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Synthesis and Biophysical Investigations of Oligonucleotides Containing Galactose-Modified DNA, LNA, and 2′-Amino-LNA Monomers

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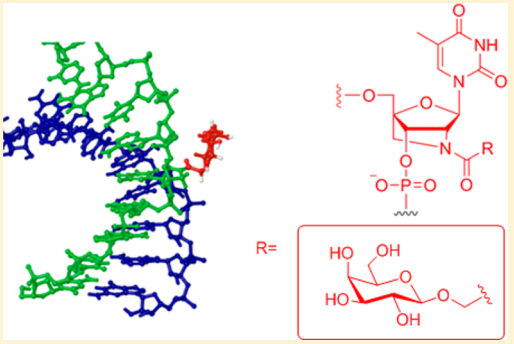
Supporting Information

ABSTRACT: Galactose-modified thymidine, LNA-T, and 2′-amino-LNA-T nucleosides were synthesized, converted into the corresponding phosphoramidite derivatives and introduced into short oligonucleotides. Compared to the unmodified control strands, the galactose-modified oligonucleotides in general, and the N2′-functionalized 2′-amino-LNA derivatives in particular, showed improved duplex thermal stability against DNA and RNA complements and increased ability to discriminate mismatches. In addition, the 2′-amino-LNA-T derivatives induced remarkable 3′-exonuclease resistance. These results were further investigated using molecular modeling studies.



INTRODUCTION

Carbohydrates and nucleic acids are two important classes of biomolecules. Direct conjugation between carbohydrates and nucleic acid components opens up new possibilities regarding optimization of biomolecular recognition processes. In nature few examples of such combined molecules exist, e.g., base J (β-D-glucosyl-hydroxymethyluridine) known in parasites like *Trypanosoma brucei* or *Leishmania major*[1] and glucosylated 5-hydroxymethyl-2′-deoxycytidine known in coliphages.[2] Until now the function of these carbohydrate modified nucleosides is still not entirely proven, but base J seems to be important in diminishing transcription and regulating gene expression,[3] while glucosylated 5-hydroxymethyl-2′-deoxycytidine enhances the stability against restriction endonuclease cleavage.[4]

For nucleic acid drug development carbohydrate-modified oligonucleotides (ONs) are of current great interest as a strategy to improve tissue targeting and cellular uptake of, e.g., small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs).[5−16] A prominent example is the recognition of galactose or *N*-acetylgalactosamine ligands by the asialoglycoprotein receptor (ASGPR) on parenchymal liver cells.[17−19] To enhance liver uptake of nucleic acid therapeutics different galactose or *N*-acetylgalactosamine bearing linker strategies have been developed.[14,18,20] The most intensively investigated building blocks are based on triantennary galactose/*N*-acetylgalactosamine units connected for example via a tris-(hydroxymethyl)aminomethane or an ethylene glycol linker to either the 3′- or the 5′-end of the desired ON.[18] In alternative approaches, the carbohydrate units have been attached on different types of nucleotides positioned either toward the ends or more centrally within the ON.[14] Another protein-carbohydrate recognition modus which has been explored is the hyaluronan-CD44 recognition. CD44 is a transmembrane glycoprotein which is involved in inflammation processes and is overexpressed in many cancers,[21] and for example have results with hyaluronan containing ONs in the context of gene silencing or PET imaging been reported.[22,23]

Different methods for the introduction of carbohydrates into ON strands have been realized. They can be installed terminally during ON synthesis either at the 5′-end via phosphoramidite chemistry, or used together with a solid support in the 3′-end as has been realized for the previous described triantennary carbohydrate linkers. The groups of Hrdlicka and Kobayashi have modified LNA-U and 2′-deoxyuridine, respectively, in the C5 position of the pyrimidine base with an acetylene linked monosaccharide under Sonogashira conditions.[24,25] As in base J, formation of a direct glycosidic bond between a carbohydrate

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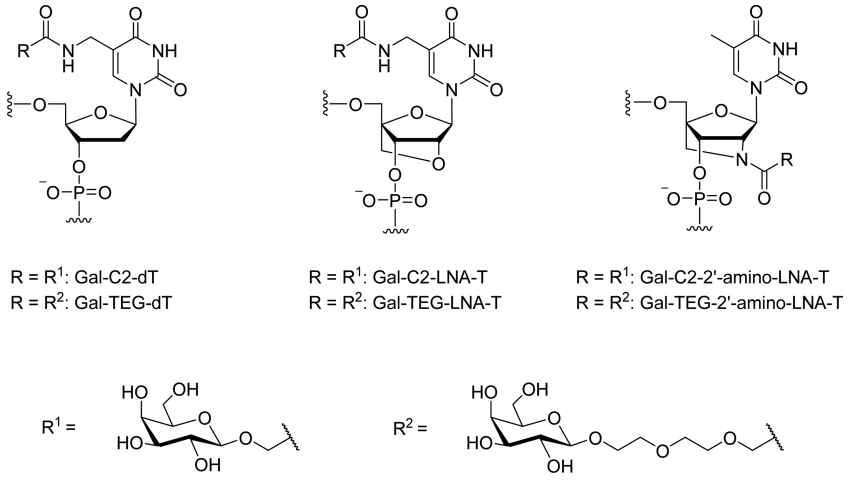
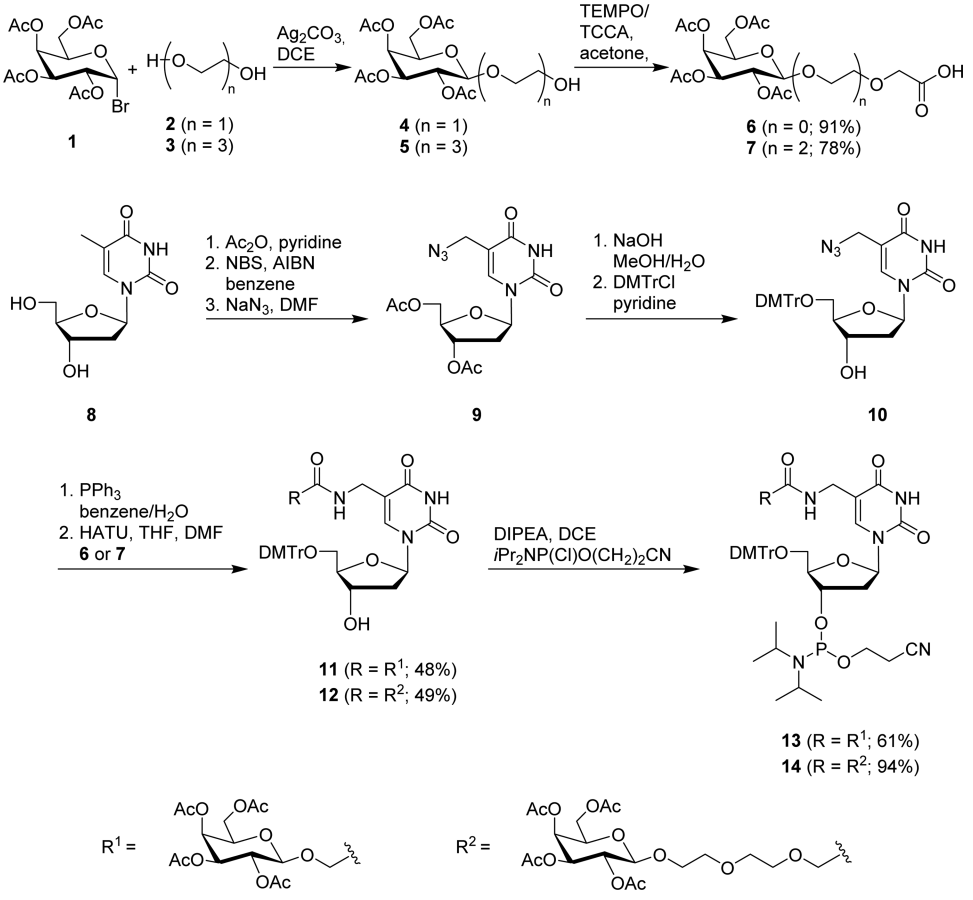


Figure 1. Structure of the six different galactose modified nucleotides studied herein.

Scheme 1. Synthesis of Galactosyl Carboxylic Acids 6 and 7 and Galactose Modified Thymidine Phosphoramidites 13 and 14[a]



[a]TEMPO = 2,2,6,6-tetramethyl-1-piperidinyloxy, TCCA = trichloroisocyanuric acid, HATU = 1-(bis(dimethylamino)methylene)-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium3-oxid hexafluorophos-phate, DMTr = 4,4′-dimethoxytrityl.

and a hydroxyl modified base of the nucleoside is also possible.[26,27] The group of Lönnberg exchanged the whole nucleobase by a triantennary carbohydrate linker using oxime and azide−alkyne cycloaddition chemistry.[28] Beigelman and co-workers introduced several galactosamines in the 2′-position of deoxyribose through an amide linker strategy.[29] Matsuda et al. used a similar approach in which they coupled one carbohydrate functionalized carboxylic acid moiety either to the 2′- or 3′-position of a ribose ring via amide bond formation.[14] Other examples include the introduction of carbohydrates during or after DNA/RNA synthesis, as exemplified by amide bond formation between a modified nucleobase and an amine carbohydrate linker during or after ON synthesis, and azide−alkyne cycloaddition reaction between an alkyne modified deoxyribose nucleotide and an azide-linked galactosamine after ON synthesis.[30−32] Very recently, formation of glucose-nucleobase pseudo base pair has been demonstrated.[33]

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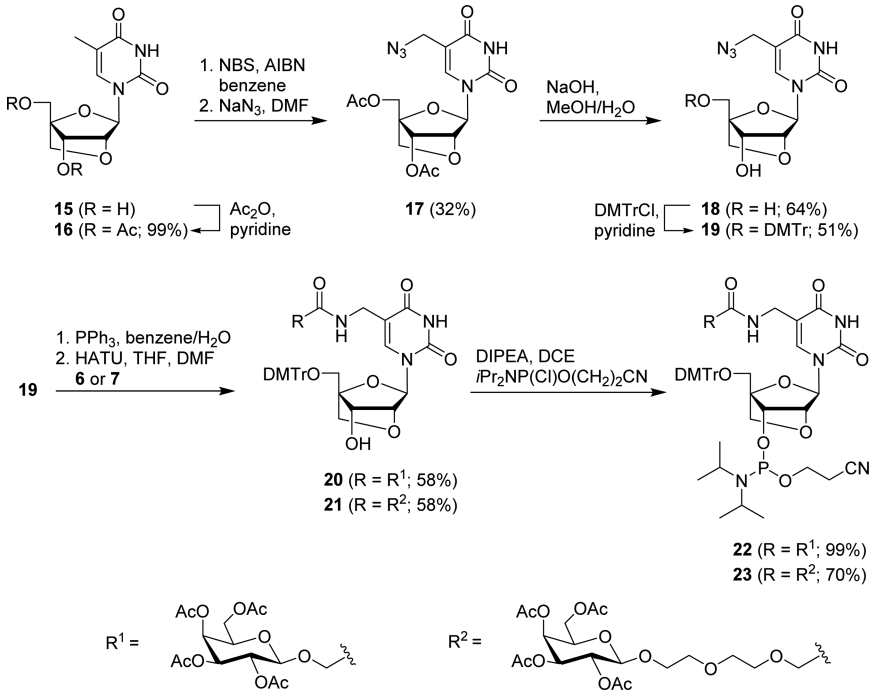
Scheme 2. Preparation of the Galactosyl Containing LNA-T Derivatives 22 and 23



Modified nucleotide monomers are widely used within ON drug development to enhance the affinity and selectivity toward complementary nucleic acids and to improve the nuclease resistance.[34−37] Locked nucleic acid (LNA) monomers in which the ribofuranose moiety is locked in a C-3′-*endo* (North type) conformation by the introduction of an oxomethylene linker between the C2′ and C4′ atoms of the ribose ring is a prominent example inducing a very significant increase in duplex and triplex stabilities.[38−41] Several structurally modified LNA analogues have been synthesized in the past few years to enhance binding specificity, stability and biodistribution. Most of these modifications have involved the linker between the C2′ and C4′ atoms as exemplified by 2′-amino-LNA, carba-LNA,[42] spirocyclopropylene-LNA[43] and methylene-carba-LNA[44] but also modifications/attachments in the C5 position of the pyrimidine base.[41,45,46] One recent example of the latter involved carbohydrate modified LNA derivatives from the Hrdlicka laboratory.[24] They compared glucose, galactose and lactose modified LNA nucleotides incorporated into ONs in view of binding activity, specificity and nuclease resistance. They used an alkyne linkage between the carbohydrate and the C5 position of LNA-U.

N2′-Functionalized 2′-amino-LNA monomers constitute another attractive class of ON analogs for further derivatization.[47,48] The substituents attached to the N2′ atom are unlike C5 modifications of the nucleobase oriented into the minor groove of a double helix, and various molecular entities have successfully been attached to 2′-amino-LNA,[49] e.g., a piperazino motif.[50]

In this paper six different galactose-modified DNA, LNA and 2′-amino-LNA derivatives have been synthesized (Figure 1). For modification at the C5-position of the thymine base of thymidine and LNA-T monomers, base-modified azido derivatives served as precursors, whereas in the case of N2′-modification of 2′-amino-LNA-T the synthesis started from known 2′-amino-LNA nucleoside derivate **24** [(1*R*,3*R*,4*R*,7*S*)-1-(4,4′-dimethoxytrityloxymethyl)-7-hydroxy-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2.2.1]heptane].[48]

RESULTS AND DISCUSSION

Synthesis of Phosphoramidites. The two desired galactosyl carboxylic acids **6** and **7** were synthesized in two steps (Scheme 1). In the first step either ethylene glycol (**2**) or triethylene glycol (**3**) were attached to acetobromo-α-D-galactose (**1**),[51,52] and in the second step the primary hydroxy groups were converted to the corresponding carboxylic acids using TEMPO/TCCA oxidation in yields of 91% (for **6**) and 78% (for **7**). 5-Azidomethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**10**) was synthesized according to known procedures[53−55] but optimized with respect to the acetyl deprotection step, for which we used sodium hydroxide in methanol/water instead of ammonium hydroxide as reported since we otherwise observed partial substitution of the azido group by an amino group. After 4,4′-dimethoxytrityl (DMTr) protection of the 5′-hydroxy group, the azido group of compound **10** was reduced to the corresponding amine using Staudinger conditions, and was subsequently coupled with the appropriate galactosyl carboxylic acid (**6** and **7**) in the presence of HATU. Finally, the galactosyl derivatives **11** and **12** were converted into the corresponding 2-cyanoethyl-*N*,*N*′-diisopropylphosphoramidites **13** and **14**, respectively, in satisfactory yields.

Similar synthetic routes were used for the two LNA derivatives (Scheme 2). Acetylation and bromination of LNA-T **15**, and conversion to the desired azide **17** was performed in 32% combined yield. Saponification followed by DMTr protection of the 5′-hydroxy group led to azido derivative **19** ready for coupling with the galactose derivatives **6** and **7**. Staudinger reduction and coupling of the two building blocks with HATU gave the desired LNA-T derivatives **20** and **21** in a moderate yield of 58% for both compounds. To prepare for automated ON synthesis, compounds **20** and **21** were

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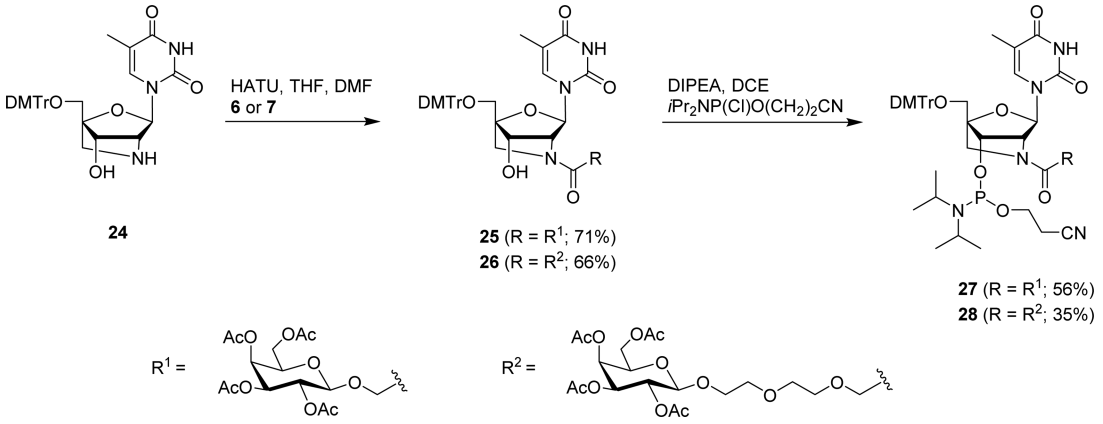
Scheme 3. Synthesis of Galactose Modified 2′-Amino-LNA-T 27 and 28 via Amide Bond Formation



converted to the respective phosphoramidite derivatives **22** and **23** in good yields by reaction with 2-cyanoethyl-*N*,*N*′-diisopropylchlorophosphoramidite reagent.

To obtain the functionalized 2′-amino-LNA-T building blocks, nucleoside **24**[48] was coupled with the corresponding galactosyl carboxylic acids **6** and **7** in the presence of HATU followed by phosphoramidite synthesis (Scheme 3) to give derivatives **27** and **28** in moderate yields.

ON Synthesis. All six phosphoramidites **13**, **14**, **22**, **23**, **27** and **28** were successfully used in automated ON synthesis on a commercial nucleic acid synthesizer. The stepwise coupling yields were 80−90% for all modified phosphoramidites (using so-called "hand coupling conditions")[56] and ∼99% for standard DNA and LNA phosphoramidites. Under standard ON deprotection conditions (32% ammonia, 50 °C, 16 h) we observed partial cleavage of the galactose from the nucleotide with the C5-functionalized monomers. This demonstrated that the amide linkage between the carbohydrate linker and the nucleobase was cleaved resulting in an aminomethyl group being present at the C5 position of the modified nucleotides. This problem was solved using so-called ultramild phosphoramidites for ON synthesis, which enabled the use of milder deprotection conditions (32% ammonia, r.t., 12 h). All ONs were purified by reversed-phase HPLC and their composition and purity (>80%) were verified by MALDI-TOF MS (Table S1, Supporting Information) and ion exchange HPLC, respectively.

Thermal Denaturation Experiments: Binding Affinity. 9-Mer DNA sequences with one or three incorporations of each of the galactose-modified monomers as well as one galactose-modified monomer and two LNA monomers were synthesized to evaluate the effect of galactose-conjugation on binding affinity (Table 1−3) and base-pairing specificity (Table 4) toward complementary DNA and RNA. In the discussion below the values obtained for the unmodified all-DNA 9-mer strand **ON1** and the LNA modified 9-mer strand **ON18** are the reference points used for comparison.

All DNA reference **ON1** displayed a thermal denaturation temperature of 31.0 °C with a DNA complementary strand and of 29.5 °C with an RNA counterpart. The results of **ON2** showed that the galactosyl-modification installed via C5 on a thymidine monomer with the shorter ethylene glycol linker (Gal-C2-) was well tolerated in a DNA/DNA duplex as the thermal denaturation temperature was comparable with that of the reference **ON1** (Table 1). A slight destabilization of −2.0 °C was observed with the RNA complement. The corresponding galactosyl-modification with the longer triethylene glycol linker (Gal-TEG-) in **ON3** was less well tolerated as the thermal denaturation temperature was reduced by −3.5 °C against a DNA complement and −5.0 °C against an RNA complement. LNA modified **ON4**-**ON9** showed increased thermal stability for duplexes against both DNA and RNA relative to reference **ON1** but the galactosyl C5-modified LNA-T monomer stabilized less than the unmodified LNA-T monomer. Also in this case the shorter linker (**ON5**) was better tolerated than the triethylene glycol one (**ON6**). All the 2′-amino-LNA-T modified ONs showed thermal denaturation temperatures higher than the C5-modified ones, in fact reflecting thermal stabilities comparable to those of **ON4** and **ON7** containing parent LNA and 2′-amino-LNA monomers, respectively.

In general similar trends were observed for the sequences containing three modifications (Table 2). However, **ON10** with three galactose moieties attached via a short linkage to the thymidine showed only slightly decreased thermal denaturation temperature while **ON11** with a triethylene glycol linker displayed more pronounced decreases in thermal denaturation temperatures. As earlier reported,[38] three incorporations of LNA-T stabilized the duplexes significantly (**ON12**), while the Gal-C2-LNA-T modification was less stabilizing (**ON13**) and

Table 1. Thermal Denaturation Temperatures of Matched Duplexes for the Sequence 5′-GTG AXA TGC[a]

	5′-GTG AXA TGC	T_m [°C]		T_m [°C]	
ON	X	DNA: 3′-CAC TAT ACG		RNA: 3′-CAC UAU ACG	
ON1	T	31.0	(ref.)	29.5	(ref.)
ON2	Gal-C2-T	31.5	(+0.5)	27.5	(−2.0)
ON3	Gal-TEG-T	27.5	(−3.5)	24.5	(−5.0)
ON4	LNA-T	37.0	(+6.0)	38.5	(+9.0)
ON5	Gal-C2-LNA-T	35.0	(+4.0)	34.5	(+5.0)
ON6	Gal-TEG-LNA-T	33.0	(+2.0)	33.0	(+3.5)
ON7	2′-amino-LNA-T	36.5	(+5.5)	37.0	(+7.5)
ON8	Gal-C2−2′-amino-LNA-T	37.0	(+6.0)	38.5	(+9.0)
ON9	Gal-TEG-2′-amino-LNA-T	36.0	(+5.0)	37.5	(+8.0)

[a] T_m values (°C) of duplexes recorded in medium salt buffer (100 mM NaCl, 0.1 mM EDTA, 5.8 mM NaH_2PO_4/Na_2HPO_4, pH 7.0) using 2.5 μM of each strand measured as the maxima of the first derivatives of the thermal melting curves (A_{260} vs T). The T_m values are an average of two measurements within ±0.5 °C. Numbers in brackets are the ΔT_m values calculated as the difference in T_m values between unmodified and modified duplexes.

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Table 2. Thermal Denaturation Temperatures of Matched Duplexes for the Sequence 5′-GXG AXA XGC[a]

	5′-GXG AXA XGC	T_m [°C]		T_m [°C]	
ON	X	DNA: 3′-CAC TAT ACG		RNA: 3′-CAC UAU ACG	
ON1	T	31.0	(ref.)	29.5	(ref.)
ON10	Gal-C2-T	30.0	(−1.0)	23.0	(−6.5)
ON11	Gal-TEG-T	21.0	(−10.0)	16.0	(−13.5)
ON12	LNA-T	45.0	(+14.0)	52.5	(+23.0)
ON13	Gal-C2-LNA-T	37.0	(+6.0)	42.5	(+13.0)
ON14	Gal-TEG-LNA-T	31.0	(±0)	37.5	(+8.0)
ON15	2′-amino-LNA-T	43.0	(+12.0)	49.5	(+20.0)
ON16	Gal-C2−2′-amino-LNA-T	44.5	(+13.5)	54.5	(+25.0)
ON17	Gal-TEG-2′-amino-LNA-T	41.0	(+10.0)	51.5	(+22.0)

[a]See footnotes Table 1.

the Gal-TEG-LNA-T (**ON14**) modification neutral relative to **ON1** against a DNA complement and slightly stabilizing with an RNA complement. Three incorporations of the galactose modifications of 2′-amino-LNA-T was very well tolerated as already observed for single incorporations.

For the results of the ONs with one central modification flanked by two LNA-T nucleotides, **ON18** was used as the reference (Table 3). In case of the thymidine nucleotides

Table 3. Thermal Denaturation Temperatures of Matched Duplexes for the Sequence 5′-GT^{L}G AXA T^{L}GC[a]

	5′-GT^{L}G AXA T^{L}GC	T_m [°C]		T_m [°C]	
ON	X	DNA: 3′-CAC TAT ACG		RNA: 3′-CAC UAU ACG	
ON18	T	39.0	(ref.)	44.5	(ref.)
ON19	Gal-C2-T	37.5	(−1.5)	39.0	(−5.5)
ON20	Gal-TEG-T	35.0	(−4.0)	34.5	(−10.0)
ON21	LNA-T	45.5	(+6.5)	52.5	(+8.0)
ON22	Gal-C2-LNA-T	42.0	(+3.0)	48.0	(+3.5)
ON23	Gal-TEG-LNA-T	40.5	(+1.5)	47.0	(+2.5)
ON24	Gal-C2−2′-amino-LNA-T	46.0	(+7.0)	54.0	(+9.5)
ON25	Gal-TEG-2′-amino-LNA-T	45.5	(+6.5)	52.5	(+8.0)

[a]See footnotes Table 1. **T^L** = LNA-T.

(**ON19** and **ON20**), C5-attachment of the galactosyl moieties destabilized slightly (DNA complement) or strongly (RNA complement). In the case of the C5-substituted LNA nucleotides (**ON22** and **ON23**), increased duplex thermal stability was observed though not to the level observed in the case of incorporation of three LNA-T residues (**ON21**). And again the triethylene glycol linker (**ON23**) induced less favorable hybridization than the ethylene glycol linker (**ON22**). In contrast, the galactosyl-modified 2′-amino-LNA-T containing ONs (**ON24** and **ON25**) displayed hybridization properties comparable to those of **ON21**. For the 2′-amino-LNA-T containing ONs, the linkage used to attach the galactosyl units had less influence on the thermal denaturation temperature than for the ONs containing C5-substituted thymidine and LNA-T nucleotides.

In summary, incorporation of C5-functionalized thymidine and LNA-T nucleotides, designed to have the attached galactose moieties pointing into the major groove, induced similar or decreased duplex stability relative to the reference duplexes. These results are in good agreement with results reported by Matsuura et al.[25] and Schlegel et al.[30] for nucleotides containing carbohydrate-modified nucleobases. On the contrary, incorporation of N2′-functionalized 2′-amino-LNA nucleotides, designed to have the attached galactosyl moieties pointing into the minor groove, showed similar or increased duplex stability relative to incorporation of the corresponding unmodified LNA nucleotide. The shorter ethylene glycol linker unit was in general better tolerated than the more flexible triethylene glycol linker unit, thus demonstrating the significant influence of the linker on the hybridization properties of carbohydrate modified ONs, as also reported for other carbohydrate-modified thymidine and LNA-U derivatives having a stabilizing alkyne linkage between the carbohydrate and the 5-position of the nucleobase.[24,25]

Thermal Denaturation Experiments: Binding Specificity. For evaluation of the mismatch discriminative properties of these galactose-modified ONs, the 9-mer sequences with one galactosyl nucleotide and two additional LNA-T nucleotides were chosen with one mismatched nucleotide opposite the galactosyl modification. All modifications showed similar or even enhanced mismatch discrimination relative to the unmodified strand **ON18**, with the discriminative power generally superior against DNA strands (Table 4). Especially for the T:C and T:G mismatches, C5-modified LNA-T and N2′-modified 2′-amino-LNA-T containing ONs showed improved binding specificity.

Table 4. Thermal Denaturation Temperatures of Mismatched Duplexes[a]

			ΔT_m [°C]			ΔT_m [°C]	
	5′-GT^{L}G AXA T^{L}GC		DNA: 3′-CAC TAT ACG			RNA: 3′-CAC UAU ACG	
ON	X	C[b]	G[b]	T[b]	C[b]	G[b]	U[b]
ON18	T	−14.5	−9.5	−14.5	−15.0	−5.5	−18.0
ON19	Gal-C2-T	−15.5	−9.0	−15.0	−12.0	−2.5	−15.5
ON20	Gal-TEG-T	−17.0	−8.0	−15.0	−9.5	±0	−13.0
ON21	LNA-T	−18.5	−12.5	−15.5	−15.5	−7.5	−16.0
ON22	Gal-C2-LNA-T	−19.5	−12.0	−16.5	−16.0	−6.5	−16.0
ON23	Gal-TEG-LNA-T	−21.0	−12.0	−18.0	−16.5	−7.5	−17.5
ON24	Gal-C2−2′-amino-LNA-T	−18.5	−16.0	−15.0	−16.0	−10.5	−15.5
ON25	Gal-TEG-2′-amino-LNA-T	−19.5	−16.5	−15.5	−15.0	−9.5	−14.5

[a]See footnotes Table 1. [b]ΔT_m values for mismatch calculated as the difference in T_m values between fully matched and mismatched duplexes. **T^L** = LNA-T.

3′-Exonuclease Stability of Galactose-Modified ONs. 3′-Exonuclease resistance studies with six different galactose containing ONs (**ON2**, **ON3**, **ON5**, **ON6**, **ON8** and **ON9**) were performed. The stability of 5′-^{32}P labeled ONs upon incubation with snake venom phosphodiesterase (SVPDE, 3′-exonuclease) was evaluated using denaturing 20% PAGE. Compared to the LNA and 2′-amino-LNA containing 9-mer sequences,[50,57] the galactose-modified sequences showed significantly enhanced nuclease resistance (Figure 2, S19 and S20, Supporting Information). Unmodified **ON1** was rapidly

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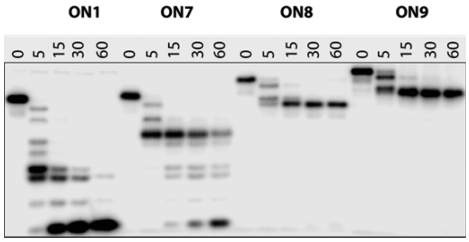

Figure 2. 20% PAGE denaturing gel showing the time-course of the 3′-exonuclease resistance analysis of 5′-^{32}P-GTGAXATGC with ON1 (X = T), ON7 (X = 2′-amino-LNA-T), ON8 (X = Gal-C2−2′-amino-LNA-T) and ON9 (X = Gal-TEG-2′-amino-LNA-T).

degraded as even 5 min incubation resulted in no intact ON and 60 min incubation in essentially complete degradation. In contrast, both C5-galactosyl-modified thymidine derivatives (**ON2** and **ON3**, Figure S19, Supporting Information) clearly enhanced the nuclease resistance, and similar improvement was observed for the C5-galactosyl-modified LNA-T containing ONs (**ON5** and **6**, Figure S20, Supporting Information). All four oligonucleotides however showed no intact ON after 15 min incubation, but after digestion of the TGC-3′ triad, further degradation was completely inhibited. This is in good agreement with previous studies on ONs containing C-5-substituted LNA pyrimidines.[24,45]

Compared to thymidine, the 2′-amino-LNA-T monomer (**ON7**) induced enhanced 3′-nucleolytic resistance (Figure 2) in general agreement with previous results for a piperazino-modified 2′-amino-LNA-T monomer.[50] After 30 min incubation, the fragment 5′-GTGAXA (**X** = 2′-amino-LNA-T monomer) was the dominant specie. However, further digestion was still observed (evaluated after 60 min digestion). For ONs containing N2-galactosyl modified 2′-amino-LNA-T, the nuclease resistance was markedly augmented (Figure 2, **ON8** and **ON9**) and the 5′-GTGAXA segment remained intact after 60 min digestion.

Molecular Modeling Studies. In order to obtain an indication of the directional preference of the galactose residue attached to LNA-T (Gal-C2-LNA-T) and 2′-amino-LNA-T (Gal-C2−2′-amino-LNA-T) within a duplex structure, we performed molecular modeling studies. The 9-mer duplex structure [5′-d(CTGATATGC):5′-r(GCAUAUCAG)] was downloaded from the protein data bank (PDB entry pdb 1HG9)[58] and was modified with Gal-C2-LNA-T and Gal-C2−2′-amino-LNA-T in the middle of the DNA strand. An AMBER* force field in Macro Model 9.1 was used to generate representative low energy structures. The model structures showed that the galactose residue attached to the base of LNA-T is located into the major groove of the double helix (Figure 3a), while the galactose residue attached to the N2′-atom of 2′-amino-LNA is oriented into the minor groove of the double helix (Figure 3b), both not causing any steric constraints to the parent duplex structure.

CONCLUSION

In conclusion, six novel galactose-containing thymidine, LNA-T and 2′-amino-LNA-T phosphoramidites have been synthesized and successfully used for solid supported automated synthesis of galactose-functionalized ONs. Especially, the galactose-modified LNA-T and 2′-amino-LNA-T containing ONs showed high duplex stability, good base-pairing specificity and pronounced resistance against 3′-exonucleolytic degradation. We have thus demonstrated that the C5-position of LNA-T, with the linkers employed, and the 2′-position of 2′-amino-LNA-T are highly suitable for attachment of carbohydrate moieties within high-affinity ONs. Attachment of other monosaccharides and oligosaccharides is planned together with evaluation of the novel constructs in the context of nucleic acid drug development.

EXPERIMENTAL SECTION

General Information. All reagents used were purchased from commercial sources and used without further purification. The solvents were dried over activated molecular sieves (3 Å, 8−12 mesh). All reactions were performed under an argon atmosphere. Dry column vacuum chromatography was carried out using silica gel 60 (particle size 0.040−0.063 mm). Thin layer chromatography (TLC) was performed using silica gel 60 F_{254} aluminum plates and visualized under UV light and by dipping in either a solution of 5% sulfuric acid in ethanol or a vanillin staining solution and heating until spots appeared. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a 400 MHz

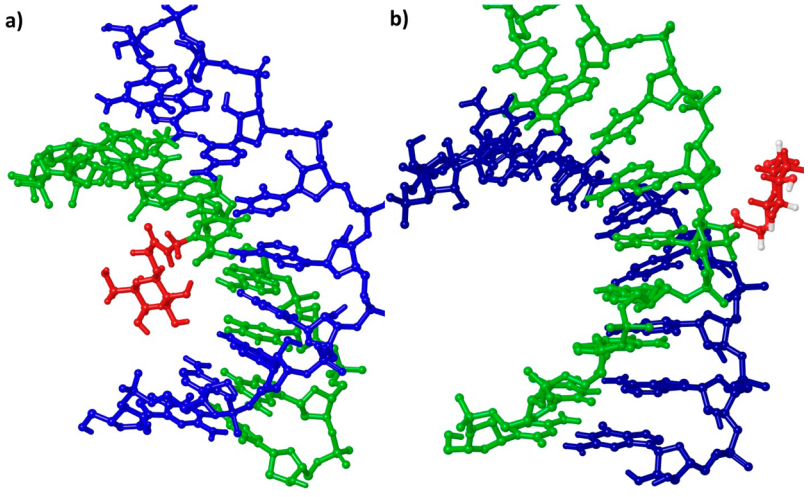

Figure 3. Snapshots from molecular dynamics simulations of 9-mer duplex [(5′-d(CTGAXATGC):5′-r(GCAUAUCAG)] modified centrally (position **X**) with Gal-C2-LNA-T (a) and Gal-C2−2′-amino-LNA-T (b). Coloring scheme: DNA strand, green; RNA strand, blue; the galactose residue with ethylene glycol linker red.

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spectrometer at 400 MHz, 101 and 162 MHz, respectively, in $CDCl_3$ and DMSO-d_6. Chemical shifts are reported in ppm (parts per million) relative to TMS (tetramethylsilane) as the internal reference, unless otherwise stated; s (singlet), d (doublet), t (triplet), dd (doublet of doublets), m (multiplet) and coupling constants (J) in Hz. NMR assignments are based on correlation spectroscopy (COSY, HSQC and HMBC) experiments and follow standard nucleoside nomenclature. Copies of ^{1}H and ^{13}C NMR spectra can be found in the Supporting Information. Systematic compound names for the bicyclic compounds are given according to von Baeyer nomenclature. High resolution mass spectra (HRMS) were performed on a TOF (time-of-flight) analyzer and were recorded in positive ion mode using electrospray ionization (ESI). PE refers to petroleum ether of distillation range 60−80 °C.

2-(2-(2-Hydroxyethoxy)ethoxy)ethyl-2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranoside (5).[52] Compound **5** was synthesized according to the procedure published by Szurmai et al. We however used Ag_2CO_3 instead of $Hg(CN)_2$ as activator.

2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyloxyacetic acid (6). Galactopyranoside **4** (1.96 g, 5.00 mmol, 1.0 equiv) was dissolved in acetone (100 mL), a saturated aqueous $NaHCO_3$ solution (18 mL) was added and the mixture cooled to 0 °C. 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO, 15 mg, 0.10 mmol, 0.02 equiv) and NaBr (48 mg, 0.47 mmol, 0.09 equiv) were added, followed by the addition of trichloroisocyanuric acid (TCCA, 2.3 g, 9.90 mmol, 2.0 equiv) portionwise over 20 min. The reaction mixture was allowed to warm to room temperature and was stirred for 16 h. The solvents were removed under reduced pressure and the resulting residue was redissolved in EtOAc (100 mL). The organic phase was washed successively with saturated aqueous NH_4Cl (50 mL) and 1 M HCl (50 mL) solution, dried (Na_2SO_4) and evaporated to dryness to afford 1.85 g of **6** which was used in the next step without further purification. HRMS (ESI) m/z 429.0988 ($[M + Na]^+$, $C_{16}H_{22}O_{12}{\cdot}Na^+$, Calc. 429.1003).

8-(2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyloxy)-3,6-dioxaoctanoic acid (7). Galactopyranoside **5** (3.70 g, 7.70 mmol, 1.0 equiv) was dissolved in acetone (70 mL), a saturated aqueous $NaHCO_3$ solution (23 mL) was added and the mixture cooled to 0 °C. TEMPO (24 mg, 0.15 mmol, 0.02 equiv) and NaBr (79 mg, 0.77 mmol, 0.1 equiv) were added, followed by the addition of TCCA (3.90 g, 16.8 mmol, 2.2 equiv) portionwise over 20 min. The resulting mixture was allowed to warm to room temperature and stirred for 16 h. The solvents were removed under reduced pressure and the resulting residue was redissolved in EtOAc (150 mL). The organic phase was washed successively with saturated aqueous NH_4Cl (75 mL) and 1 M HCl (75 mL) solution and the water phase was back-extracted with EtOAc (100 mL). The combined organic phase was dried (Na_2SO_4) and evaporated to dryness to afford 2.97 g (78% yield) of compound **7**. HRMS (ESI) m/z 517.1504 ($[M + Na]^+$, $C_{20}H_{30}O_{14}{\cdot}Na^+$, Calc. 517.1528); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.42 (br s, 1H, COOH), 5.25 (dd, 1H, J = 3.4 Hz, 0.7 Hz, H4), 5.14 (dd, 1H, J = 10.5 Hz, 3.6 Hz, H3), 4.93 (dd, 1H, J = 10.3 Hz, 8.0 Hz, H2), 4.74 (d, 1H, J = 8.0 Hz, H1), 4.18 (dd, 1H, J = 6.3 Hz, 5.6 Hz, H5), 4.14−3.99 (m, 4H, 2 × H6, CH_2O), 3.83−3.75 (m, 1H, CH_2O), 3.67−3.48 (m, 9H, CH_2O), 2.12 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.91 (s, 3H, OAc); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.5 (COOH), 169.9 (OAc), 169.8 (OAc), 169.4 (OAc), 169.1 (OAc), 100.0 (C1′), 70.2 (C3′), 69.8 (CH_2O), 69.7 (CH_2O), 69.3 (CH_2O), 68.6 (C5′), 68.4 (C2′), 67.6 (CH_2O), 67.3 (C4′), 61.3 (C6′), 20.4 (OAc), 20.4 (OAc), 20.3 (OAc), 20.3 (OAc).

5-(Azidomethyl)-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine (10).[54] Compound **10** was synthesized starting from thymidine (**8**) in five steps according to known procedures. 3′,5′-Di-*O*-acetyl-5-azidomethyl-2′-deoxyuridine (**9**)[53,55] was deprotected using 2 M NaOH in MeOH/H_2O instead of concentrated aqueous ammonia as earlier reported.[53]

5′-*O*-(4,4′-Dimethoxytrityl)-5-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyloxyacetami-do)methyl-2′-deoxyuridine (11). 5-Azidomethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**10**, 117 mg, 0.20 mmol, 1.0 equiv) was dissolved in benzene/H_2O (15 mL/800 μL) and PPh_3 (136 mg, 0.52 mmol, 2.6 equiv) was added. The reaction mixture was stirred at 60 °C for 16 h. The solvents were removed under reduced pressure, and the resulting residue was coevaporated with toluene (3 × 2 mL) and dried under vacuum for 1 h. The residue was dissolved in a mixture of anhydrous DMF (8 mL) and anhydrous *N*,*N*-diisopropylethylamine (DIPEA, 60 μL, 0.34 mmol, 1.7 equiv) and carboxylic acid **6** (130 mg of the intermediate synthesized from **4**) were added. 1-(Bis(dimethylamino)methylene)-1H-1,2,3-triazolo[4,5-*b*]pyridinium3-oxidhexafluorophos-phate (HATU, 122 mg, 0.32 mmol, 1.6 equiv) dissolved in anhydrous DMF (2 mL) was added dropwise over 10 min and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was diluted with EtOAc (25 mL) and washed with saturated aqueous $NaHCO_3$ (2 × 20 mL), H_2O (20 mL), and brine (2 × 20 mL). The organic phase was dried (Na_2SO_4) and evaporated to dryness and the residue purified by silica gel column chromatography (0−8.5%, MeOH/CH_2Cl_2, v/v) to afford compound **11** as a yellow foam (90.3 mg, 48% yield). R_f = 0.32 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) m/z 970.3217 ($[M + Na]^+$, $C_{47}H_{53}N_3O_{18}{\cdot}Na^+$, Calc. 970.3216); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.96 (br s, 1H, NH), 7.70 (s, 1H, H6), 7.43−7.38 (m, 2H, Ar), 7.34−7.27 (m, 6H, Ar), 7.23−7.17 (m, 1H, Ar), 6.96 (t, 1H, J = 6.3 Hz, NH), 6.86−6.80 (m, 4H, Ar), 6.25 (t, 1H, J = 6.8 Hz, H1′), 5.39 (d, 1H, J = 3.3 Hz, Gal-H4), 5.22 (dd, 1 H, J = 10.6 Hz, 8.0 Hz, Gal-H2), 5.03 (dd, 1H, J = 10.5 Hz, 3.3 Hz, Gal-H3), 4.49 (d, 1H, J = 8.0 Hz, Gal-H1), 4.49−4.44 (m, 1H, H3′), 4.18 (d, 1H, J = 15.4 Hz, CH_2O), 4.15−4.09 (m, 2H, Gal-H6), 4.03 (d, 1H, J = 15.4 Hz, CH_2O), 4.01−3.96 (m, 1H, H4′), 3.92 (dd, 1H, J = 6.9 Hz, 6.6 Hz, Gal-H5), 3.84−3.68 (m, 2H, CH_2C5), 3.77 (s, 6H, 2 × OMe), 3.44−3.33 (m, 2H, H5′), 2.39 (ddd, 1H, J = 13.8 Hz, 6.3 Hz, 4.0 Hz, H_a2'), 2.26 (ddd, 1H, J = 13.7 Hz, 13.7 Hz, 6.6 Hz, H_b2'), 2.16 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.98 (s, 3H, OAc); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6 (OAc), 170.4 (OAc), 170.2 (OAc), 170.0 (OAc), 168.7 (CONH), 162.9 (C4), 158.8 (DMTr), 150.2 (C2), 144.6 (Ar), 139.1 (C6), 135.7 (Ar), 135.6 (Ar), 130.2 (Ar), 128.2 (Ar), 128.1 (Ar), 127.2 (Ar), 113.4 (Ar), 110.9 (C5), 101.1 (Gal-C1), 87.0 (DMTr), 85.9 (C4′), 85.3 (C1′), 72.3 (C3′), 71.1 (Gal-C5), 70.7 (Gal-C3), 68.9 (Gal-C2), 68.5 (CH_2O), 67.0 (Gal-C4), 63.8 (C5′), 61.3 (Gal-C6), 55.4 (OMe), 40.7 (C2′), 36.3 (CH_2−C5), 20.9 (OAc), 20.8 (OAc), 20.8 (OAc), 20.7 (OAc).

5′-*O*-(4,4′-Dimethoxytrityl)-5-(8-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyloxy)-3,6-dioxaoctanamido)methyl-2′-deoxyuridine (12). Compound **10** (483 mg, 0.82 mmol, 1.0 equiv) was dissolved in benzene/H_2O (15 mL/400 μL) and PPh_3 (562 mg, 2.14 mmol, 2.6 equiv) was added. The reaction mixture was stirred at 60 °C for 16 h. The solvents were removed under reduced pressure and the resulting residue was coevaporated with toluene (3 × 2 mL) and dried under vacuum for 1 h. Carboxylic acid **7** (490 mg, 0.99 mmol, 1.2 equiv) was dissolved in anhydrous THF (10 mL), and DIPEA (290 μL, 1.66 mmol, 2.0 equiv) and HATU (376 mg, 0.99 mmol, 1.2 equiv) dissolved in anhydrous DMF (2 mL) were added. The reaction mixture was stirred at room temperature for 10 min. The residue obtained above was dissolved in anhydrous THF (5 mL) was added dropwise, and the resulting mixture was stirred at room temperature for 1.5 h. EtOAc (50 mL) was added and washing was performed with saturated aqueous $NaHCO_3$ (2 × 25 mL), H_2O (25 mL), and brine (2 × 25 mL). The water layer was back-extracted with EtOAc (50 mL), and the combined organic phases were dried (Na_2SO_4) and evaporated to dryness, and the residue purified by silica gel column chromatography (0−5%, MeOH/CH_2Cl_2, v/v) to afford compound **12** as a yellow foam (412 mg, 49% yield). R_f = 0.15 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) m/z 1058.3694 ($[M + Na]^+$, $C_{51}H_{61}N_3O_{20}{\cdot}Na^+$, Calc. 1058.3741); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (br s, 1H, NH), 7.69 (s, 1H, H6), 7.43−7.39 (m, 2H, Ar), 7.34−7.25 (m, 7H, Ar), 7.23−7.17 (m, 1H, NH), 6.86−6.81 (m, 4H, Ar), 6.25 (t, 1H, J = 6.6 Hz, H1′), 5.38 (dd, 1H, J = 3.5 Hz, 0.8 Hz, Gal-H4), 5.20 (dd, 1H, J = 10.5 Hz, 7.9 Hz, Gal-H2), 5.10 (dd, 1H, J = 10.5 Hz, 3.4 Hz, Gal-H3), 4.61 (d, 1H, J = 7.9 Hz, Gal-H1), 4.51−4.44 (m, 1H, H3′), 4.20 (dd, 1H, J = 11.4 Hz, 6.7 Hz, Gal-H_a6), 4.14−4.08 (m, 1H, Gal-H_b6), 4.00−3.93 (m, 3H, H4′, Gal-H5, CH_2O), 3.89−3.72 (m, 3H, CH_2C5, CH_2O), 3.77 (s, 6H, 2 × OMe), 3.70−3.55 (m, 6H, CH_2O), 3.45−

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3.34 (m, 2H, H5′), 2.63 (d, 1H, J = 3.7 Hz, OH), 2.39 (ddd, 1H, J = 13.7 Hz, 6.3 Hz, 4.0 Hz, H_a2′), 2.26 (ddd, 1H, J = 13.7 Hz, 13.5 Hz, 6.8 Hz, H_b2′), 2.13 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.03 (s, 3H, OAc), 1.98 (s, 3H, OAc); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8 (OAc), 170.4 (OAc), 170.4 (OAc), 170.1 (OAc), 169.9 (CONH), 163.2 (C4), 158.8 (ArC), 150.1 (C2), 144.6 (Ar), 139.1 (C6), 135.8 (Ar), 135.7 (Ar), 130.2 (Ar), 128.2 (Ar), 128.1 (Ar), 127.2 (Ar), 113.4 (Ar), 111.1 (C5), 101.4 (Gal-C1), 87.0 (DMTr), 85.7 (C4′), 85.2 (C1′), 72.2 (C3′), 71.2 (Gal-C5), 71.0 (CH_2O), 70.7 (Gal-C3), 70.6 (CH_2O), 70.5 (CH_2O), 70.5 (CH_2O), 69.2 (CH_2O), 69.1 (Gal-C2), 67.3 (Gal-C4), 63.8 (C5′), 61.5 (Gal-C6), 55.4 (OMe), 40.6 (C2′), 35.9 (CH_2C5), 20.9 (OAc), 20.8 (OAc), 20.8 (OAc), 20.8 (OAc).

3′-*O*-(2-Cyanoethoxy(diisopropylamino)phosphanyl)-5′-*O*-(4,4′-dimethoxytrityl)-5-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyloxyacetamido)methyl-2′-deoxyuridine (13). Compound **11** (339 mg, 0.36 mmol, 1.0 equiv) was dissolved in anhydrous 1,2-dichloroethane (15 mL), and DIPEA (300 μL, 1.76 mmol, 4.9 equiv) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (112 μL, 0.50 mmol, 1.4 equiv) were added. The reaction mixture was stirred at room temperature for 2 h. The solution was diluted with EtOAc (50 mL) and washed with saturated aqueous $NaHCO_3$ (25 mL) and brine (25 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated to dryness under reduced pressure. First the residue was purified by silica gel column chromatography (0−4.5%, MeOH/CH_2Cl_2, v/v) and then dissolved in EtOAc (2.5 mL) and precipitated with PE (120 mL), affording compound **13** as a white foam (252 mg, 61% yield). R_f = 0.3 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) m/z 1170.4282 ([M + Na]$^+$, $C_{56}H_{70}N_5O_{19}P{\cdot}Na^+$, Calc. 1170.4295); ^{31}P NMR (162 MHz, $CDCl_3$) δ 148.9, 148.7.

3′-*O*-(2-Cyanoethoxy(diisopropylamino)phosphanyl)-5′-*O*-(4,4′-dimethoxytrityl)-5-(8-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyloxy)-3,6-dioxaoctanamido)methyl-2′-deoxyuridine (14). Compound **12** (178 mg, 0.17 mmol, 1.0 equiv) was dissolved in anhydrous 1,2-dichloroethane (8 mL), and DIPEA (150 μL, 0.88 mmol, 5.2 equiv) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (60 μL, 0.27 mmol, 1.6 equiv) were added. The reaction mixture was stirred at room temperature for 2 h. The solution was diluted with EtOAc (50 mL) and washed with saturated aqueous $NaHCO_3$ (25 mL) and brine (25 mL). The organic phase was dried (Na_2SO_4) and concentrated to dryness under reduced pressure. The residue was precipitated (2.5 mL EtOAc/120 mL PE) to afford compound **14** as a white foam (192 mg, 94% yield). R_f = 0.4 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) m/z 1258.4861 ([M + Na]$^+$, $C_{60}H_{78}N_5O_{21}P{\cdot}Na^+$, Calc. 1258.4819); ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.0, 148.7.

(1*R*,3*R*,4*R*,7*S*)-7-Acetoxy-1-acetoxymethyl-3-thymin-1-yl-2,5-dioxabicyclo[2.2.1]heptane (16). Nucleoside **15** (10.0 g, 37.0 mmol, 1.0 equiv) was dissolved in anhydrous THF (100 mL) and the solution was cooled to 0 °C whereupon 4-dimethylaminopyridine (DMAP, 452 mg, 3.70 mmol, 0.1 equiv), pyridine (5 mL) and acetic anhydride (10.5 mL, 111 mmol, 3.0 equiv) were added. The reaction mixture was stirred at room temperature for 2 h. The solvents were removed under reduced pressure and the residue was redissolved in CH_2Cl_2 (100 mL) and washed with H_2O (3 × 100 mL) and saturated aqueous $NaHCO_3$ (2 × 100 mL). The organic phase was dried (Na_2SO_4) and evaporated to dryness under reduced pressure to afford compound **16** (13.0 g, 99% yield) as a white foam. R_f = 0.5 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) m/z 377.0956 ([M + Na]$^+$, $C_{15}H_{18}N_2O_8{\cdot}Na^+$, Calc. 377.0955); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (br s, 1H, NH), 7.43 (d, 1H, J = 1.2 Hz, H6), 5.70 (s, 1H, H1′), 4.90 (s, 1H, H3′), 4.66 (s, 1H, H2′), 4.49 (d, 1H, J = 12.9 Hz, H_a5″), 4.40 (d, 1H, J = 12.9 Hz, H_b5″), 4.02 (d, 1H, J = 8.3 Hz, H_a5′), 3.97 (d, 1H, J = 8.3 Hz, H_b5′), 2.17 (s, 3H, OAc), 2.14 (s, 3H, OAc), 1.96 (d, 3H, J = 1.2 Hz, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0 (OAc), 169.8 (OAc), 163.4 (C4), 149.7 (C2), 133.7 (C6), 110.8 (C5), 87.2 (C1′), 85.5 (C4′), 78.0 (C2′), 71.8 (C3′), 71.1 (C5″), 58.7 (C5′), 20.7 (OAc), 20.7 (OAc), 12.9 (CH_3).

(1*R*,3*R*,4*R*,7*S*)-7-Acetoxy-1-acetoxymethyl-3-(5-azidomethyl)uracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (17). Nucleoside **16** (2.50 g, 7.06 mmol, 1.0 equiv), *N*-bromo-succinimide (2.51 g, 14.1 mmol, 2.0 equiv) and azobis(isobutyronitrile) (AIBN, 140 mg, 0.85 mmol, 0.12 equiv) were placed in a 250 mL round-bottom flask. Benzene (100 mL) was added and the resulting mixture was stirred under reflux for 3 h. The solvent was removed under reduced pressure and the residue was redissolved in anhydrous DMF (20 mL). Sodium azide (902 mg, 13.8 mmol, 2.0 equiv) was added and the reaction mixture was stirred at 90 °C for 12 h. The reaction mixture was diluted with EtOAc and washed with cold H_2O (3 × 100 mL), saturated aqueous $NaHCO_3$ (2 × 100 mL), and brine (2 × 100 mL). The organic phase was dried (Na_2SO_4,) and evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (20−40%, EtOAc/PE, v/v) to afford compound **17** as a white foam (900 mg, 32% yield). R_f = 0.5 (EtOAc/PE, 60:40, v/v); HRMS (ESI) m/z 418.0952 ([M + Na]$^+$, $C_{15}H_{17}N_5O_8{\cdot}Na^+$, Calc. 418.0969); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.89 (br s, 1H, NH), 7.66 (s, 1H, H6), 5.71 (s, 1H, H1′), 4.86 (s, 1H, H3′), 4.69 (s, 1H, H2′), 4.49 (d, 1H, J = 12.9 Hz, H_a5′), 4.39 (d, 1H, J = 12.9 Hz, H_b5′), 4.24−4.15 (m, 2H, CH_2N_3), 4.03 (d, 1H, J = 8.3 Hz, Ha5″), 3.98 (d, 1H, J = 8.3 Hz, H_b5″), 2.19 (s, 3H, OAc), 2.14 (s, 3H, OAc); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3 (OAc), 169.8 (OAc), 162.1 (C4), 149.3 (C2), 136.2 (C6), 109.8 (C5), 87.5 (C1′), 86.0 (C4′), 78.1 (C2′), 72.0 (C3′), 71.1 (C5″), 58.6 (C5′), 47.4 (CH_2N_3), 20.9 (OAc), 20.6 (OAc).

(1*R*,3*R*,4*R*,7*S*)-3-(5-(Azidomethyl)uracil-1-yl)-7-hydroxy-1-hydroxymethyl-2,5-dioxabicyclo[2.2.1]heptane (18). Nucleoside **17** (1.00 g, 2.50 mmol. 1.0 equiv) was dissolved in MeOH/H_2O (3:1, 12 mL) and the mixture was cooled to 0 °C. 2 M NaOH (4 mL) was added and the reaction mixture was stirred at room temperature for 30 min, neutralized with Dowex-50 WX 2 (H^+ form) and filtered. The filtrate was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (3−6%, MeOH/CH_2Cl_2, v/v) to afford compound **18** as a white foam (502 mg, 64% yield); R_f = 0.5 (MeOH/CH_2Cl_2, 10:90, v/v); HRMS (ESI) m/z 334.0766 ([M + Na]$^+$, $C_{11}H_{13}N_5O_6{\cdot}Na^+$, Calc. 334.0758); ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.61 (br s, 1H, NH), 7.91 (s, 1H, H6), 5.68 (d, 1H, J = 4.3 Hz, 3′−OH), 5.43 (s, 1H, H1′), 5.14 (t, 1H, J = 5.8 Hz, 5′−OH), 4.17 (s, 1H, H3′), 4.12−4.04 (m, 2H, CH_2N_3), 3.90−3.83 (m, 2H, H2′, H_a5″), 3.83−3.74 (m, 2H, $H_2$5′), 3.66 (d, 1H, J = 7.8 Hz, H_b5″); ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.0 (C4), 149.7 (C2), 138.7 (C6), 107.4 (C5), 89.1 (C4′), 86.4 (C1′), 78.9 (C3′), 71.0 (C5″), 68.8 (C2′), 56.2 (C5′), 46.9 (CH_2N_3).

(1*R*,3*R*,4*R*,7*S*)-3-(5-(Azidomethyl)uracil-1-yl)-1-(4,4′-dimethoxytrityloxymethyl)-7-hydroxy-2,5-dioxabicyclo[2.2.1]-heptane (19). Nucleoside **18** (950 mg, 3.05 mmol, 1.0 equiv) was dissolved in anhydrous pyridine (10 mL), and DMTrCl (1.25 g, 3.66 mmol, 1.2 equiv) was added to the solution. The reaction mixture was stirred at room temperature for 4 h. EtOH (7 mL) was added and the mixture evaporated to dryness under reduced pressure. The residue was redissolved in EtOAc (100 mL) and the mixture was extracted with H_2O (2 × 100 mL), saturated aqueous $NaHCO_3$ (2 × 100 mL), and brine (2 × 100 mL). The organic phase was dried (Na_2SO_4) and cocentrated under reduced pressure. The residue was purified by silica gel column chromatography (40−60%, EtOAc/PE, v/v) to afford compound **19** as a white foam (950 mg, 51% yield). R_f = 0.5 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) m/z 636.2036 ([M + Na]$^+$, $C_{32}H_{31}N_5O_8{\cdot}Na^+$, Calc. 636.2065); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.57 (br s, 1H, NH), 7.81 (s, 1H, H6), 7.47−7.40 (m, 2H, Ar), 7.34−7.21 (m, 7H, Ar), 6.90−6.79 (m, 4H, Ar), 5.60 (s, 1H, H1′), 4.46 (s, 1H, H2′), 4.25 (d, 1H, J = 4.5 Hz, H3′), 3.90 (d, 1H, J = 8.2 Hz, H_a5″), 3.86−3.72 (m, 8H, H_b5″, CH_aH_b-N_3, OMe), 3.67 (d, 1H, J = 13.7 Hz, CH_aH_b-N_3), 3.55−3.51 (m, 2H, $H_2$5′), 3.28 (d, 1H, J = 4.9 Hz, 3′−OH); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.0 (C4), 158.8 (ArC), 149.7 (C2), 144.4 (Ar), 137.9 (C6), 135.4 (Ar), 135.3 (Ar), 130.1 (Ar), 128.1 (Ar), 127.2 (Ar), 113.4 (Ar), 109.2 (C5), 88.3 (C4′), 87.1 (C1′), 86.9 (DMTr), 79.3 (C2′), 71.8 (C5″), 70.3 (C3′), 58.3 (C5′), 55.3 (OMe), 47.2 (CH_2N_3).

(1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-3-(5-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyloxy-acetamido)methyluracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (20). Nucleoside **19** (200 mg, 0.33 mmol, 1.0 equiv) was dissolved in a

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mixture of benzene (6 mL) and H_2O (130 μL) and PPh_3 (225 mg, 0.86 mmol, 2.6 equiv) was added. The reaction mixture was stirred at 60 °C for 16 h. The solvents were removed under reduced pressure and, the crude amine was coevaporated with anhydrous toluene (3 × 3 mL) and dried under vacuum for 1 h, ready for conjugation. In parallel, the carboxylic acid **6** (161 mg, 0.40 mmol, 1.2 equiv) was dissolved in anhydrous THF (3 mL), and DIPEA (110 μL, 0.63 mmol, 1.9 equiv) and HATU (150 mg, 0.39 mmol, 1.2 equiv) dissolved in anhydrous DMF (1 mL) were added slowly. The resulting mixture was stirred at room temperature for 20 min. The crude amine dissolved in anhydrous THF (2 mL) was added dropwise, and the mixture stirred at room temperature for 1.5 h. EtOAc (50 mL) was added, and the organic phase was washed with saturated aqueous $NaHCO_3$ (2 × 25 mL), H_2O (25 mL) and brine (2 × 25 mL). The aqueous layer was back-extracted with EtOAc (50 mL), and the combined organic phase was dried (Na_2SO_4) and concentrated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (0−4%, MeOH/CH_2Cl_2, v/v) to afford compound **20** as a yellow foam (183 mg, 58% yield). R_f = 0.13 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) *m/z* 998.3120 ([M + Na]$^+$, $C_{48}H_{53}N_3O_{19}$·Na$^+$, Calc. 998.3165); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (br s, 1H, NH), 7.83 (s, 1H, H6), 7.51−7.45 (m, 2H, Ar), 7.40−7.18 (m, 7H, Ar), 7.04−6.98 (m, 1H, NH), 6.88−6.82 (m, 4H, Ar), 5.63 (s, 1H, H1′), 5.39 (dd, 1H, *J* = 3.4 Hz, 0.9 Hz, Gal-H4), 5.21 (dd, 1H, *J* = 10.6 Hz, 7.9 Hz, Gal-H2), 5.04 (dd, 1H, *J* = 10.6 Hz, 3.4 Hz, Gal-H3), 4.49 (d, 1H, *J* = 7.9 Hz, Gal-H1), 4.34 (s, 1H, H2′), 4.25 (d, 1H, *J* = 5.6 Hz, H3′), 4.22 (d, 1H, *J* = 15.5 Hz, CH_2O), 4.14−4.08 (m, 2H, Gal-H6), 4.04 (d, 1H, *J* = 15.5 Hz, CH_2O), 3.96−3.82 (m, 5H, 6′-H_2, 5″-H_2, Gal-H5, CH_2−C5), 3.78 (s, 6H, 2 × OMe), 3.58 (d, 1H, *J* = 11.3 Hz, H_a5′), 3.52 (d, 1H, *J* = 11.3 Hz, H_b5′), 3.22−3.17 (m, 1H, OH), 2.16 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.99 (s, 3H, OAc); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7 (OAc), 170.4 (OAc), 170.2 (OAc), 170.2 (OAc), 169.9 (CONH), 162.9 (C4), 158.8 (Ar), 149.7 (C2), 144.6 (Ar), 138.3 (C6), 135.6 (Ar), 135.5 (Ar), 130.3 (Ar), 128.2 (Ar), 128.2 (Ar), 127.2 (Ar), 113.5 (Ar), 110.1 (C5), 101.2 (Gal-C1), 88.1 (DMTr), 87.0 (C4′), 86.9 (C1′), 79.6 (C2′), 72.0 (C5″), 71.1 (Gal-C2), 70.7 (C3′), 70.7 (Gal-C3), 69.0 (Gal-C5), 68.6 (CH_2O), 67.0 (Gal-C4), 61.2 (Gal-C6), 58.9 (C5′), 55.4 (OMe), 36.8 (CH_2−C5), 20.9 (OAc), 20.8 (OAc), 20.8 (OAc), 20.7 (OAc).

(1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-3-(5-(8-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3,6-dioxaoctanamido)methyluracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (21). Nucleoside **19** (200 mg, 0.33 mmol, 1.0 equiv) was dissolved in a mixture of benzene (6 mL) and H_2O (130 μL) and PPh_3 (222 mg, 0.85 mmol, 2.6 equiv) was added. The resulting mixture was stirred at 60 °C for 16 h. The solvents were removed under reduced pressure and, the crude amine was coevaporated with anhydrous toluene (2 × 5 mL) and dried under vacuum for 1 h, ready for conjugation. In parallel, the carboxylic acid **7** (177 mg, 0.36 mmol, 1.1 equiv) dissolved in anhydrous THF (3 mL), and DIPEA (113 μL, 0.65 mmol, 2.0 equiv) and HATU (136 mg, 0.36 mmol, 1.1 equiv) dissolved in anhydrous DMF (2 mL) were added slowly. After 30 min the crude amine dissolved in anhydrous THF (3 mL) was added to the reaction mixture and stirred at room temperature for 90 min. The reaction mixture was diluted with EtOAc (100 mL), washed with saturated aqueous $NaHCO_3$ (2 × 50 mL) and brine (2 × 50 mL) and concentrated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (2−3%, MeOH/CH_2Cl_2, v/v) to afford compound **19** as a white foam (200 mg, 58% yield). R_f = 0.4 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) *m/z* 1086.3713 ([M + Na]$^+$, $C_{52}H_{61}N_3O_{21}$·Na$^+$, Calc. 1086.3690); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.21 (br s, 1H, NH), 7.79 (s, 1H, H6), 7.49−7.47 (m, 2H, Ar), 7.43−7.33 (m, 5H, Ar, NH), 7.32−7.28 (m, 2H, Ar), 7.25−7.18 (m, 1H, Ar), 6.88−6.81 (m, 4H, Ar), 5.65 (s, 1H, H1′), 5.38 (dd, 1H, *J* = 3.3 Hz, 0.8 Hz, Gal-H4), 5.19 (dd, 1H, *J* = 10.4 Hz, 7.9 Hz, Gal-H2), 5.10 (dd, 1H, *J* = 10.4 Hz, 3.4 Hz, Gal-H3), 4.61 (d, 1H, *J* = 7.9 Hz, Gal-H1), 4.36 (s, 1H, H2′), 4.22 (d, 1H, *J* = 5.3 Hz, H3′), 4.19 (dd, 1H, *J* = 11.2 Hz, 6.6 Hz, Gal-H_a6), 4.11 (dd, 1H, *J* = 11.2 Hz, 6.8 Hz, Gal-H_b6), 4.01−3.88 (m, 8H, Gal-H5, CH_2O), 3.78−3.76 (m, 7H, H_a,5″, OMe), 3.66−3.59 (m, 8H, H_a5′, H_b5″, CH_2O), 3.52 (d, 1H, *J* = 11.0 Hz, H_b5′), 3.25 (d, 1H, *J* = 5.4 Hz, 3′−OH), 2.14 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.03 (s, 3H, OAc), 1.98 (s, 3H, OAc); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6 (OAc), 170.4 (OAc), 170.3 (CONH), 170.3 (OAc), 169.7 (OAc), 163.1 (C4), 149.5 (C2), 137.9 (C6), 158.7, 144.5, 135.6, 135.4, 130.1, 128.1, 128.0, 127.0 (Ar), 113.3 (Ar), 110.2 (C5), 101.4 (Gal-C1), 88.0 (C4′), 87.0 (C1′), 86.7 (DMTr), 79.6 (C2′), 71.9 (C5″), 71.1 (C3′), 70.9 (Gal-C3), 70.7 (Gal-C5), 70.5, 70.4 (OCH_2), 69.1 (Gal-C2), 69.0 (OCH_2), 67.1 (Gal-C4), 61.4 (Gal-C6), 59.2 (C5′), 55.2 (OMe), 36.4 (CH_2NH), 20.8 (OAc), 20.7 (OAc), 20.6 (OAc), 20.6 (OAc).

(1*R*,3*R*,4*R*,7*S*)-7-(2-Cyanoethoxy(diisopropylamino)-phosphinoxy)-1-(4,4′-dimethoxytrityloxymethyl)-3-(5-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyloxyacetamido)methyl-uracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (22). Nucleoside **20** (160 mg, 0.16 mmol, 1.0 equiv) was dissolved in anhydrous 1,2-dichloroethane (7 mL), and DIPEA (140 μL, 0.82 mmol, 5.1 equiv) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (50 μL, 0.22 mmol, 1.4 equiv) were added. The reaction mixture was stirred at room temperature for 2 h. The solution was diluted with EtOAc (50 mL) and washed with saturated aqueous $NaHCO_3$ (25 mL) and brine (25 mL). The organic phase was dried (Na_2SO_4) and concentrated to dryness under reduced pressure. The residue was precipitated (2.0 mL EtOAc/120 mL PE) to afford compound **22** as a white foam (186 mg, 99% yield). R_f = 0.4 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) *m/z* 1198.4252 ([M + Na]$^+$, $C_{57}H_{70}N_5O_{20}P$·Na$^+$, Calc. 1198.4244); ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.5, 148.6.

(1*R*,3*R*,4*R*,7*S*)-7-(2-Cyanoethoxy(diisopropylamino)-phosphinoxy)-1-(4,4′-dimethoxytrityloxymethyl)-3-(5-(8-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3,6-dioxaoctanamido)methyl-uracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (23). Nucleoside **21** (500 mg, 0.47 mmol, 1.0 equiv) was dissolved in anhydrous 1,2-dichloroethane (20 mL), and DIPEA (400 μL, 2.30 mmol, 4.9 equiv) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (150 μL, 0.66 mmol, 1.4 equiv) were added. The reaction mixture was stirred at room temperature for 6 h. The solution was diluted with EtOAc (100 mL) and washed with saturated aqueous $NaHCO_3$ (2 × 50 mL) and brine (2 × 50 mL). The organic phase was dried (Na_2SO_4) and concentrated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (1−2%, MeOH/CH_2Cl_2, v/v) to afford compound **23** as a white foam (417 mg, 70% yield). R_f = 0.4 (2.5%, MeOH/CH_2Cl_2, v/v); HRMS (ESI) *m/z* 1286.4714 ([M + Na]$^+$, $C_{61}H_{78}N_5O_{22}P$·Na$^+$, Calc. 1286.4768); ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.48, 148.61.

(1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-5-*N*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyloxyacetamido)-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2.2.1]heptane (25). Carboxylic acid **6** (156 mg, 0.38 mmol, 1.1 equiv) was dissolved in anhydrous THF (8 mL), and DIPEA (120 μL, 0.69 mmol, 2.0 equiv) and HATU (146 mg, 0.38 mmol, 1.1 equiv) dissolved in anhydrous DMF (1 mL) were added. The reaction mixture was stirred at room temperature for 10 min. A solution of 2′-amino-LNA-T **24**[48] (200 mg, 0.35 mmol, 1.0 equiv) dissolved in anhydrous DMF (2 mL) was added dropwise, and the resulting mixture stirred at room temperature for 1.5 h. EtOAc (50 mL) was added, and washing was performed with saturated aqueous $NaHCO_3$ (2 × 25 mL) and brine (2 × 25 mL). The combined aqueous phase was back-extracted with EtOAc (50 mL), and the combined organic layers were dried (Na_2SO_4) and concentrated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (0−3.25%, MeOH/CH_2Cl_2, v/v) to afford compound **25** as a yellow foam (242 mg, 71% yield). R_f = 0.2 (MeOH/CH_2Cl_2, 1:20, v/v); HRMS (ESI) *m/z* 982.3196 ([M + Na]$^+$, $C_{48}H_{53}N_3O_{18}$·Na$^+$, Calc. 982.3216); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.77 (br s, 1H, NH), 7.63 (s, 1H, 6-H_I), 7.60 (s, 1H, 6-H_{II}), 7.50−7.42 (m, 2H, Ar), 7.38−7.12 (m, 12H, Ar), 6.91−6.79 (m, 5H, Ar), 5.55 (s, 1H, 1′-H_I), 5.50 (s, 1H, 1′-H_{II}), 5.38 (d, 1H, *J* = 3.5 Hz, Gal-H_I4), 5.35 (d, 1H, *J* = 3.3 Hz, Gal-H_{II}4), 5.21 (dd, 1H, *J* = 10.4 Hz, 8.1 Hz, Gal-H2), 5.01 (dd, 1H, *J* = 10.0 Hz, 3.5 Hz, Gal-H3), 4.84 (s, 1H, H2′), 4.68 (d, 1H, *J* = 8.1 Hz, Gal-H1), 4.63 (d, 1H, *J* = 13.5 Hz, CH_2O), 4.35−3.90 (m, 7H, H3′, 5″-H_2, Gal-H5, Gal-6-H_2, CH_2O), 3.80 (s, 6H, OMe), 3.53 (s, 6H, OMe), 3.52−3.43 (m, 2H, 5′-H_2), 2.35 (br s, 1H, OH), 2.14 (s, 3H, OAc), 2.06 (s, 3H,

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OAc), 2.02 (s, 3H, OAc), 1.98 (s, 3H, OAc), 1.66 (s, 3H, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6 (OAc), 170.2 (OAc), 170.1 (OAc), 169.9 (OAc), 168.0 (CONH), 163.6 (C4), 159.0 (Ar), 150.1 (C2), 144.5 (Ar), 135.6 (Ar), 135.4 (Ar), 134.5 (C6), 130.2 (Ar), 130.2 (Ar), 129.3 (Ar), 129.2 (Ar), 128.4 (Ar), 128.2 (Ar), 127.3 (Ar), 125.5 (Ar), 113.6 (Ar), 113.4 (Ar), 110.8 (C5), 102.1 (Gal-C1), 88.4 (DMTr), 87.2 (C4′), 87.0 (C1′), 77.4, 71.5, 70.9, 70.6, 70.5, 69.0, 67.2, 62.9, 61.3, 59.4 (C2′, C3′, C5′, C5″, Gal-C2, Gal-C3, Gal-C4, Gal-C5, Gal-C6, CH_2O), 55.4 (OMe), 21.0 (OAc), 20.8 (OAc), 20.8 (OAc), 20.7 (OAc), 12.7 (CH_3).

(1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-5-*N*-(8-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3,6-dioxaoctanamido)-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2.2.1]heptane (26). Carboxylic acid **7** (475 mg, 0.96 mmol, 1.1 equiv) was dissolved in anhydrous THF (16 mL), and DIPEA (300 μL, 1.72 mmol, 2.0 equiv) and HATU (365 mg, 0.96 mmol, 1.1 equiv) dissolved in anhydrous DMF (2 mL) were added. The reaction mixture was stirred at room temperature for 10 min. A solution of 2′-amino-LNA-T **24**[48] (500 mg, 0.87 mmol, 1.0 equiv) dissolved in anhydrous DMF (4 mL) was added dropwise, and the resulting mixture was stirred at room temperature for 1.5 h. EtOAc (100 mL) was added and washing was performed with saturated aqueous $NaHCO_3$ (2 × 50 mL) and brine (2 × 50 mL). The combined aqueous phase was back-extracted with EtOAc (50 mL), and the combined organic phase was dried (Na_2SO_4) and concentrated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (1−6%, MeOH/CH_2Cl_2, v/v) to afford compound **26** as a yellow foam (601 mg, 66% yield). R_f = 0.2 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) *m/z* 1070.3723 ([M + Na]$^+$, $C_{52}H_{61}N_3O_{20}$·Na$^+$, Calc. 1070.3741); ^1H NMR (400 MHz, $CDCl_3$) δ 8.94 (br s, 1H, NH), 7.60−7.57 (m, 1H, 6-H), 7.50−7.44 (m, 2H, Ar), 7.39−7.20 (m, 9H, Ar), 6.88−6.81 (m, 5H, Ar), 5.52 (s, 1H, 1′-H_I), 5.47 (s, 1H, 1′-H_{II}), 5.36 (dd, 1H, *J* = 3.4 Hz, 0.9 Hz, Gal-4-H), 5.27 (dd, *J* = 9.7 Hz, 3.2 Hz, 1 H, Gal-3-H_{II}), 5.17 (dd, 1H, *J* = 10.4 Hz, 8.1 Hz, Gal-2-H_{II}), 5.10 (dd, 1H, *J* = 10.4 Hz, 7.8 Hz, Gal-2-H_I), 5.03 (dd, 1H, *J* = 10.4 Hz, 3.2 Hz, Gal-3-H_I), 4.95 (s, 1H, H2′), 4.55 (d, 1H, *J* = 13.6 Hz, CH_2O), 4.54 (d, 1H, *J* = 7.8 Hz, Gal-1-H), 4.33−3.89 (m, 7H, H3′, 5″-H_2, Gal-H5, Gal-6-H_2, CH_2O), 3.84 (s, 3H, OMe), 3.83 (s, 3H, OMe) 3.79 (s, 6H, OMe), 3.76−3.47 (m, 12H, 5′-H_2, CH_2O), 2.03 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.67 (d, 3H, *J* = 0.8 Hz, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7 (OAc), 170.5 (OAc), 170.4 (OAc), 169.9 (OAc), 169.1 (CONH), 163.9 (C4), 158.8 (Ar), 150.2 (C2), 144.6 (Ar), 135.7 (Ar), 135.5 (Ar), 134.7 (C6), 130.3 (Ar), 130.2 (Ar), 128.2 (Ar), 128.2 (Ar), 127.2 (Ar), 113.5 (Ar), 110.4 (C5), 101.2 (Gal-C1), 88.2 (DMTr), 87.4 (C4′), 86.8 (C1′), 77.4, 71.5, 70.9, 70.8, 70.8, 70.5, 70.0, 69.1, 68.8, 67.1, 63.3, 61.5, 59.7 (C2′, C3′, C5′, C1″, Gal-C2, Gal-C3, Gal-C4, Gal-C5, Gal-C6, CH_2O), 55.4 (OMe), 20.8 (OAc), 20.7 (OAc), 20.7 (OAc), 20.6 (OAc), 12.7 (CH_3).

(1*R*,3*R*,4*R*,7*S*)-7-(2-Cyanoethoxy(diisopropylamino)-phosphinoxy)-1-(4,4′-dimethoxytrityloxymethyl)-5-*N*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyloxyacetamido)-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2.2.1]heptane (27). Nucleoside **25** (241 mg, 0.25 mmol, 1.0 equiv) was dissolved in anhydrous 1,2-dichloroethane (9 mL), DIPEA (210 μL, 1.23 mmol, 4.9 equiv) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (200 μL, 0.90 mmol, 3.6 equiv) were added. The reaction mixture was stirred at room temperature for 2.5 h. The solution was diluted with EtOAc (50 mL) and washed with saturated aqueous $NaHCO_3$ (25 mL) and brine (25 mL). The organic phase was dried (Na_2SO_4) and concentrated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (1.5−5%, MeOH/CH_2Cl_2, v/v) and precipitation (2.5 mL EtOAc/120 mL PE) to afford compound **27** as a white foam (166 mg, 56% yield). R_f = 0.2 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) *m/z* 1182.4300 ([M + Na]$^+$, $C_{57}H_{70}N_5O_{19}P$·Na$^+$, Calc. 1182.4295); ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.8, 149.8, 149.1, 148.0.

(1*R*,3*R*,4*R*,7*S*)-7-(2-Cyanoethoxy(diisopropylamino)-phosphinoxy)-1-(4,4′-dimethoxytrityloxymethyl)-5-*N*-(8-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3,6-dioxaoctanamido)-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2.2.1]heptane (28). Nucleoside **26** (272 mg, 0.26 mmol, 1.0 equiv) was dissolved in anhydrous 1,2-dichloroethane (12 mL), and DIPEA (220 μL, 1.29 mmol, 5.0 equiv) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (120 μL, 0.54 mmol, 2.1 equiv) were added. The reaction mixture was stirred at room temperature for 1.5 h and additional 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (120 μL, 0.54 mmol, 2.1 equiv) was added and the reaction mixture was stirred at room temperature for 2 h. The solution was diluted with EtOAc (50 mL) and washed with saturated aqueous $NaHCO_3$ (25 mL) and brine (25 mL). The organic phase was dried (Na_2SO_4) and concentrated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (1−6%, MeOH/CH_2Cl_2, v/v) and precipitation (2 mL EtOAc/120 mL PE) to afford compound **28** as a white foam (115 mg, 35% yield). R_f = 0.3 (MeOH/CH_2Cl_2, 5:95, v/v); HRMS (ESI) *m/z* 1270.4818 ([M + Na]$^+$, $C_{61}H_{78}N_5O_{21}P$·Na$^+$, Calc. 1270.4819); ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.8, 149.3, 148.3.

Synthesis and Purification of Oligonucleotides. ON synthesis was performed on a DNA synthesizer (PerSeptive BioSystems Expedite 8909) in either 0.2 or 1.0 μmol scale (CPG support) using ultramild phosphoramidites from Glen Research. The galactose modified phosphoramidite monomers were incorporated via hand-coupling[56] using 5-[3,5-bis(trifluoromethyl)phenyl]-*H*-tetrazole (0.25 M, anhydr. MeCN) as activator and 20 min coupling time. The oligonucleotides were deprotected with 32% anhydrous ammonia for 12 h at room temperature. All modified ONs were purified by RP-HPLC using a C18-column (5 μm, 100 mm × 19 mm). The composition of the ONs were confirmed by MALDI MS analysis (Table S1, Supporting Information) and purities (>80%) by analytical IE-HPLC.

Thermal Denaturation Studies. Thermal denaturation studies were carried out on a UV/vis Spectrophotometer using synthetic quartz 10 mm path length cuvettes. The ONs (2.5 μm per strand) were dissolved in medium salt buffer [NaCl (100 mM), EDTA (0.1 mM), 5.8 mM NaH_2PO_4/Na_2HPO_4, pH 7.0], heated to 90 °C for 10 min and then slowly cooled to 5 °C. UV absorbance at 260 nm as a function of time was recorded and the thermal denaturation temperatures (T_m) were determined as the maxima of the first derivative of the resulting curve (Table S2−S13, Supporting Information). The T_m values are an average of two measurements within ±0.5 °C. ΔT_m values were calculated as the difference in T_m values between unmodified and modified duplexes.

Nuclcease Resistance Assays. Nuclease resistance assays regarding the oligonucleotide stability against snake venom phosphodiesterase I from *Crotalus adamanteus* (Pharmacia Biotech) were performed by incubating 3 μM of 5′-^{32}P-labeled ON with 6.7 ng/μL phosphodiesterase I in 100 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$ (20 μL) at 21 °C. Initial aliquots (3 μL, 0 min) were drawn immediately prior to adding the enzyme. At time points 5, 10, 15, 30, and 60 min aliquots (3 μL) were taken and added to tubes containing 2 μL ice-cold loading buffer (95% formamide, 20 mM EDTA, xylene cyanol, and bromophenol blue). All samples were heated to 80 °C for 2 min to stop nucleolytic activity and then resolved on 20% denaturing polyacrylamide electrophoresis gels with 7 M urea which were visualized by autoradiography on a Typhoon Trio Variable Mode Imager.

Molecular Modeling. Molecular modeling was performed with Macro Model v9.1 from Schrödinger. All calculations were conducted with AMBER* force field and the GB/SA water model. The dynamic simulations were performed with stochastic dynamics, a SHAKE algorithm to constrain bonds to hydrogen, time step of 1.5 fs and simulation temperature of 300 K. Simulation for 0.5 ns with an equilibration time of 150 ps generated 250 structures, which all were minimized using the PRCG method with convergence threshold of 0.05 kJ/mol. The starting structures were generated by incorporation of first a standard LNA-T nucleotide into the duplex structure (DNA:RNA; Protein data bank (PDB) entry pdb 1HG9)[58] followed by dynamic simulation. Further modification of the resulting energy minimized structure was performed to give the duplexes containing the modified nucleotides Gal-C2-LNA-T or Gal-C2−2′-amino-LNA-T which were subjected to repeated calculations.

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■ ASSOCIATED CONTENT

Supporting Information

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NMR spectra for all new compounds; MS data for new ONs; T_m curves (PDF)

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Notes

The authors declare no competing financial interest.

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