HHS Public Access

Author manuscript

Glycoconj J. Author manuscript; available in PMC 2016 October 01.

Published in final edited form as:

Glycoconj J. 2015 October ; 32(7): 549–556. doi:10.1007/s10719-015-9597-3.

Design and syntheses of hyaluronan oligosaccharide conjugates as inhibitors of CD44-Hyaluronan binding

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Abstract

Hyaluronan (HA) is an integral component of the extracellular matrix. Its interactions with a cell surface receptor CD44 has been shown to play important roles in a variety of biological events including cell proliferation and metastasis. As multivalent CD44-HA binding is critical for downstream signaling, compounds that can selectively disrupt the complex formation of HA polysaccharide with CD44 can serve as useful probes of CD44 mediated cellular events as well as potential leads for novel therapeutics. Herein, we report the synthesis of several series of HA conjugates to target the HA binding pocket of CD44. As a small library of HA disaccharide derivatives failed to exhibit any inhibitory activities, we focused on HA tetrasaccharide based analogs. Traditional synthetic strategies towards HA oligosaccharides involve the construction of backbone from the corresponding monosaccharide building blocks, which can be quite tedious. In order to expedite the synthesis, we designed a new synthetic route taking advantage of the ability of hyaluronidase to generate large quantities of HA tetrasaccharide through digestion of HA polysaccharides. The HA tetrasaccharide obtained was utilized to prepare multiple *S*-linked HA analogs bearing aromatic groups at the reducing end glycan. One such compound containing an *m*-benzyl phenyl moiety exhibited significant inhibition of CD44-HA binding. Our approach provides a new direction towards the design of HA based CD44 antagonists.

Keywords

CD44; Hyaluronan; Inhibitor design; Synthesis

Introduction

Hyaluronan (HA) is a non-sulfated negatively charged linear polysaccharide, which is composed of repeating units of di-saccharide: D-glucuronic acid (β1→3) *N*-acetyl-D-glucosamine (β1→4) [1, 2]. Among many HA binding proteins [3], a major receptor of HA

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Electronic supplementary material The online version of this article (doi:10.1007/s10719-015-9597-3) contains supplementary material, which is available to authorized users.

Compliance with ethical standards The authors declare that there are no potential conflicts of interests. All authors agree with the submission.

is CD44, a cell surface glycoprotein involved in signal transduction, cell adhesion and migration [4, 5]. The interactions of CD44 and HA have been shown to be important for tumor cell proliferation, metastasis and the development of multidrug resistance [6–9].

Due to the polymeric nature of HA, it can crosslink multiple CD44 receptors on cell surface, resulting in the activation of kinases and downstream cellular signaling [10]. The polyvalent interactions between HA and CD44 are critical for the formation of the signaling complexes. An HA oligosaccharide can potentially compete with endogenous HA polysaccharide for CD44 binding, resulting in disassembly of the signaling complexes [11]. This in turn can lead to the inhibition of CD44 dependent tumor cell proliferation and drug resistance [12, 8].

Reagents that can disrupt the binding of CD44 and HA can serve as useful probes of CD44 functions as well as potential leads for the development of novel therapeutics [12, 8, 11]. From culture broth of fungus, several glycolipids have been isolated that can inhibit CD44-HA binding with IC_{50} values around 20 μM, although the binding sites are not known [13–15]. Through fragment screening, Finzel and coworkers identified several tetrahydroisoquinolines, which bound with CD44 with Kd in the mM range [16]. Crystallographic characterization showed that these molecules bound to CD44 in a site adjacent to HA binding pocket, which interfered with CD44 interactions with polymeric HA. Besides these studies, an alternative approach to develop CD44 antagonists is to design HA analogs targeting HA binding pocket of CD44.

The crystal structures of hyaluronan binding domain (HABD) of CD44 and HA octasaccharides (HA_8) have been reported [17]. HA_8 binds to a groove on the surface of CD44 (Kd=125 μM) with four glycan units shown to make main contacts with CD44 [17]. The interactions are dominated by hydrogen bonds and hydrophobic interactions. Close examination of the crystal structure of CD44 HABD revealed the presence of an empty hydrophobic pocket formed by tyrosine 46 (Tyr46), Tyr83, isoleucine 111 (Ile111), threonine 116 (Thr116) and Tyr119 adjacent to the HA binding pocket (shown in orange color in Fig. 1). Studies have shown that binding affinities of carbohydrate ligands to receptors can be significantly improved by introducing aromatic groups to strengthen cation-π and hydrophobic-hydrophobic interactions with the proteins [18–20]. Herein, we explored the possibility of conjugating hydrophobic motifs into HA oligosac-charides to enhance CD44 binding affinity.

Result and discussion

Synthesis and CD44 binding of a HA disaccharide library

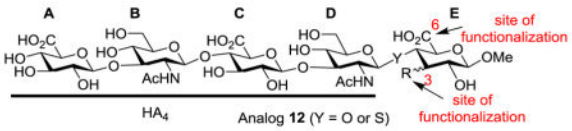
Due to the synthetic ease, our initial approach focused on a library of HA disaccharide (HA_2) analogs, which were prepared from a common HA_2 precursor **1**. The synthesis of **1** started from coupling of monosaccharides **2** [21] and **3** producing disaccharide **4** in 70 % yield (Scheme 1). The PMB group in compound **4** was removed by DDQ oxidation, and the resulting primary hydroxyl group was oxidized to carboxylic acid [22]. Subsequent removal of benzoyl (Bz) and phthalimido (Phth) groups followed by acylation of the free hydroxyl groups and methyl ester protection led to disaccharide **1**. Various hydrophobic functionalities were then conjugated to compound **1** through the copper catalyzed azide-

alkyne cycloaddition reactions [23]. After ester hydrolysis, HA_2 analogs **5**–**11** were obtained.

In order to assay CD44 binding by HA_2 analogs, a competitive enzyme linked immunosorbent assay (ELISA) was established by incubating a CD44 HABD/IgGFc chimera onto anti-IgG antibody coated 96-well plates [24]. Compared to the direct absorption of CD44 onto the uncoated plates, this layout helped to orient the CD44 HABD for HA binding. HA poly-saccharide (MW~16 kDa) was functionalized with biotin (biotin-HA) [25] and the abilities of the HA_2 analogs to inhibit biotin-HA binding to CD44 were measured by ELISA. Unfortunately, none of the HA_2 showed any inhibitory effects. This was presumably due to the short length of the glycan units. In addition, docking of the disaccharides onto CD44 crystal structure showed that the aromatic groups on the di-saccharide analogs could not reach the hydrophobic pocket of interest. These considerations prompted us to investigate HA analogs with longer backbones.

Design, synthesis and screening of HA_4 based conjugates

HA analog **12** was initially designed as its ring E can provide multiple potential sites including the C-3 (either axial or equatorial) and C-6 positions for modifications. The analogs were evaluated manually *in silico* inserting **12** into the co-crystal structure of CD44 HABD-HA_8 (2JCQ) using Pymol and overlaying the HA_4 portion of the backbone (glycan A → D in analog **12**) onto HA_8 in the structure. C-3-axial position of the E ring was deemed suitable for directing extra functionality into the hydrophobic pocket, while maintaining the hydrogen bond between the carboxylic group of glycan E and CD44. The C-3-equatorial and C-6 positions were also determined to be suitable functionalization sites with minimum impacts on HA_4 interactions with CD44 HABD.



Assembly of oligosaccharides such as **12** can be a highly challenging task. While chemical syntheses of a variety of HA oligosaccharides have been accomplished [26–29], it is still very tedious to build up the oligosaccharide from the corresponding monosaccharide building blocks. To expedite the synthesis, we explored a new strategy, where a HA tetrasaccharide would be obtained through enzymatic digestion of HA polysaccharide and utilized for glyco-assembly.

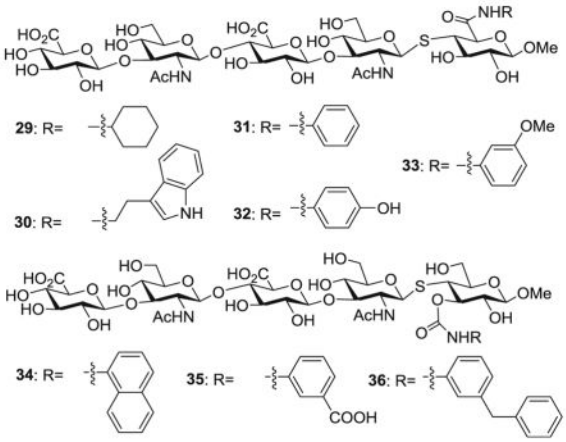
In order to prepare the HA tetrasaccharide, HA polysaccharide was treated with a hyaluronidase at pH 5.2 in sodium acetate buffer (Scheme 2) [30, 31]. This reaction was performed on an 80 g scale yielding 50 g of HA tetrasaccharide **13**. Due to the low cost of hyaluronidase (~ $100/g) and HA polysaccharide ($2/g), this is an attractive approach to readily access the needed material. The HA tetrasaccharide **13** was methylated and acetylated yielding fully protected tetrasaccharide **14** in 35 % yield.

With HA tetrasaccharide **14** in hand, its conversion to analog **12** was explored by first transforming **14** to a glycosyl donor. Treatment of **14** with HBr and acetic acid generated the α-glycosyl bromide **15**, which was converted to thioglycoside donor **16** (Scheme 3a). However, when either donor **15** or **16** was subjected to glycosylation, glycosyl oxazoline was obtained as the major side product with no desired glycosides, which is a common occurrence with 2-acetamide containing donors [32]. To overcome this problem, *N*,*N*-di-acetyl imide [33] donor **17** was prepared, which did not undergo productive glycosylations with several thiophilic promoters (Scheme 3b). Besides glycosyl bromide and thioglycoside, we examined the transformation of **14** to either glycosyl chloride or trichloroacetimidate donors, which were not successful.

With the difficulties in formation of *O*-glycosides, the alternative of using *S*-linkage was explored. *S*-Glycosides have better hydrolytic stabilities than the corresponding *O*-glycosides, while maintaining similar conformations [34–36]. To establish the feasibility, a glycosyl thiol **19** was prepared as a model donor. Coupling reaction between **19** and triflate **20** failed to yield the desired thioglycoside **21** (Scheme 4a) . In stead, thioaziridine **22** was formed as the major product. This was presumably due to the attack of azide by the glycosyl thiolate to form S-N bond, followed by intramolecular displacement of the triflate (Scheme 4b). To avoid this problem, acceptor **23** was tested next, which gave mainly the elimination product **24**. Interestingly, switching the configuration of 3-*O*-Lev from axial to equatorial (acceptor **25**) led to the formation of the *S*-linked disaccharide **26** in 56 % yield.

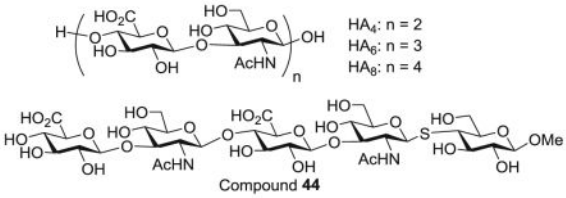
In order to prepare *S*-linked HA analogs, HA tetrasaccharide **15** was treated with tetrabutylammonium thioacetate (TBASAc) and thioacetic acid (HSAc) to form glycosyl thioacetate. HSAc was used to adjust the acidity of the reaction mixture to minimize the oxazoline formation. The *S*-acetate was removed selectively with NaOMe at −40 °C to give glycosyl thiol **28** in 30 % overall yield from **15**. (Scheme 5).

With the glycosyl thiol **28** in hand, two series of analogs (**29**–**33** and **34**–**36**) were designed based on the co-crystal structure of CD44 and HA_8 [17]. Due to the difficulties encountered with the C-3 axial alloside (Scheme 4), we focused on functionalizing C-3 equatorial and C-6 positions of the reducing end glucoside or glucuronate. These analogs were manually overlaid onto HA_8 in the crystal structure to maintain the conformation of the glycan backbone in the binding pocket (Figure S1). The strategically added functional groups were deemed to potentially be able to introduce new π-π stacking, hydrophobic interaction and/or H-bonding with CD44.



The synthesis of compounds **29**–**36** is outlined in Scheme 6. The triflate bearing galactosides **37** and **38** were prepared from commercially available methyl galactoside **39** and the two regio-isomers were separated (Scheme 6a). Glycosyl thiol **28** was coupled with galactoside **38** followed by TBDPS removal to generate **42** (Scheme 6b). The resulting free OH in **42** was oxidized to carboxylic acid [37], which was then coupled with a variety of amines. Subsequent ester hydrolysis produced analogs **29**–**33**. Similarly, glycosyl thiol **28** was coupled with galactoside **37** and transformed to compound **43** (Scheme 6c). The 3-OH of **43** was conjugated to several isocyanates, which followed by deprotection led to conjugates **34**–**36**.

With the HA analogs **29**–**36** in hand, their abilities to inhibit the binding of biotinylated HA polysaccharide to CD44 HABD were analyzed using the competitive ELISA first at a single concentration (240 μM) together with several HA oligosaccharides. As shown in Fig. 2a, out of all synthetic compounds, **36** exhibited strongest inhibition indicating that the *m*-benzyl phenyl moiety linked to 3-*O* position of the reducing end glycan was beneficial for CD44 interactions. Compared to the unconjugated **44**, the stronger affinity of **36** suggested the hydrophobic moiety was important. The inhibitory activity of **36** was stronger than that of HA_4, comparable to HA_6 but weaker than HA_8 in this assay. Next, the abilities of **36**, HA_6 and HA_8 to inhibit CD44-HA binding were measured at multiple concentrations, which yielded IC50 values of 322 μM (**36**), 213 μM (HA_6) and 168 μM (HA_8) (Fig. 2b).



Based on the modelling results, both the *m*-benzyl and phenyl groups in compound **36** can contact the hydrophobic pocket near the HA binding site. The phenyl group possesses hydrophobic interactions with Ile 111 (Figure S1H). The *m*-benzyl group has hydrophobic interactions with Tyr 119 and T-shaped π-π stacking with Tyr 46. The methylene group between two aromatic rings presents the two rings in an arrangement that both rings can

fully contact this hydrophobic pocket. Compared to **36**, compounds **29**~**35** have fewer hydrophobic contacts with the pocket than **36** (Figure S1, A~G).

Conclusion

Several series of HA conjugates have been designed to target the HA binding pocket of CD44 and to inhibit the interaction of CD44 with HA. The HA_2 library did not exhibit any appreciable activities. As assembly of higher HA oligosaccharides could be very tedious, a new synthetic approach towards HA oligosaccharides was developed by taking advantage of the ability of hyaluronidase to produce HA tetrasaccharides by digesting HA polysaccharides. The HA tetrasaccharides generated by hyaluronidase were utilized to synthesize multiple HA conjugates. Compared to the traditional chemical synthesis [26–29], this strategy was more efficient as it utilized readily available sources of materials (HA polysaccharide and hyaluronidase) and obviated the need to assemble the tetrasaccharide through glycosylation reactions. Furthermore, with the simplified protective group patterns utilized, deprotection of the final product was much simpler than the lengthy and at times unpredictable deprotection procedures encountered in traditional synthesis [38, 28]. Among all conjugates synthesized, analog **36** bearing the *m*-benzyl phenyl carbamate moiety gave the strongest inhibition of CD44-HA polysaccharide binding. While its inhibitory activity is still modest, the structure of **36** provides a lead in designing HA based CD44 inhibitors, which demonstrates strategically placed aromatic rings can improve the potency. Further optimization of HA conjugates is underway to enhance the affinity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for financial support from the Department of Chemistry, Michigan State University as well as the National Institute of General Medical Sciences, NIH (R01GM072667) and the National Science Foundation (CHE-1507226).

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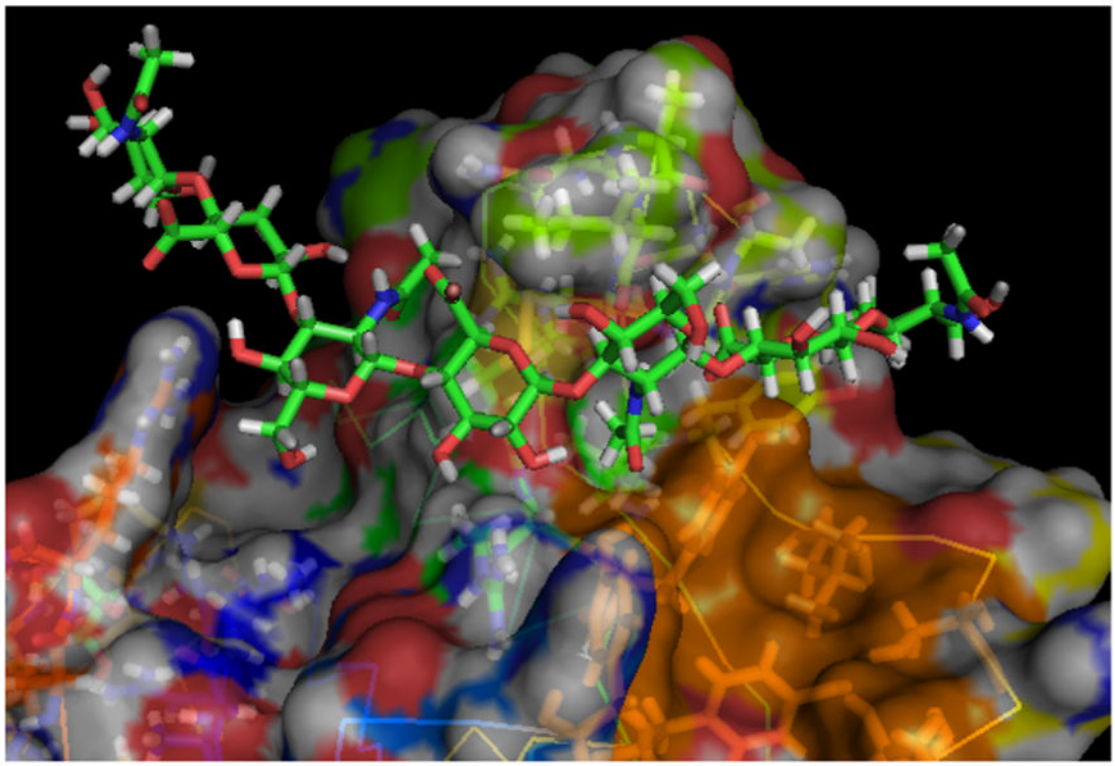


Fig. 1.

The hydrophobic pocket in CD44-HA_8 co-crystal structure highlighted in orange color. The image was generated from the co-crystal structure (PDB code: 2JCQ) using Pymol

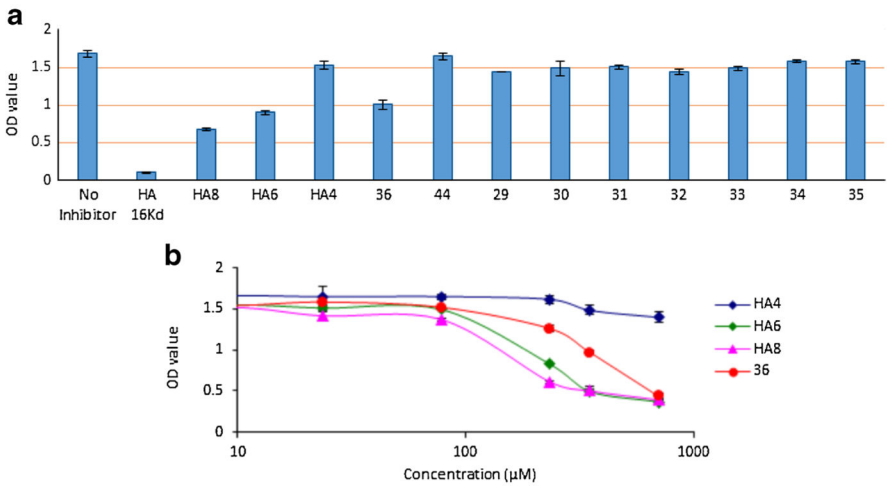
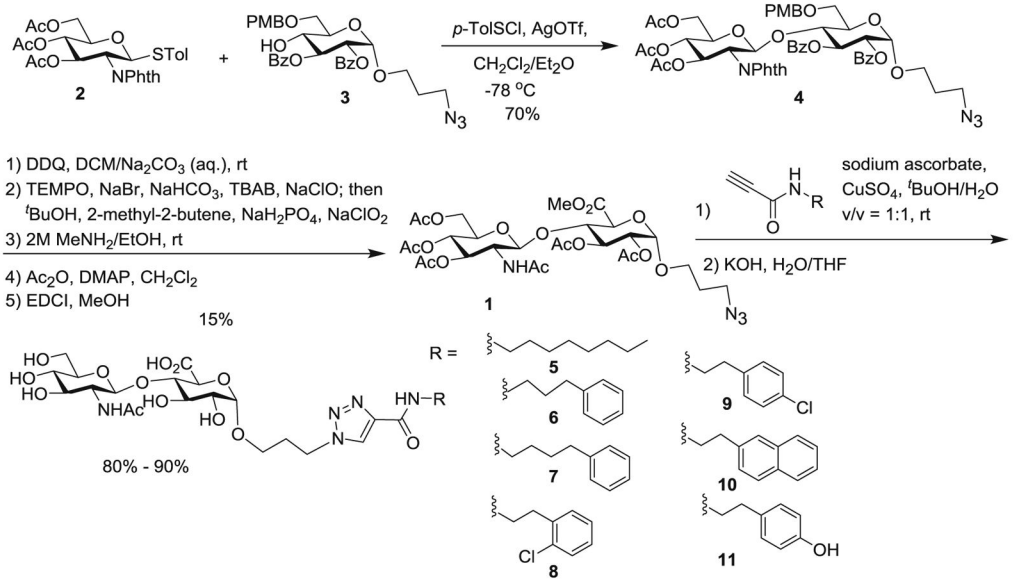
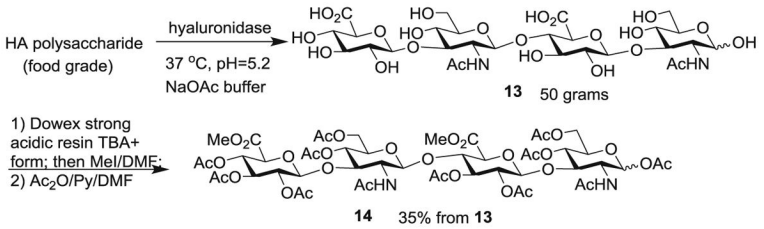


Fig. 2.

a Comparison of activities of various HA analogs at 240 μM to inhibit CD44-HA binding through the competition ELISA. **b** Inhibition curves of compound **36**, HA_4, HA_6 and HA_8

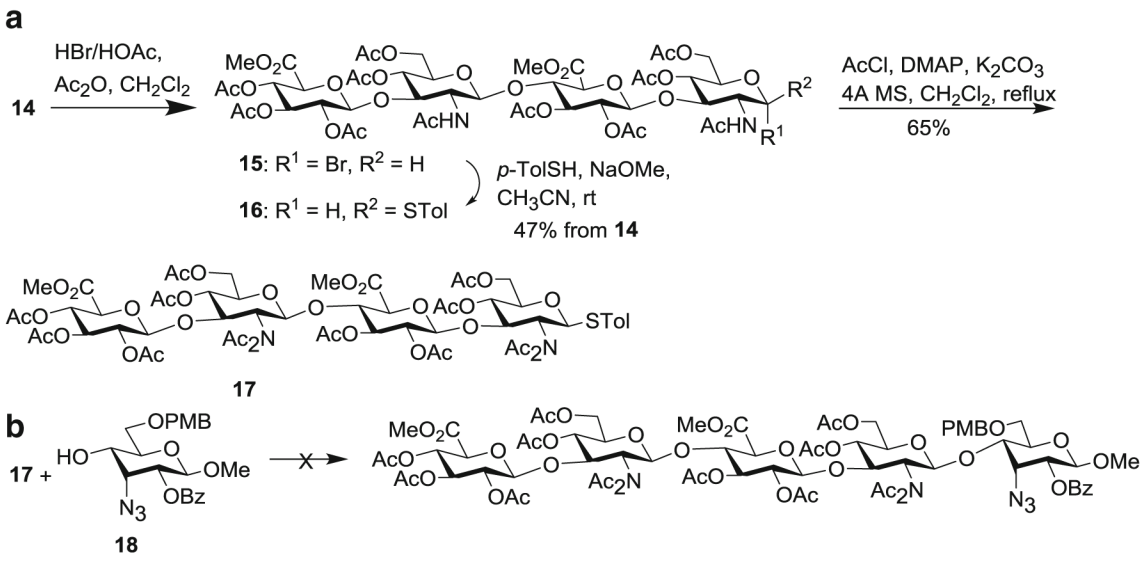


Scheme 1.
Synthesis of HA_2 analogs



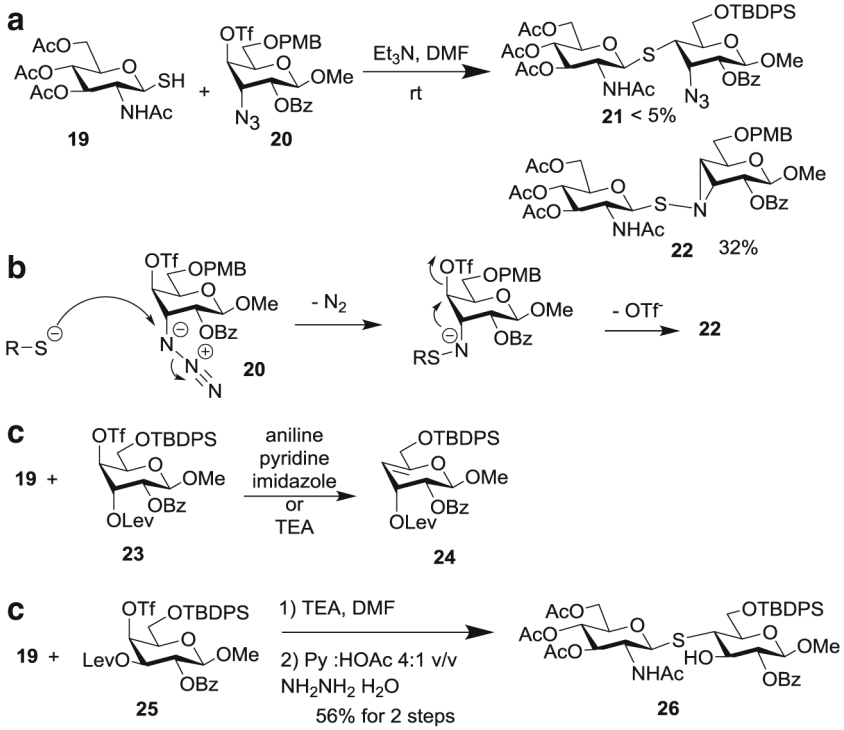
Scheme 2.

Synthesis of tetrasaccharide **14**



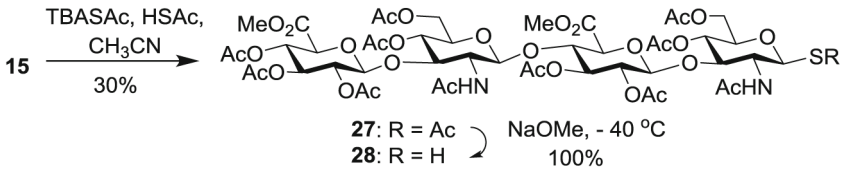
Scheme 3.

aSynthesis of tetrasaccharide donor **17. b** Unsuccessful glycosylation of **18** by donor **17**

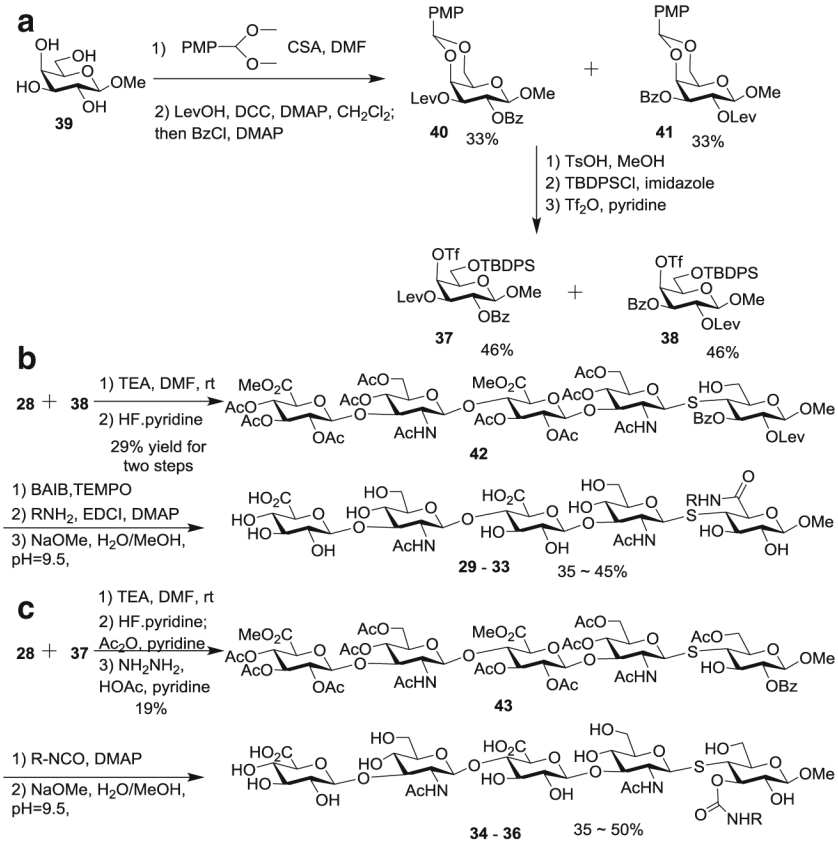


Scheme 4.

Model study for formation of *S*-linked glycans



Scheme 5.
Synthesis of glycosyl thiol **28**



Scheme 6.

Synthesis of HA conjugates **29**–**36**