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A convenient synthesis of 5′-triantennary *N*-acetyl-galactosamine clusters based on nitromethanetrispropionic acid

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ABSTRACT

A convenient method for the synthesis of several triantennary GalNAc clusters based on a nitromethanetrispropionic acid core was developed. The synthetic approach involves pentafluorophenolic ester intermediates which can be used in a one-pot, seven reaction procedure to quickly prepare a variety of triantennary GalNAc conjugated ASOs. The GalNAc clusters were conjugated to the 5′-end of an antisense oligonucleotide and evaluated for activity in primary mouse hepatocytes where they showed ~10-fold improvement in activity.

Antisense based therapeutics continue to evolve rapidly, with over 35 antisense oligonucleotides (ASOs) advancing in the clinic and many more poised to do so in the near future.[1–5] Because the biological targets for more than half of these ASOs are expressed predominantly in liver hepatocytes,[2] the asialoglycoprotein (ASGPR) receptor—a receptor expressed on all mammalian hepatocyte cell surfaces and which is highly specific for *N*-acetyl-galactosamine (GalNAc) terminated glycans—has been utilized to facilitate specific ASO uptake into this critical cell type.[6–10] Recently, we have demonstrated that these triantennary GalNAc conjugated ASOs can improve the biological activity 7–10 fold in rodents.[9]

The most commonly used triantennary GalNAc clusters[9,17] implement Kempen's[11] 2-amino-2-hydroxymethyl-1,3-propanediol (Tris, Fig. 1) as the core branching moiety,[6–10] although other triantennary GalNAc clusters are also known.[12] Because the typical GalNAc cluster synthesis requires over 20 steps from commercially available starting materials,[10] we sought to construct a highly simplified GalNAc cluster starting with nitromethanetrispropionic acid (Triacid, **1**)—a commercially available and inexpensive compound widely used as a precursor to construct various polymers.[13,14] As shown in Figure 1, Triacid **1** is structurally extended compared to Tris, deriving from simple Michael additions to nitromethane, while the Tris linker must be further elaborated to the Trisacid structure to achieve an appropriately substituted core structure suitable for functionalization. This difference in structural complexity manifests itself in an increased number of synthetic transformations.

With the core chosen, pentafluorophenolic esters (Pfp) of the triacid-derived clusters were prepared for use in ASO conjugations (vide infra). Coupling of **1** to N-Boc-1,3-diaminopropane using standard HBTU coupling conditions gave compound **2** in 85% yield. After Boc removal, C-5 GalNAc acid **4**[10] was coupled to the resultant cluster under identical conditions to give cluster **5**. After Raney Nickel reduction, coupling to the mono benzyl ester of glutaric acid, and hydrogenation, the pentafluorophenolic ester **7** was obtained.

To further simplify the structure of the GalNAc cluster we sought to remove the internal diamine from the cluster, which required substituting an amino functional group for the carboxylic acid moiety in the cluster's center. This strategy is outlined in Scheme 2. First, tetra-*O*-acetyl GalNAc (**8**) was conjugated to N-CBz-aminohexanol to give precursor **9** using conditions identical to that for the preparation of **4**. Next, **1** was treated with pentafluorophenol trifluoroacetate to give the Pfp triester **10**. Conveniently, **10** is a stable crystalline solid and can be prepared in large quantities. Moreover, we found that hydrogenating **10** over 20% Pd/C in EtOAc/MeOH resulted in no reaction over at least 12 h. The identification of this 'hydrogenation resistant' carboxylic acid activating group led us to the idea of a one-pot coupling reaction. Therefore, treating 1 equiv of **10** with 3.1 equiv of **9** under catalytic hydrogenation conditions gave a 95% yield of cluster **11**, with no observed reduction of the triester **10**. This proved to be a very

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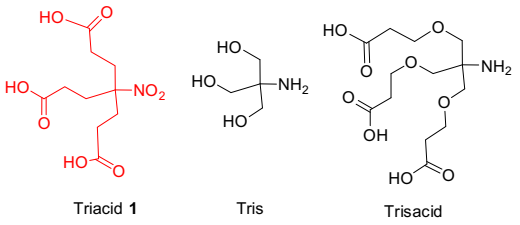


Figure 1. Structures of the Triacid linker, Tris and the Triacid linkers.

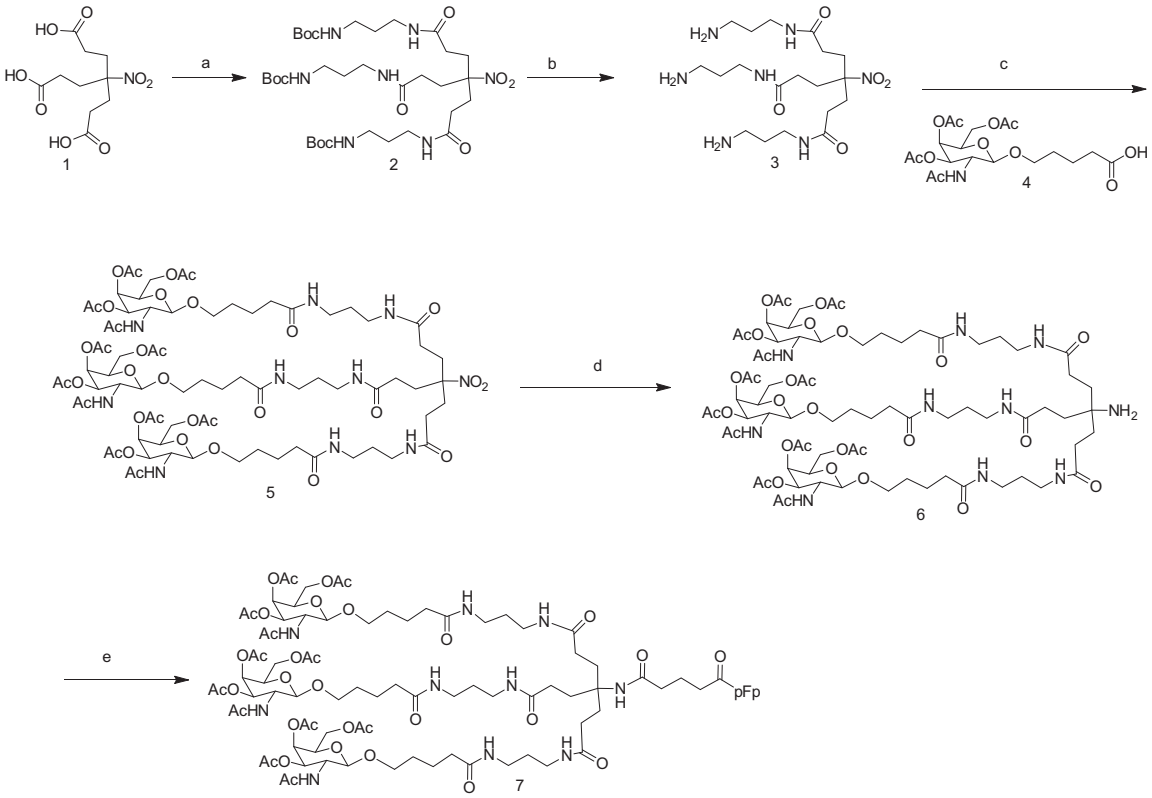
convenient way to prepare an advanced cluster intermediate in a relatively few number of steps. Installing the glutaric acid moiety, followed by Pfp ester formation resulted in the preparation of cluster **13**.

To simplify the synthesis further, while eliminating the Raney Nickel reduction step, we sought to prepare compound **12** using another modification of our one-pot coupling strategy; this is outlined in Scheme 3. Commercially available compound **14**, derived from **1** in two steps, was coupled with the mono benzyl ester of glutaric acid, **15**, to give the advanced intermediate **16**. Analogous to (**1** → **10**) outlined in Scheme 2, Pfp ester **17** was prepared. Conveniently, this is also isolated as a solid, and can be prepared in large quantities. Treatment of **17** with **9** under catalytic hydrogenation conditions (analogous to **9** + **10** → **11** in Scheme 2) gave cluster **12** in a 90% yield, thereby accomplishing; (a) three CBz deprotections, (b) three amide bond formations, and (c) one debenzylation, all in a single pot within one hour. This methodology allows for the construction of triacid-derived ASO GalNAc conjugates in a few short steps from commercially available starting materials.

A triantennary GalNAc conjugated **ASO 2** (Scheme 4) was constructed by coupling the pentafluorophenolic ester **7** to the 5′-end of a hexylamino linked ASO using methodology recently described.[15] **ASO 3–8** (Fig. 2) were synthesized in an analogous manner. For example, the Pfp esters of the clusters used to prepare **ASO 3–4** were made using the procedure analogous to that in Scheme 1, in comparable yields, from the C-6 GalNAc acid analog of compound **4**[10] and N-Boc-1,4-diaminobutane and conjugated as in Scheme 4. The clusters used to prepare **ASO 5–8** were constructed in an analogous manner to Scheme 2 and conjugated as in Scheme 4.

We evaluated **ASO 1–8**, ASOs which target mouse scavenger receptor B1 (SRB1), in freshly plated mouse primary hepatocytes, cells which retain good ASGPR expression and activity against non-conjugated ASOs under free-uptake conditions (i.e., without the need for a transfection reagent).[16] As shown in Table 1, all of the GalNAc-conjugated ASOs (**ASO 2–8**, IC_{50} = 20–70 nM) were about ~10-fold more active than the unconjugated ASO (**ASO 1**, IC_{50} = 250 nM).

It is interesting that the shorter linkers (**ASO 6** and **7**) performed as well as the longer linkers (**ASO 2–5**, **8**). A model (based on NMR and molecular modeling of asialoglycoprotein receptors from rabbit hepatocytes) for the optimal distance of triantennary GalNAc moieties has been previously proposed by Lee et al.[17,18] This model suggests that distances of 14–20 Å from the branching point to the terminal sugar residues are preferred, and it seems most cluster designs reported in the literature to date are consistent with this model and contain 14–16 atoms between the branching point and GalNAc sugar residues.[9,10,19] Clearly, a much shorter linker is easily tolerated, as only 7 atoms are between the branching point and GalNAc sugar residues of **ASO 6**.



Scheme 1. Reagents and conditions: (a) N-Boc-1,3-diaminopropane, HBTU, DIEA, DMF, rt, 16 h, 85%; (b) TFA, DCM, rt, 24 h, 85%; (c) HBTU, DIEA, DMF, rt, 16 h, 65%; (d) Raney-Ni, EtOH, H_2, rt, 24 h, 86%; (e) (i) glutaric acid benzyl ester (see compound **15**, Scheme 3), HBTU, DIEA, DMF, 16 h, rt, 37%; (ii) 20% Pd/C, H_2, EtOAc/MeOH, 1 h, 97%; (iii) pentafluorophenol trifluoroacetate, DCM, DIEA, 1 h, rt, 96%. Pfp structure: see Scheme 2.