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Letter

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Chemogenesis of an Antiangiogenic Glycosaminoglycan

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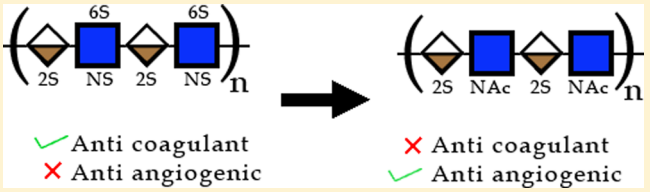
Supporting Information

ABSTRACT: In this letter we report a facile chemical conversion of heparin, a potent anticoagulant with minimal antiangiogenic activity, into an effective antiangiogenic glycosaminoglycan through optimized chemical approaches. This work highlights the potential for industrial scale production of a therapeutic anticancer glycosaminoglycan.



KEYWORDS: *Acharan sulfate, angiogenesis, heparan sulfate, synthesis, heparin*

Glycosaminoglycans (GAGs) such as heparan sulfate (HS) and chondroitin sulfate (CS) are linear polyanionic molecules typically linked to proteins. Because of their molecular diversity, they are involved in a variety of pathophysiological processes including cell signaling and development, growth and morphogenesis, angiogenesis, inflammation, and tumor progression.[1−3]

Acharan sulfate is a sulfonated GAG from the giant African snail *Achatina fulica*.[4] It has a unique structure that is unlike heparin or heparan sulfate. In naturally occurring glycosaminoglycans, sulfate groups may be located on the 2-*N*, 3-*O*, and 6-*O* positions of glucosamine residues, as well as the 2-*O* position of uronic acid residues. Heparin is primarily composed of trisulfated disaccharides of 2*N*-sulfamido-6-*O*-sulfo-α-D-glucopyranoside (1 → 4)-2-*O*-sulfo-α-L-idopyranosyluronate, whereas heparan sulfate is primarily composed of monosulfated disaccharides of 2*N*-sulfamido-α-D-glucopyranoside(1 → 4)-β-D-glucopyranosyluronate. Conversely, the primary structure of acharan sulfate is composed of 2*N*-acetamido-α-D-glucopyranoside (1 → 4)-2-*O*-sulfo-α-L-idopyranosyluronate. This sequence is rarely produced in human tissue as epimerization of glucuronic acid to iduronic acid requires the presence of neighboring *N*-sulfo groups.[5]

On the basis of its unique structure, acharan sulfate is a potentially valuable molecular medicine for treating cancers. Previously, several studies have discussed heparin's inherent anticancer properties.[6] However, unfractionated heparin is a potent anticoagulant and cannot be used for cancer therapy due to bleeding complications. The presence of 3-*O*, 6-*O*, and *N*-sulfo groups on heparin allow it to bind to antithrombin through a unique pentasaccharide sequence.[6] Unlike heparin, acharan sulfate is nonanticoagulant and mitigates fibroblast growth factor (FGF) signaling without binding to FGF directly.[7] Acharan sulfate lacks all three critical sulfate groups required for binding antithrombin and acting as an anticoagulant. Additionally, acharan sulfate effectively prevents vascular endothelial growth factor-induced (VEGF) angiogenesis in models of inflammation.[8] Furthermore, acharan sulfate shows no toxicity in vitro when tested at concentrations as high as 5 mg mL^{-1}.[7]

Current methods for procuring acharan sulfate require tedious isolation from snail tissue. Additionally, there is a possibility of contamination with other sulfated polysaccharides when isolating acharan sulfate directly from tissue. To overcome these challenges, there is a need for a chemical or chemoenzymatic process to synthesize acharan sulfate.

In this letter, we report a facile preparation of a GAG similar to acharan sulfate from heparin, a widely available polysaccharide that can be obtained in large quantities (Scheme 1). This GAG is synthesized from the pyridinium salt of heparin in three steps including: complete 6-*O* desulfation, *N*-desulfation, and *N*-acetylation. As reported previously, there is a concomitant removal of 3-*O*-sulfates when heparin is subjected to 6-*O*- or *N*-desulfation.[9,10] The presence of repeating disaccharides of *N*-acetyl-D-glucosamine (GlcNAc) and 2-*O*-sulfo-iduronic acid in the synthesized polymer was confirmed by nuclear magnetic resonance (NMR) and strong anion exchange high performance liquid chromatography (SAX-HPLC) analysis (Figure S1 and Table S1, Supporting Information). The molecular weight of the resultant polymer was analyzed on size exclusion chromatography (SEC)-HPLC (Figure S2, Supporting Information). As expected, desulfation of the low molecular weight heparin precursor leads to a removal of sulfate residues and a reduction in the ability to attract water molecules. Additionally, since heparin was utilized as the precursor, the final product has a very high iduronic acid content as confirmed by NMR analysis (Figure S5, Supporting Information).

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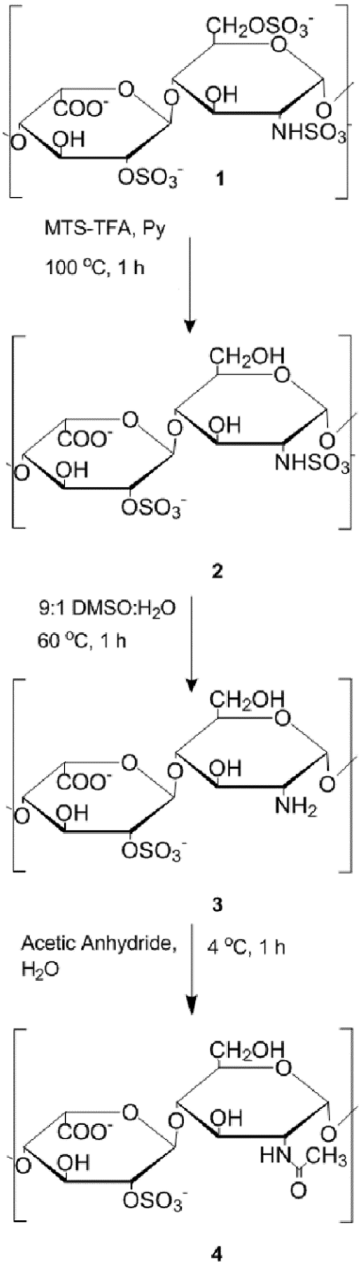
Scheme 1. Synthesis of an Acharan Sulfate-Like GAG from Heparin



Complete 6-*O*-desulfation results in the generation of a C6-hydroxyl group on the GlcNAc residues, in agreement with resonances for H6 a,b (^{1}H NMR, 3.90 ppm; ^{13}C NMR, 59.84 ppm). In comparison, the H6 a,b proton signals resonate at ∼4.3 ppm in the heparin starting material. Upon complete de-*N*-sulfation and *N*-acetylation, a new signal appears at 2.09 ppm ^{1}H and 22.07 ppm ^{13}C, in agreement with the *N*-acetyl CH_3 group. The H2 proton signal of the newly formed GlcNAc residue is shifted downfield to 4.03 ppm (53.76 ppm ^{13}C) from the 3.3 ppm signal observed for that of $GlcNHSO_4$.

The proton signal corresponding to the sulfonated O<u>H</u>−C_2 appears at 4.29 ppm confirming the presence of 2-*O*-sulfated hydroxyl group on the IdoA residues. The structural assignment of the synthetic acharan sulfate-like molecule (Table S1, Supporting Information) is in agreement with the published data.[4,11] However, the mimetic differs from natural acharan sulfate due to the lower abundance of 2-*O*-sulfate groups present on the polymer backbone. Natural acharan sulfate has more than 90 percent 2-*O* sulfated disaccharide residues, whereas heparin, the precursor for the current synthesis, also includes several disaccharides that do not contain 2-*O* sulfates.[12]

To confirm the antiangiogenic activity of the newly synthesized GAG, a robust in vitro tube formation assay was utilized (Figure 1). In this assay, vascular endothelial cells were grown on matrigel where they form tube-like structures. The efficacy of antiangiogenic compounds can be determined by examining their effects on tube branching, number, and length. It is evident that the synthesized acharan sulfate-like GAG was effective at 1 and 2 mg/mL concentrations. Observing the

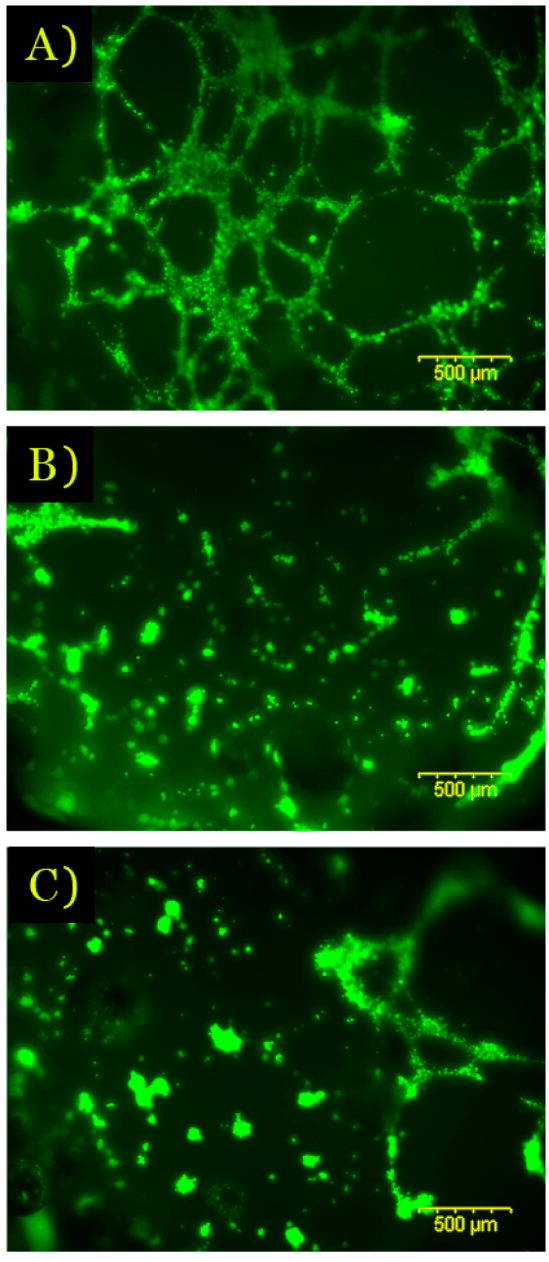


Figure 1. Inhibition of BLMVEC tube formation upon treatment with the acharan sulfate-like polysaccharide. Representative panels are (A) control tube formation, (B) 1 mg/mL mimetic, and (C) 2 mg/mL mimetic. Experiments were performed three times in duplicate wells.

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branching, number, and length of tubes shows that, upon treatment, endothelial cells formed very few tubes and that the majority of cells form clusters that are not connected to a branched network. In contrast, heparin had a negligible effect on tube formation at 1 and 2 mg/mL concentrations (Figure S3, Supporting Information). Additionally, the mimetic was less effective at 0.5 mg/mL (Figure S4, Supporting Information). It is likely that increasing the 2-*O* sulfate content of the mimetic will likely enhance its antiangiogenic potential.

For decades, it has been known that preventing tumor-associated angiogenesis is an effective method for controlling cancer growth.[13] While several angiogenesis inhibitors such as Avastin have been developed previously, these therapies typically target singular molecular functions.[14] Proteoglycans, present on cell membranes and in the extracellular matrix, are integral components in tumor progression and angiogenesis. In contrast to typical antiangiogenic therapeutics, proteoglycan-based therapeutics have the potential to affect several signaling pathways involved in tumor-associated angiogenesis, invasion, and metastasis.[15] However, although several GAG-based therapeutic agents such as PI-88 have been discovered, very few of them have been utilized to treat patients.[16,17] One of the primary reasons for the lack of clinical application of GAG-based therapeutics is their tedious synthesis, frequently involving several low-yield steps and difficult purification techniques.[18] Additionally, naturally derived therapeutic GAGs such as acharan sulfate cannot be obtained in sufficient quantities to be utilized in the clinic.

In this letter, we provide a novel and simple method for synthesizing an antiangiogenic acharan sulfate-like GAG from heparin, a readily available and widely used clinical anticoagulant. The newly synthesized polymer is biologically active at low concentrations, effectively inhibits tube formation in vitro, and can be produced in large quantities. Because of its similarity to acharan sulfate, the mimetic is also likely to be non-anticoagulant. Further optimization and preclinical evaluation of this molecule is currently underway to determine its in vivo efficacy. Further studies are necessary to determine the exact molecular mechanism of action of the synthetic acharan sulfate mimetic.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, SAX-HPLC analysis, effect of heparin on tube formation, effect of other acharan sulfate concentrations, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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