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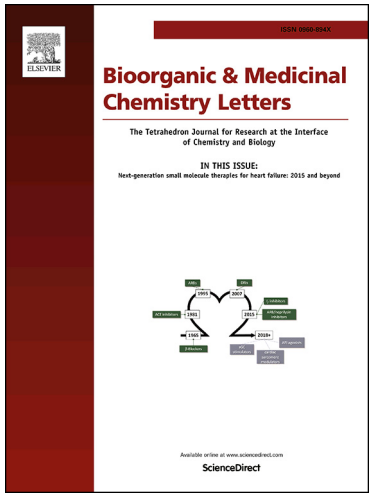

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Synthesis of cationic glucosamino nucleic acids for stabilizing oligonucleotides

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Keywords

Glucosamine; Nucleic acids; Oligonucleotides; Pyranosyl nucleosides; Zwitterionic nucleotides

Abstract

Glucosamino nucleic acids (GANAs) bearing a β-*N*-glycoside bond between carbon 1 of the glucosamine and the nucleobase nitrogen were synthesized and incorporated into oligonucleotides (4′,6′-GANA and 3′,6′-GANA). The thermal stability of oligonucleotide duplexes containing the GANA zwitterionic nucleotides was also investigated.

Chemically modified oligonucleotides (ONs) are currently attracting intense interest because they are tools in molecular biology, gene probes for diagnosis, and potential drugs. Many chemical modification strategies have been employed to improve the nuclease stability, RNA-binding affinity, and pharmacokinetic properties of ONs for therapeutic applications. In particular, the sugar moieties are often modified to increase nuclease resistance or increase affinity for the complementary target. Several series of modified nucleosides with a six-membered carbohydrate moiety instead of the five-membered sugar ring contained in natural nucleosides have been synthesized and incorporated into ONs (Figure 1).[1] Examples include homo-DNAs,[2] hexitol nucleic acids (HNA),[3] mannitol nucleic acids (MNA),[4] and altritol nucleic acids (ANA).[4] Herdewijn[5,6] and Eschenmoser[7,8] developed pioneering synthetic access to ONs containing hexopyranosyl and dideoxyhexopyranosyl nucleosides. Moreover, ONs that contain pyranosyl nucleoside analogues may form stable duplexes.[9]

Glucosamine, an amino derivative of glucose, has a typical pyranose ring structure. The polysaccharides chitosan and chitin contain glucosamine as a structural element that has high membrane affinity and low toxicity.[10] The positive charge on glucosamine can also neutralize the negatively charged phosphates on ONs. Therefore, we have focused our research on the thermal stability of ONs containing glucosamino nucleic acids (GANAs). We have previously synthesized glucosamine derivatives with thymine at the 6-position for an RNAi study.[11] Here, we synthesize the thymine analogue (GANA-T) bearing a β-*N*-glycoside bond between carbon 1 of the glucosamine and nitrogen 1 of the nucleobase thymine to prepare ONs with two types of linkage between the glucosamine and natural nucleotides, 4′,6′-linked ONs and 3′,6′-linked ONs (Figure 1). We investigated the hybridization of ONs containing GANA-T either in the center or at the end of the sequence.

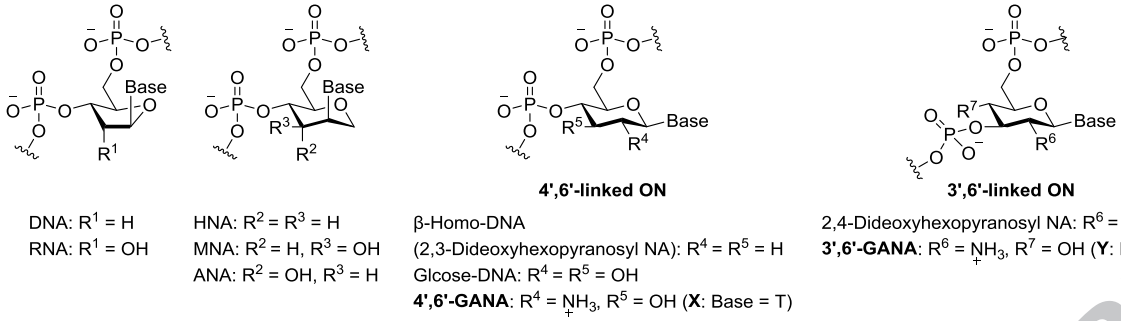
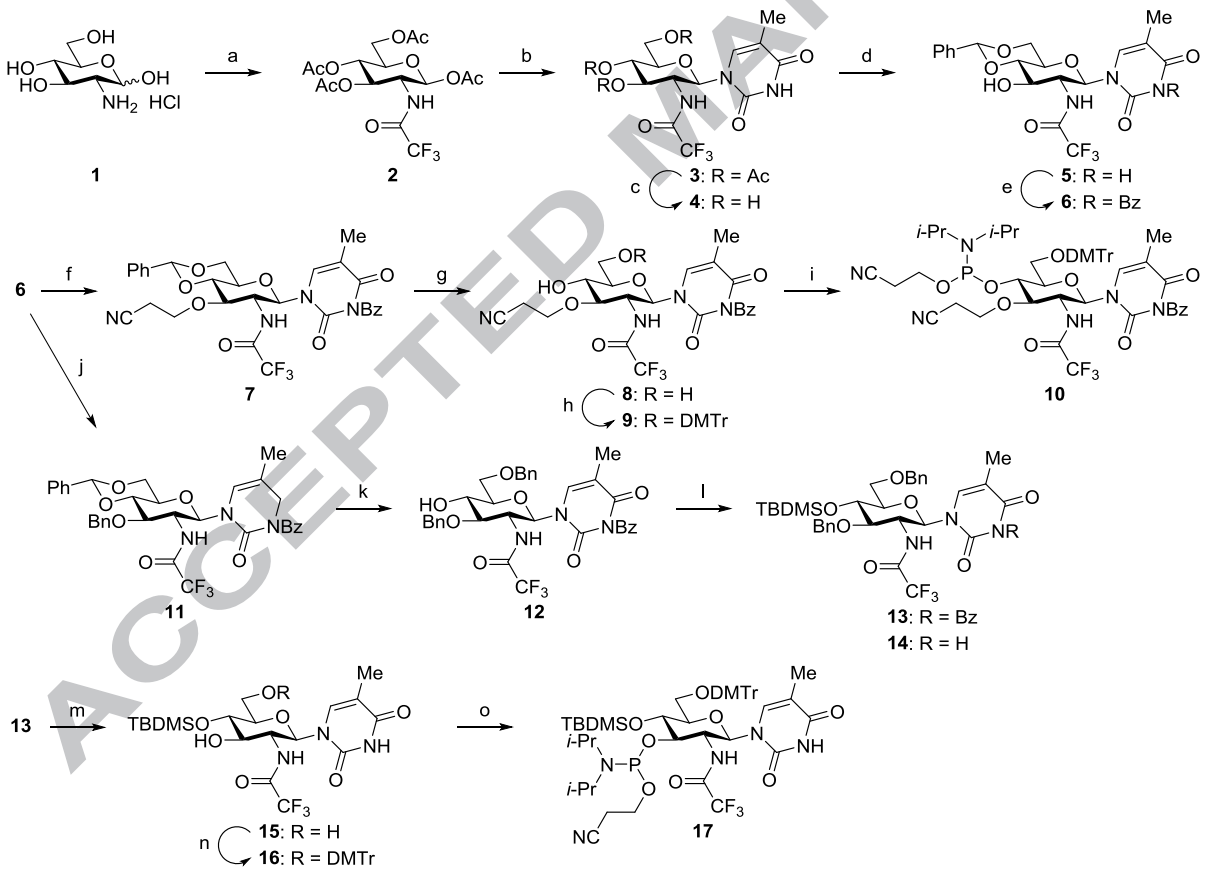


Figure 1. Partial structures of DNA, RNA, and chemically modified ONs bearing various pyranosyl nucleoside analogs.

The synthetic route of the GANA-T derivatives, thymine analogues of 4′,6′-GANA (**X**) and 3′,6′-GANA (**Y**) (Base = T in Figure 1), is shown in Scheme 1. First, thymine was introduced into the anomeric position of **2**[12,13], which was easily prepared from glucosamine hydrochloride (**1**), using trimethylsilyl trifluoromethanesulfonate (TMSOTf) to obtain **3** with complete stereoselectivity. After all acetyl groups were removed by treatment with NaOMe, the 4'- and 6'-positions of the sugar moiety were protected by a benzylidene group, and subsequent benzoyl protection of the N^3 position of the thymine moiety produced key intermediate **6**. We chose cyanoethyl as the protecting group for the free 3′- or 4′-hydroxyl group in the phosphoramidite derivatives. Compound **6** was treated with acrylonitrile to afford **7**. The benzylidene acetal was removed by treatment with (±)-10-camphorsulfonic acid (CSA) to give **8** in 81% yield. Next, the 6′-hydroxyl group of **8** was protected by a 4,4′-dimethoxytrityl (DMTr) group to give corresponding 6′-DMTr derivative **9**, which was phosphitylated with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite to produce **10**. In addition, **6** was treated with benzyl bromide and sodium hydride in DMF to afford corresponding benzyl compound **11** in 80% yield. Subsequent regioselective reductive ring opening of the benzylidene acetal[14] gave 3′,6′-dibenzyl-protected derivative **12**. Cyanoethyl protection of 4′-hydroxyl group failed; therefore, we protected the 4′-OH with a *tert*-butyldimethylsilyl (TBDMS) group, obtaining corresponding 4′-silyl-protected derivative **13**, although partial debenzoylation occurred. Next, the benzyl groups and *N*-benzoyl group of thymine were removed by hydrogenation with $Pd(OH)_2/C$ in EtOH. Finally, DMTr protection of the primary 6′-hydroxyl group

of **15** followed by phosphitylation reactions provided phosphoramidite derivative **17**.

To investigate the hybridization properties of GANA-T-modified ONs with a complementary strand, a set of dT15-mers modified with GANA-T at the 5′-end, at the center, or at the 3′-end (ONs **19**–**23**) were synthesized by an automated nucleic acid synthesizer using phosphoramidite derivative **10** or **17** (Table 1). The structures of these nucleic acid oligomers were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis. In the hybridization experiments, the natural dT_{15} (ON **18**) and each synthetic GANA-T-modified ON (ONs **19**–**23**) were incubated with complementary 15-mer DNA (dA_{15}) and 15-mer RNA (rA_{15}) (Table 1).



Scheme 1. Reagents and conditions: (a) i) CF_3COOEt, Et_3N, MeOH, rt; ii) Ac_2O, pyridine, rt, 99%; (b) $(TMS)_2$-thymine, TMSOTf, MeCN, reflux, 74%; (c) NaOMe, MeOH, rt, 99%; (d) $PhCH(OMe)_2$, CSA, MeCN, reflux, 79%; (e) i) TMSCl, pyridine, rt; ii) BzCl, rt, iii) H_2O, rt, 75%; (f) acrylonitrile, Cs_2CO_3, *t*-BuOH, DMF, rt, 49%; (g) CSA, MeOH, rt, 81%; (h) DMTrCl, pyridine, rt, 44%; (i) (*i*-Pr_2N)P(Cl)O$(CH_2)_2$CN, *i*-Pr_2NEt, CH_2Cl_2, rt, 77%; (j) BnBr, NaH, DMF, −20 °C, 80%; (k) CF_3CO_2H,

$(CF_3CO)_2O$, Et_3SiH, CH_2Cl_2, rt, 52%; (l) TBDMSCl, imidazole, DMF, 80 °C, 36% for **13** and 41% for **14**; (m) H_2, $Pd(OH)_2/C$, EtOH, rt, 54%; (n) DMTrCl, pyridine, rt, 48%; (o) (*i*-Pr_2N)P(Cl)O$(CH_2)_2$CN, *i*-Pr_2NEt, CH_2Cl_2, rt, 56%.

Table 1. T_m values of double-stranded ONs containing GANA-T.

No.of ON	Sequence	dA_{15}	rA_{15}
		T_m (°C)	T_m (°C)
ON **18**	5'-d(TTTTTTTTTTTTTTT)-3'	37.4	36.6
ON **19**	5'-d(**X**TTTTTTTTTTTTTT)-3'	35.9 (−1.5)	33.8 (−2.8)
ON **20**	5'-d(TTTTTTT**X**TTTTTTT)-3'	26.2 (−11.2)	24.7 (−11.9)
ON **21**	5'-d(TTTTTTTTTTTTTTT**X**)-3' [a]	37.5 (+0.1)	35.8 (−0.8)
ON **22**	5'-d(**Y**TTTTTTTTTTTTTT)-3'	35.7 (−1.7)	33.2 (−3.4)
ON **23**	5'-d(TTTTTTT**Y**TTTTTTT)-3'	23.9 (−13.5)	24.9 (−11.7)

[a]The structure is the same as that of 5'-d(TTTTTTTTTTTTTTT**Y**)-3'.

[b]The values in parentheses indicate ΔT_m relative to the unmodified duplex.

[c]Measurements were carried out in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), and 100 mM NaCl, with 3.0 μM of each ON.

The T_m value of the modified ON bearing GANA-T at the 3′-end (ON **21**) with dA_{15} and rA_{15} was similar to that of unmodified dT_{15} (ΔT_m +0.1 and −0.8 °C, respectively). The binding affinities of modified ONs with GANA-T at the 5′-end (ONs **19** and **22**) toward the complementary DNA and RNA were lower than that of the corresponding 3′-GANA-T-modified ON (**21**). The ΔT_m values were similar for the DNA and RNA complementary duplexes of 4′,6′-linked (**X**) ON **19** and 3′,6′-linked (**Y**) ON **22** containing GANA-T at the 5′-end. The thermal stabilities of the duplexes of ONs containing GANA-T in the center (ON **20** and ON **23**) with complementary DNA or RNA were lower than those of the unmodified natural duplexes in all strands. The stability of ON **20**/rA_{15} (T_m = 24.7 °C) was similar to that of ON **23**/rA_{15} (T_m = 24.9 °C). In contrast, the binding affinities for the DNA complementary duplexes of 4′,6′-linked (**X**) ON **20** and 3′,6′-linked (**Y**) ON **23** containing GANA-T in the center were different. The stability of ON **20**/dA_{15} (T_m = 26.2 °C) was greater than that of ON **23**/dA_{15} (T_m = 23.9 °C). The stability of duplexes with DNA complements were slightly greater than those of duplexes with RNA complements in ONs with GANA-T at the 3′- or 5′-end. However, the DNA/DNA duplex with **Y** in the center (ON **23**/dA_{15}) was slightly less stable than the DNA/RNA duplex with **Y** in the center (ON **23**/rA_{15}). DNA/DNA duplexes usually possess B-type duplex geometries under physiological conditions, whereas RNA/DNA duplexes have A-type duplex geometries. Thus, the

GANA-T conformation in ONs should differ between A- and B-type duplexes, and the thymine moiety in GANA-T, especially **X**, can form more favorable base pairs with the complementary adenine base of the opposite strand in B-type DNA/DNA duplexes than in A-type DNA/RNA duplexes.

Table 2. T_m values of duplexes formed between ONs and single-stranded DNA or RNA.

No.of ON	d(A_7-Z-A_7)				r(A_7-Z-A_7)			
	Z = A	Z = T	Z = C	Z = G	Z = A	Z = U	Z = C	Z = G
ON **18**	37.4	27.8	27.9	28.4	36.6	24.5	22.2	31.5
ON **20**	26.2	18.1	16.7	18.5	24.7	18.9	14.6	24.2
ON **23**	23.9	21.0	21.2	22.3	24.9	19.7	18.2	23.9

[a]Measurements were carried out in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), and 100 mM NaCl, with 3.0 μM of each ON.

To measure the base recognition activity of **X** or **Y** in ONs, we investigated the binding affinity of ON **20** with **X** and ON **23** with **Y** for single-stranded DNA [d(A_7-Z-A_7)] bearing a deoxyribonucleoside (Z = A, G, C, or T) or RNA [r(A_7-Z-A_7)] bearing a ribonucleoside (Z = A, G, C, or U) at the center of each strand. The T_m values are summarized in Table 2. In general, ON **20** with **X** and ON **23** with **Y** formed the most stable duplexes with ONs bearing guanine in the opposite position [d(A_7-G-A_7) and r(A_7-G-A_7)], with the exception of complementary sequences (Z = A). Remarkably, the duplex formed between ON **20** and r(A_7-G-A_7) furnished a T_m value of 24.2 °C, which is only 0.5 °C lower than that obtained for the corresponding ON **20**/rA_{15} duplex (T_m = 24.7 °C). In addition, the ΔT_m value for the duplexes ON **23**/r(A_7-G-A_7) and ON **23**/rA_{15} is 1.0 °C. This result may be explained by a G-T wobble base pair forming in DNR/RNA duplexes. In contrast, the T_m values of ON **23** with single base mismatches in the DNA counter strands [d(A_7-Z-A_7)] (Z = T, C, and G) were close to those of unmodified dT_{15} (ON **18**) (ΔT_m −2.9, −2.7, and −1.6 °C, respectively). Thus, ON **23** was relatively insensitive to single-base mismatches in the DNA counter strand.

In conclusion, we have described a robust synthetic route for new GANAs bearing a β-*N*-glycoside bond between carbon 1 of the glucosamine and the nitrogen of the nucleobase. Several thymidine oligomers bearing two types of linkage between the glucosamine nucleoside (**X** and **Y**) and natural nucleoside were synthesized. Substituting these glucosamine nucleotides into the center of a 15-mer had a greater effect on duplex stability than end-substitution. The T_m experiments showed that the base recognition activity of **X** or **Y** in ONs differs between DNA/DNA duplexes and DNA/RNA duplexes.

Further investigation of the properties of GANA-T is currently in progress and the results will be reported in due course.

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Highlights

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