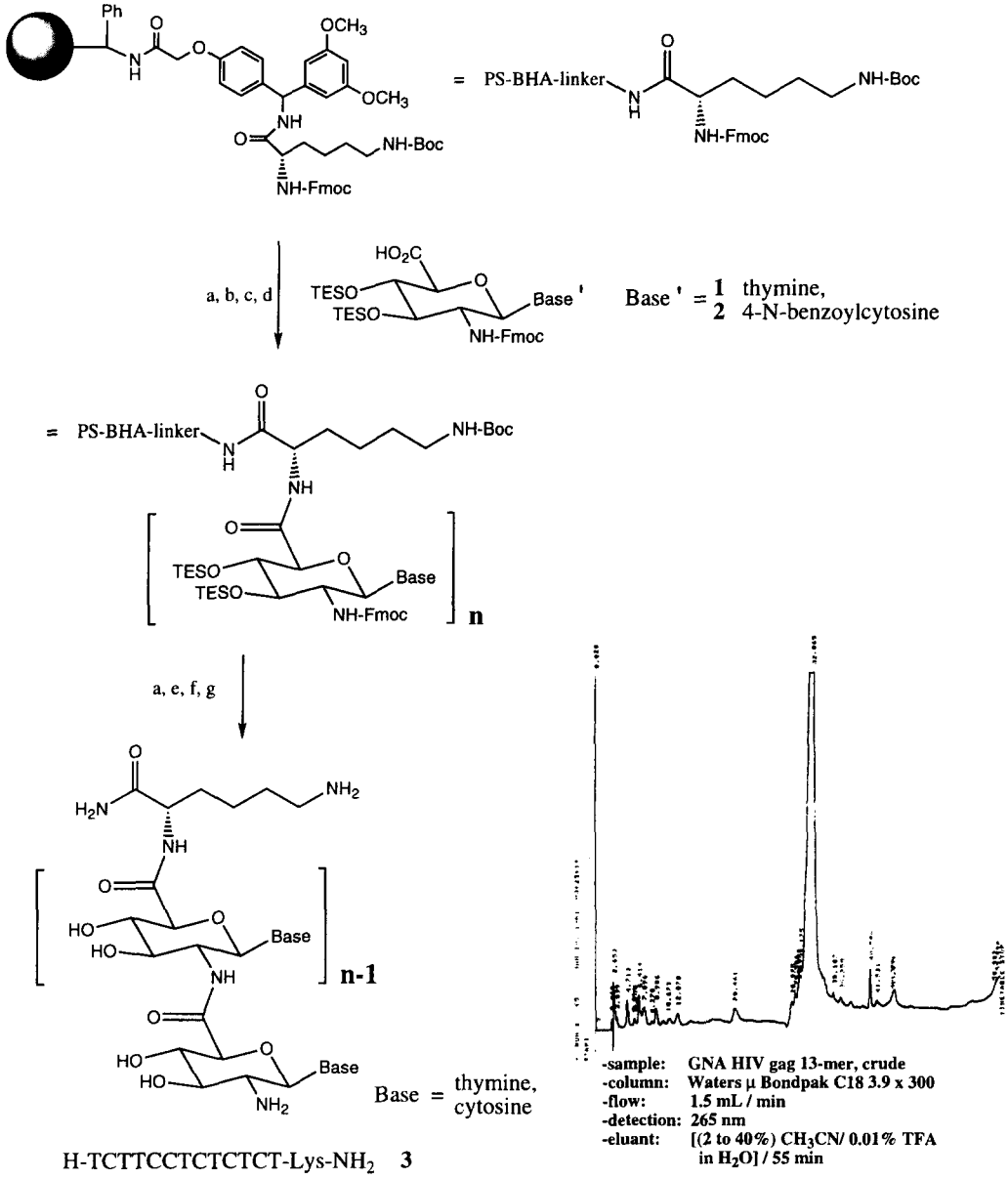


Figure 1 GNA

A homopyrimidine sequence (H-TCTTCCTCTCTCT-Lys-NH_2 **3**) complementary to a homopurine sequence in the HIV *gag* gene was synthesized on BHA[3] resin derivatized with the TFA labile Knorr linker. This was done according to standard solid phase N-Fmoc-type peptide chemistry[4] using TES-protected thymine and cytosine building blocks, **1** and **2**, respectively.[5] Each residue (5 eq) was coupled twice using HATU with excess DIPEA in DMF. Coupling yields varied between 65% and 100% and averaged greater than 90% per residue. The completed sequence was cleaved from the resin by 3 x 30 min treatments with TFA / CH_2Cl_2 (1:1). Upon evaporation of the combined filtrates, the residue was treated with 30% NH_4OH at rt for 12 h to remove the cytosine N-benzamide groups. Evaporation of the solvent yielded the highly water soluble oligomer which was purified by RP HPLC; characterization by MALDI-TOF MS indicated the expected molecular ion (3737 for M+1).

The synthesis of a 10-mer oligomer (H-TCACTAGATG-Lys-NH_2 **4**)[6] containing all four nucleobases was effected similarly using di-*tert*-butyldimethylsilyl protected building block amino acids.[7] After cleavage from the solid phase followed by deprotection of the nucleobase protecting groups with NH_3 saturated EtOH, the silyl groups were removed by treatment with TBAF. Oligomer **4** was isolated by passage through a NAP-25 size exclusion column and subsequently, by elution from RP HPLC. Lysine was included at the carboxamide termini of both oligomers to allow for comparison to the reported binding properties of lysine capped PNA oligomers.[8]



Scheme

a) 20% piperidine in DMF, 3 treatments for 1, 5 and 5 min; b) preactivation of GNA monomer acid 5.0 eq 0.06 M DMF, HATU 5.0 eq, DIPEA 10 eq, then shake with resin 20 min, rt, rinse, repeat coupling c) capping with 5% Ac_2O / DMF 5 min; d) repeat sequence of a, b, c for each GNA residue n times; e) TFA / CH_2Cl_2 (1:1), 3 treatments for 20 min at rt; f) 33% NH_4OH, rt, 12 h; g) RP HPLC

Table: Melting Temperatures and Thermodynamic Constants

	duplex	duplex type	T_m up (down)	ΔH	ΔS
1[9]	5'-TCTCTCTCCTTCT-3' 3'-AGAGAGAGGAAGA-5'	**5** DNA **6** DNA	44.2 (43.4)	84.6	240.3
2	5'-TCTCTCTCCTTCT-3' 3'-AGAGAGAGGAAGA-5'	**5** DNA **7** RNA	55.1 (54.8)	102.3	284.4
3	H_2N-Lys-TCTCTCTCCTTCT-H 3'-AGAGAGAGGAAGA-5'	**3** GNA (p) **6** DNA	46.7 (47.2)	34.5	80.8
4	H_2N-Lys-TCTCTCTCCTTCT-H 3'-AGAGAGAGGAAGA-5'	**3** GNA (p) **7** RNA	48.8 (48.4)	74.4	203
5	H_2N-Lys-TCTCTCTCCTTCT-H 5'-AGAGAGAGGAAGA-3'	**3** GNA (ap) **8** DNA	19.2 & 67.2 (18.7 & 66.4)	*	*
6	H_2N-Lys-TCTCTCTCCTTCT-H 5'-AGAGAGAGGAAGA-3'	**3** GNA (ap) **9** RNA	54.1 (53.7)	70.0	187
7	H_2N-Lys-TCTCTCTCCTTCT-H 5'-AGAGAGA**A**GAAGA-3'	**3** GNA (ap) **10** RNA G to A mm	45.7 (44.8)	56.6	151
8	H_2N-Lys-TCTCTCTCCTTCT-H 5'-AGAGAGA**C**GAAGA-3'	**3** GNA (ap) **11** RNA G to C mm	43.2 (42.9)	58.8	158
9	H_2N-Lys-TCTCTCTCCTTCT-H 5'-AGAGAGA**T**GAAGA-3'	**3** GNA (ap) **12** RNA G to T mm	48.3 (47.6)	68.6	187
10	3'-TCACTAGATG-5' 5'-AGTGATCTAC-3'	**13** DNA **14** DNA	35.8 (35.9)	60.5	169
11	3'-TCACTAGATG-5' 5'-AGTGATGTAC-3'	**13** DNA **15** RNA	34.3 (33.9)	72.5	208
12	H_2N-Lys-TCACTAGATG-H 5'-AGTGATCTAC-3'	**4** GNA (ap) **15** RNA	21.5 & 58.5 (22.0 &56.6)	*	*
13	H_2N-Lys-TCACTAGATG-H 5'-AGTGATCTAC-3'	**4** GNA (ap) **14** DNA	45.9 (45.4)	31.3	70.1
14	H_2N-Lys-TCACTAGATG-H 5'-AGTGAT**G**TAC-3'	**4** GNA (ap) **16** DNA C to G mm	41.3 (42.0)	21.9	42.0
15	H_2N-Lys-TCACTAGATG-H 5'-AGTGAT**A**TAC-3'	**4** GNA (ap) **17** DNA C to A mm	35.3 (38.0)	13.2	16.7
16	H_2N-Lys-TCACTAGATG-H 5'-AGTGAT**T**TAC-3'	**4** **4** GNA (ap) **18** **18** DNA	36.4 (38.0)	15.6	23.0

Buffer: 100 mM NaCl, 10 mM Na phosphate, 0.1 mM EDTA, pH 7.0. Total strands concentration: 3 μM; ramp rate: 0.5°C/min; T_m in °C; Notations: (p) and (ap) indicate parallel and antiparallel orientations respectively; mm indicates a base mismatch noted in bold and underscored; H_2N- indicates the carboxamide terminus, -H, the amino terminus. ΔH is in kcal/mol, ΔS in cal/(mol x deg K). The signs of ΔH and ΔS are negative for duplex formation. * Melting curves do not fit the two-state model.

The binding affinities and selectivities of these oligomers to DNA and RNA targets were determined according to measurement of duplex formation and melting temperatures (Table). Thermodynamic constants were obtained from fits of melting curve data to a two-state model with linear sloping baselines.[10] Based on the T_m's of Entries 3, 4, 5, 6, it appears that Oligo **3** prefers to bind to RNA aligned such that the amino terminus is "5'-like" (conventional oligonucleotide expresssion). The T_m=54.1°C of Entry 6 indicates that GNA sequences bind to RNA with an overall affinity comparable to that of DNA to RNA (Entry 2). The T_m's of Entries 7, 8, 9 reflect the change in binding affinity as a result of introduction of a base-pair mismatch in the target RNA strand. A drop in the melting temperature in each of the three mismatch cases (-5.9 to -10.9°C) indicates a selectivity in the recognition of RNA targets by GNA, consistent with a Watson-Crick binding model. When **3** was incubated with human and bovine serum (37°C), no degradation was observed within the detection limits of an HPLC monitoring method during greater than 24 hours.

The mixed GNA sequence **4** bound to complementary antiparallel DNA at T_m=45.9°C (Entry 13), 10°C higher than analogous DNA/DNA or DNA/RNA meltings. Antiparallel PNA of the same sequence binds to DNA

with a T_m of 51.0°C.[6] Single DNA mismatches to an internal G residue in the GNA strand (Entries 14, 15, 16) resulted in decreases of the melting temperature (-4.6 to -10.6°C). This behavior is again consistent with Watson-Crick binding. In the case of the double melts (Entries 5 and 12), relative hypochromicities of only 0.95 were observed in contrast to those of approximately 0.8 for single melt curves. Homopyrimidine GNA **3** in absence of a complementary strand showed a melting-like transition at 20°C, which we presume to represent a low affinity self-aggregation. Such a transition occurs with a shift in the UV absorption maximum from 270 nm to 260 nm.

In all cases, no hysterisis in the melting curves was observed. Hysterisis in the melting curves of *some* PNA sequences has been observed and is thought to be related to the slow kinetics of duplex transition.[11] For melt curves of GNA/DNA which showed a single transition consistent with a two-state model, all showed smaller incremental changes in OD as a function of temperature compared to those for DNA/DNA and DNA/RNA. Thus, the smaller changes in entropy calculated for GNA/DNA and GNA/RNA melting transitions as compared to those for DNA/DNA and DNA/RNA are consistent with the idea that GNA oligomer conformation is already organized and requires little adjustment for binding. The absence of a single melting transition in the case of sequence **4** binding to complementary antiparallel RNA (Entry 12) indicates the need to further elucidate the binding and recognition properties of GNA.

In conclusion, we have demonstrated a general method for the construction of a highly novel, water soluble, and stable oligonucleotide analog that mimics some binding properties of DNA and RNA. These results are an interesting contrast to those reported for artificial homo-DNA and pyranosyl-RNA, hexose oligonucleotide analogs which do not bind DNA or RNA.[12] The study of GNA binding to DNA and RNA oligonucleotides may contribute to the mechanistic understanding of Watson-Crick base-pair recognition. The results of antisense biological activity assays of GNA constructs will be reported elsewhere.

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3 Abbreviations: BHA resin: benzhydrylamine resin; TFA: trifluoroacetic acid; eq: equivalent; TES: triethylsilyl; HATU: O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; N,N-diisopropylethylamine; rt: room temperature; RP HPLC: reversed phase HPLC; MALDI-TOF: matrix-assisted laser desorption ionization-time of flight mass spectrometry; TBAF: tetrabutylammonium fluouride; T_m: melting temperature; OD: optical density.

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5 Synthesis report in progress.

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7 Synthesis reported in the preceding communication.

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9 The DNA / DNA duplex composed of strands having the reverse orientation (i.e., 3'-TCTCTCTCCTTCT-5' binding to 5'-AGAGAGAGGAAGA-3' was calculated to have a melting temperature of 44°C by Oligo Software Package. Similarly, the analogous RNA / RNA duplex was calculated to melt at 52°C.

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