Bioconjugate Chemistry

Communication

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Efficient Synthesis and Biological Evaluation of 5′-GalNAc Conjugated Antisense Oligonucleotides

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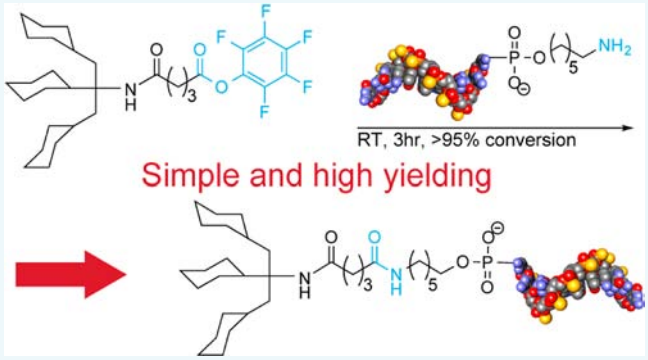
Supporting Information

ABSTRACT: Conjugation of triantennary *N*-acetyl galactosamine (GalNAc) to oligonucleotide therapeutics results in marked improvement in potency for reducing gene targets expressed in hepatocytes. In this report we describe a robust and efficient solution-phase conjugation strategy to attach triantennary GalNAc clusters (mol. wt. ∼2000) activated as PFP (pentafluorophenyl) esters onto 5′-hexylamino modified antisense oligonucleotides (5′-HA ASOs, mol. wt. ∼8000 Da). The conjugation reaction is efficient and was used to prepare GalNAc conjugated ASOs from milligram to multigram scale. The solution phase method avoids loading of GalNAc clusters onto solid-support for automated synthesis and will facilitate evaluation of GalNAc clusters for structure activity relationship (SAR) studies. Furthermore, we show that transfer of the GalNAc cluster from the 3′-end of an ASO to the 5′-end results in improved potency in cells and animals.



Antisense oligonucleotides (ASOs) hybridize to complementary mRNA in cells by Watson−Crick base-pairing and modulate their processing to produce a pharmacological effect.[1] Chemical evolution of ASOs that degrade mRNA via RNase H mediated hydrolysis has resulted in second generation "gapmer" designs, which entail the use of a phosphorothioate (PS) modified backbone and a DNA gap-region flanked by 2′-furanose-modified nucleosides.[2] The DNA gap-region supports RNase H mediated cleavage of complementary RNA, while the 2′-modified nucleotides enhance RNA-binding affinity and metabolic stability.[1] One second generation ASO, Kynamro, which targets the apolipoprotein B-100 mRNA, was recently approved by the FDA for the treatment of familial homozygous hypercholestermia.[3]

Increasing ASO potency in animal models has been a long-term goal and has in recent years been accomplished by modifying gapmer ASOs with high affinity modifications.[4,5] We recently showed that gapmer ASOs modified with triantennary GalNAc at the 3′-end showed ∼10-fold improved potency in hepatocytes.[6] Similarly, 3′-GalNAc modification of siRNA enhanced delivery to hepatocytes and improved potency in vivo.[7−10] In both cases, the 3′-GalNAc conjugates were synthesized by preloading solid support with the GalNAc cluster followed by automated synthesis on a DNA synthesizer (Scheme 1B). This approach, however, can have limitations including reduced loading of the solid support for ASO synthesis resulting in lower yields and increased consumption of phosphoramidites, slow SAR cycles for evaluating new clusters, enhanced structural complexity of the chiral 3-hydroxyprolinol adaptor used for attaching the GalNAc cluster to the solid support, potential complications with qualifying modified support for commercial synthesis and requirement for the cluster to be stable toward reagents used for oligonucleotide synthesis and deprotection.

To address these potential limitations, we have developed an efficient solution-phase methodology for conjugating GalNAc clusters to the 5′-end of ASOs. The conjugation method is straightforward and requires only a small (3-fold) excess of GalNAc cluster. Furthermore, a direct comparison of 3′- and 5′-GalNAc ASO conjugates showed that the 5′-GalNAc conjugate is quickly metabolized to the 5−10−5 MOE (2′-*O*-methyloxyethyl RNA) gapmer *in vivo* and exhibit slightly enhanced potency in cells and in animals.

Conjugation Approaches. Multiple approaches exists for conjugating moieties to ASOs in solution.[11] Reported conjugation methods include disulfide exchanges,[12,13] Diels−Alder cycloaddition, and maleimide−thiol conjugation,[14,15] but generally require laborious multistep procedures and result in low yields. With these points in mind we evaluated multiple approaches for conjugation of a GalNAc cluster to the 5′-end of ASOs. Notable failed approaches include the following (Scheme S1): (1) Coupling a GalNAc-cluster phosphoramidite

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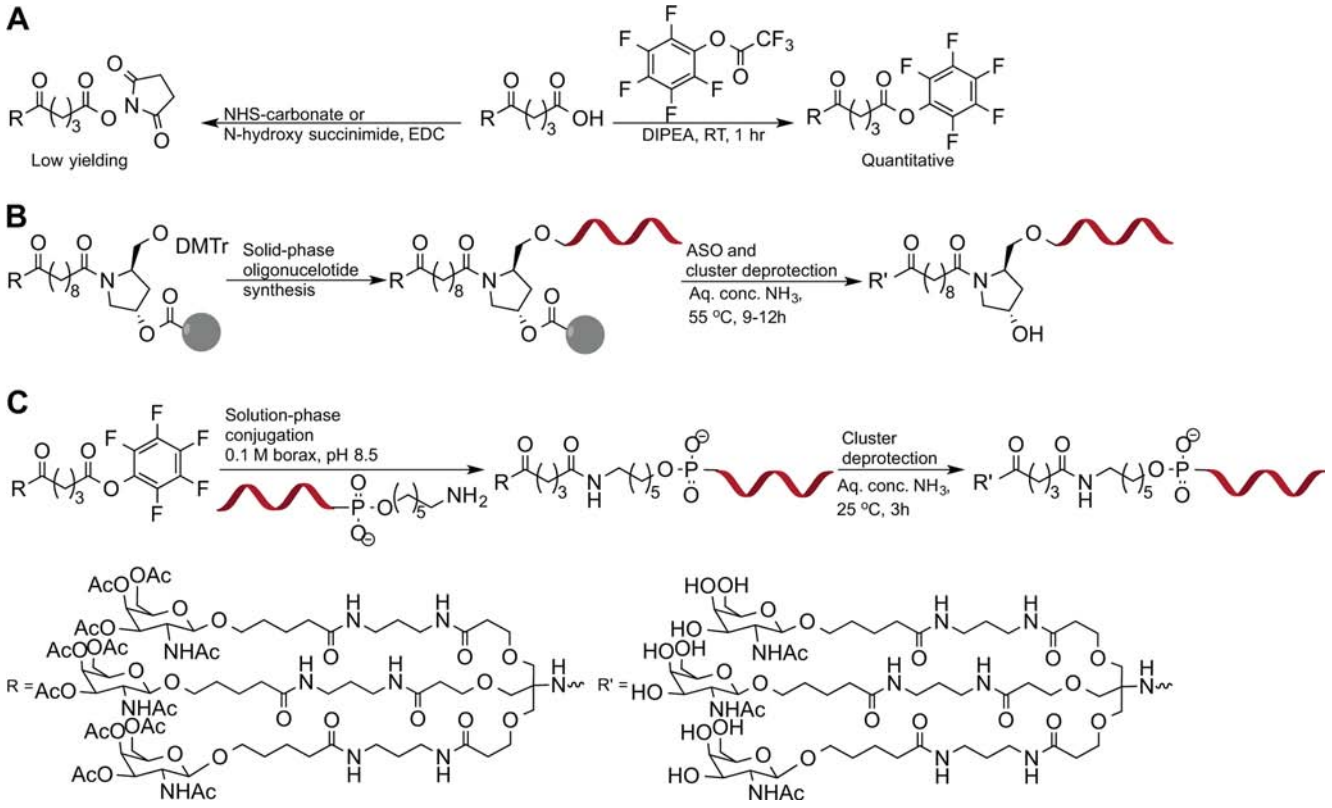
Scheme 1[a]



[a]A: Synthesis of GalNAc NHS and PFP ester. B: Synthesis of 3′-GalNAc modified ASO using GalNAc loaded resin and growing oligonucleotide chain using solid-phase oligonucleotide synthesis followed by deprotection. C: Synthesis of 5′-GalNAc modified ASOs by reaction of a 5′-hexylamino modified ASO with PFP ester activated GalNAc cluster followed by deprotection of the acetyl groups using aqueous ammonia.

Table 1. Optimization of Conjugation Conditions[a]

entry	ASO conc.	%DMSO	T (°C)	conversion
1	0.1 mM	6	25	37%
2	0.2 mM	9	25	48%
3	0.4 mM	17	25	67%
4	1.1 mM	50	25	>99%
5	2.2 mM	100	25	75%
6	0.4 mM	17	50	69%

[a]ASO (50 nmol) dissolved in 0.1 M sodium tetraborate, pH 8.5, and 3 equiv of GalNAc PFP ester dissolved in DMSO were mixed for 3 h and conversion determined by LCMS.

Table 2. GalNAc Conjugation Efficiency Is Independent of ASO Sequence

target[a]	backbone[b]	conversion	isolated yield
SRB-1	PS	100%	53%
SRB-1	PO/PS	100%	55%
A1AT	PS	100%	48%
FXI	PO/PS	100%	47%
Apo CIII	PO/PS	100%	64%

[a]SRB-1: scavenger receptor B-1. A1AT: alpha-1 antitrypsin. FXI: coagulation factor XI. Apo CIII: apolipoprotein CIII. [b]PS: full phosphorothioate backbone. PO/PS: mixed phosphate and phosphorothioate backbone. For sequences and chemistry information, see Table S1.

reagent to the 5′-end of an ASO using standard solid-phase phosphoramidite chemistry[16] failed to give any measurable product; (2) coupling *O*-acetyl protected GalNAc cluster with a free carboxylic acid to 5′-HA ASOs using peptide coupling reagents in solution or on solid support resulted in mediocre conversion or predominantly *N*-acetylation,[17,18] respectively; and (3) maleimide modified GalNAc cluster failed to couple to 5′-thiol modified ASO.

Next, conjugation of a GalNAc cluster with an activated carboxylic acid to a 5′-HA ASO in solution was attempted. The most commonly used activation group is a *N*-hydroxysuccinimide (NHS) ester; however, synthesis of a GalNAc NHS ester was problematic. Reaction of GalNAc carboxylic acid with *N*,*N*′-disuccinimidyl carbonate in base was slow and low-yielding. The reaction between GalNAc carboxylic acid and NHS using EDC as activator was slow and low-yielding and purification of the product by silica gel chromatography was problematic due to the limited hydrolytic stability and polar nature of the GalNAc NHS ester (Scheme 1A). Instead GalNAc carboxylic acid was reacted with pentafluorophenol-trifluoracetate (PFP-TFA) in the presence of diisopropylethylamine (DIPEA) resulting in clean conversion to the corresponding PFP ester (Scheme 1A).[19] The slight excess of PFP-TFA used for activation and the pentafluorophenol generated as byproduct from the reaction are either volatile or hydrolyzed upon workup and soluble in the aqueous washes. As a result, no further purification of the highly polar PFP-activated GalNAc cluster was required prior to conjugation. Coupling PFP ester activated GalNAc to a 5′-HA ASO in solution results in clean conversion (Scheme 1C). Conjugation was performed by mixing 5′-HA ASO with GalNAc PFP ester in a sodium tetraborate buffer at pH 8.5, and reaction is

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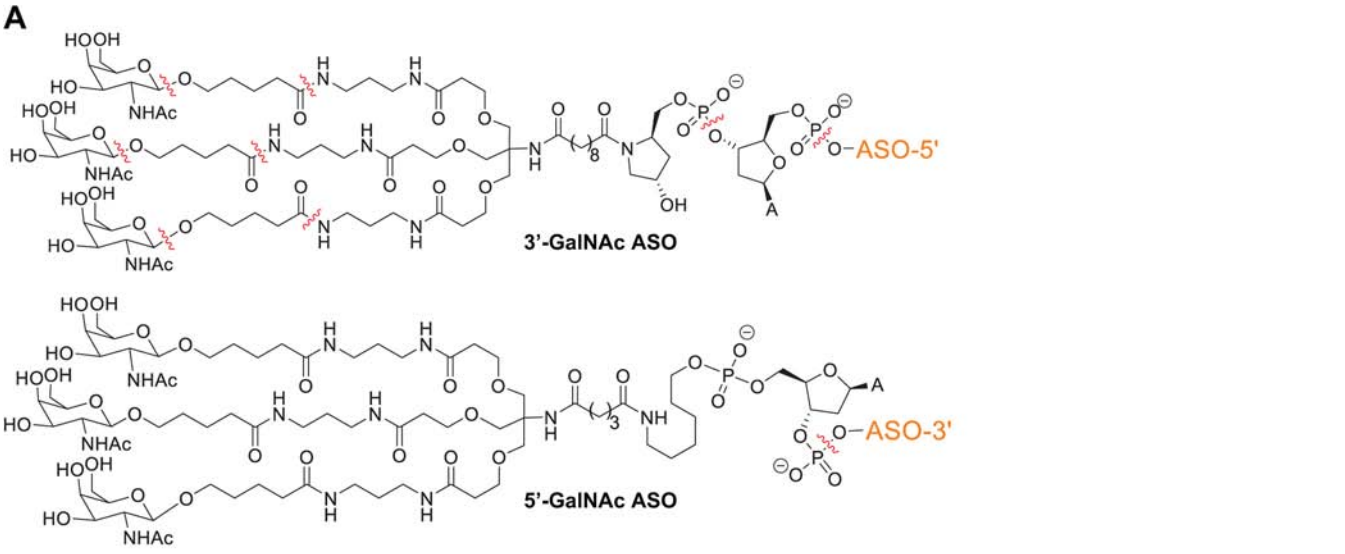

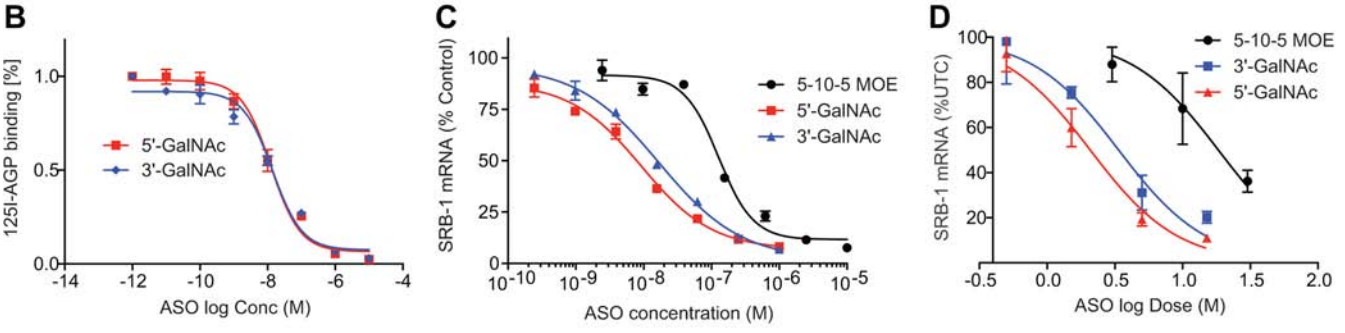

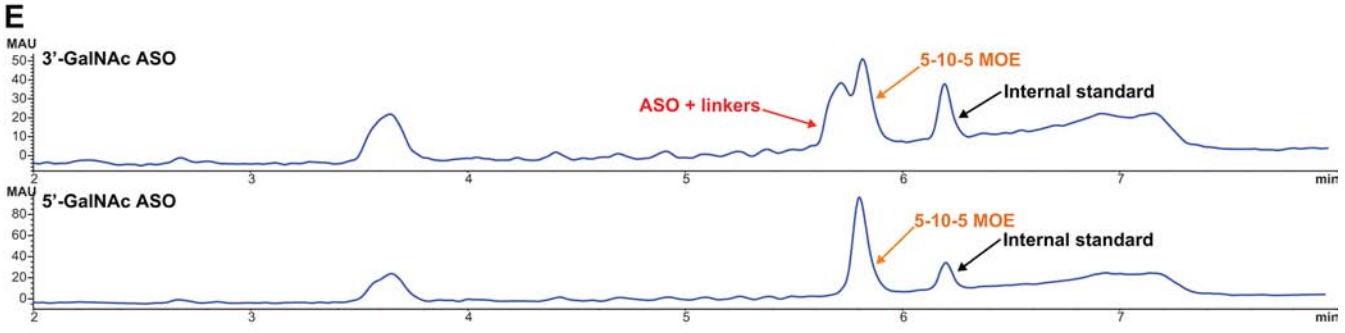

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Design	Sequence (5'-3')	ASGPR binding K_i (nM)	*in vitro* IC_{50} (nM)	*in vivo* ED_{50} (mg/kg)
5-10-5 MOE	GsCsTsTsCsAsGsTsCsAsTsGsAsCsTsTsCsCsTsT	N/A	130	19
3'-GalNAc	GsCsTsTsCsAsGsTsCsAsTsGsAsCsTsTsCsCsTsToA	10	17	3.4
5'-GalNAc	AoGsCsTsTsCsAsGsTsCsAsTsGsAsCsTsTsCsCsTsT	7	9	2.1

Figure 1. Biological evaluation of GalNAc conjugated ASOs. A: Chemical structures of 3′- and 5′-GalNAc conjugates. B: Binding to ASGPR in mouse primary hepatocytes. C and D: Dose−response curves in mice primary hepatocytes and C57B/6 mice, respectively. E: Metabolic stability in mice 72 h post-treatment; cleavage points identified by mass spectrometry are shown by red lines in Figure 1A. F: Summary of biologic evaluation. Color code: black = DNA and orange = MOE nucleosides, s = phosphorothioate and o = phosphate linkage.

complete in less than 3 h (Figure S1). Removal of the *O*-acetyl groups was performed in aqueous (aq.) concentrated (conc.) ammonia for 3 h without generating any measurable byproducts.

Conjugation Optimization. Initial conjugation conditions employed large excess of the PFP activated GalNAc cluster (50 equiv). These conditions resulted in complete conjugation, i.e., this conjugation is not prone to overaddition, e.g., to one of the exocyclic amines of the nucleobases (see Figure S1). To allow screening of a range of new SAR GalNAc clusters and to qualify this conjugation for future use in manufacture of drug substance, the conjugation required optimization to allow fewer equivalents of GalNAc PFP ester to be used. The conjugation conditions adapted from an NHS activated

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fluorophore conjugation protocol results in an ASO concentration of 0.1 mM.[20] This is approximately a 1000-fold lower concentration than what is generally recommended for organic synthesis, therefore a concentration dependence of conjugation efficiency was evaluated. Increasing concentration from 0.1 mM to 1.1 mM results in improvement of conjugation efficiency from 37% to >99% when using 3 equiv of GalNAc PFP ester (Table 1 and Figure S2). Use of buffer is necessary to give full conversion (Table 1 entry 5) and heating does not seem to improve conversion significantly (entry 6) likely due to PFP ester hydrolysis as a competing reaction. As shown, increasing ASO concentration is important to improve conjugation conversion, but it is important to note that ASOs generally cannot be dissolved to higher than 10−20 mM since concentrated ASO solutions become very viscous. To determine how large an excess of GalNAc PFP ester cluster is needed, 1.5 and 3 equiv of cluster were tested (Figure S3). 1.5 equiv gave 70% conversion while 3 equiv resulted in full conversion, suggesting that 2−3 equiv of GalNAc PFP ester is required for complete consumption of the starting 5′-HA ASO.

Conjugation Versatility. To evaluate the effect of ASO sequence on conjugation efficiency, five different 5′-HA ASOs were conjugated to the GalNAc PFP ester. All five conjugations resulted in quantitative yields based on HPLC profiles (Table 2, Figure S4) showing that ASO sequence has minimal effect on the conjugation reaction. Furthermore, the conjugation reaction was scaled up to 1.5 g of ASO to give quantitative conversion by HPLC analysis of the reaction mixture. Yield of purified ASO was 62% although conversion by LC was complete, but the lower isolated yield was a result of losses during purification of the conjugate by ion-exchange and reverse-phase HPLC.

Biological Evaluation. 3′- and 5′-conjugated GalNAc ASOs were compared in biological assays (Figure 1). A 2′-deoxyadenosine phosphodiester was inserted between the ASO and the GalNAc conjugate to facilitate metabolic cleavage of the conjugate (Figure 1A) and to minimize any potential effects of the small difference in linker structure on conjugate activity. GalNAc binds to the asialoglycoprotein receptor (ASGPR) expressed on hepatocytes;[21,22] thus, to test the properties of 3′- and novel 5′-conjugated ASOs we measured binding to ASGPR using primary mouse hepatocytes. Each GalNAc modified ASO binds ASGPR with similar low nanomolar affinity (Figure 1B). Then, potency in cells and animals was investigated. Both GalNAc modified ASOs are significantly more potent than unconjugated 5−10−5 MOE gapmer ASO (>5-fold improvement); however, 5′-conjugated ASO is 2-fold more potent in cell culture and 1.5-fold more potent in animals relative to the 3′-conjugated ASO (Figure 1C,D). To further investigate the properties of 3′- and 5′-GalNAc modified ASOs, drug was extracted from mice liver 72 h postinjection. Interestingly, the 5′-GalNAc modified ASO is fully metabolized to liberate the parent 5−10−5 MOE after 72 h, while the 3′-GalNAc modified ASO still has various linker moieties attached to the MOE gapmer (Figure 1A,E). It is conceivable that the rapid metabolic release of the ASO from the conjugate might be responsible for the enhanced potency observed with the 5′-GalNAc conjugated ASO. Thus, the 5′-GalNAc conjugate acts as a hepatocyte targeting pro-drug which is metabolized to release the parent ASO in the liver.

Conclusion. An efficient method for the conjugation of PFP activated GalNAc cluster to 5′-HA ASOs was developed. The use of PFP ester activated GalNAc cluster allows straightforward conjugation to 5′-HA ASOs. This conjugation methodology requires less than 3 equiv of GalNAc PFP ester and conjugation efficiency is independent of ASO sequences. Furthermore, direct comparison of 3′- and 5′-conjugated GalNAc ASOs shows that the 5′-conjugated ASO is more potent in primary hepatocytes as well as in animals and the 5′-conjugated ASO is quickly and cleanly metabolized *in vivo* to liberate the parent ASO. The ability to append GalNAc clusters to 5′-HA ASOs in solution will facilitate SAR studies to help define the optimal structural requirements for GalNAc conjugated ASOs. These studies are currently ongoing and the results will be reported in due course.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, conjugation attempts, LC profiles for GalNAc conjugations and characterization data for GalNAc PFP ester. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00265.

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Notes

The authors declare no competing financial interest.

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