

Arylsulfatase K is the Lysosomal 2-Sulfoglucuronate Sulfatase

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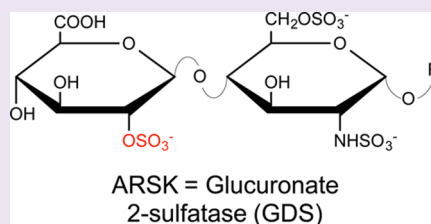
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S Supporting Information

ABSTRACT: The degradation of glycosaminoglycans (GAGs) involves a series of exolytic glycosidases and sulfatases that act sequentially on the nonreducing end of the polysaccharide chain. Enzymes have been cloned that catalyze all of the known linkages with the exception of the removal of the 2-*O*-sulfate group from 2-sulfoglucuronate, which is found in heparan sulfate and dermatan sulfate. Here, we show using synthetic disaccharide substrates that arylsulfatase K is the glucuronate-2-sulfatase. Arylsulfatase K acts selectively on 2-sulfoglucuronate and lacks activity against 2-sulfoiduronate, whereas iduronate-2-sulfatase (IDS) desulfates synthetic disaccharides containing 2-sulfoiduronate but not 2-sulfoglucuronate. As arylsulfatase K has all of the properties expected of a lysosomal enzyme, we conclude that arylsulfatase K is the long sought lysosomal glucuronate-2-sulfatase, which we designate GDS.



Animal cells express plasma membrane and extracellular matrix proteoglycans, which consist of sulfated glycosaminoglycans (GAGs) covalently attached to proteoglycan core proteins. The major sulfated GAGs include heparan sulfate, chondroitin sulfate, and dermatan sulfate. Assembly initiates by the attachment of xylose to specific serine residues, followed by assembly of a linkage tetrasaccharide (D-glucuronic acid β 1–3-D-galactose β 1–4 D-xylose). The GAG chains then polymerize by the alternating addition of D-N-acetylhexosamine and D-glucuronate residues. A family of sulfotransferases installs sulfate groups at various positions along the chains, and an epimerase converts a subset of D-glucuronate residues to L-iduronate.¹ Domains containing sulfated sugar residues and iduronic acid confer the capacity to interact with various protein ligands, including growth factors, chemokines, lipoproteins, and membrane receptors.² GAG–protein interactions facilitate a wide range of processes crucial during development and important for normal physiology.^{3,4}

Proteoglycans are highly dynamic, undergoing partial desulfation and heparanase cleavage at the cell surface, shedding from the cell surface by proteolysis and endocytosis.⁴ The latter process results in the delivery of the proteoglycan and any associated ligands to the lysosome where they undergo degradation.⁵ Degradation of the GAG chains occurs in a sequential manner from the nonreducing end of the chain and requires the action of multiple exoglycosidases and exosulfatases, and in the case of heparan sulfate an N-acetyltransferase.⁶ Loss of any one of these enzymes arrests degradation and

causes accumulation of undegraded GAGs and GAG fragments in the lysosome. In humans, defects in lysosomal GAG degradation result in a group of diseases called mucopolysaccharidoses (MPS), a type of lysosomal storage disorder (LSD) characterized by developmental and neurological defects, and in severe cases death in the first or second decade.^{7,8}

The enzymatic activity of all eukaryotic sulfatases is strictly dependent on a unique post-translational modification in which an active site cysteine within a conserved sequence is converted into a C α -formylglycine residue by the formylglycine-generating enzyme (FGE).^{9–11} To date, 17 different sulfatases that act on GAGs, sulfated lipids, and other sulfated substrates have been described in humans.^{12–16} Many of these sulfatases exhibit activity *in vitro* against small sulfated aromatic pseudosubstrates, such as *p*-nitrocatechol sulfate, *p*-nitrophenyl sulfate, and 4-methylumbelliferyl sulfate and thus are classified as arylsulfatases. Deficiencies in seven of the eight known lysosomal sulfatases result in different forms of lysosomal storage. Arylsulfatase A deficiency causes metachromatic leukodystrophy, whereas loss of galactosamine-4-sulfatase (arylsulfatase B), galactosamine-6-sulfatase, glucosamine-6-sulfatase, sulfamidase, and iduronate-2-sulfatase (IDS) cause

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different types of mucopolysaccharidoses.⁶ We recently showed that arylsulfatase G acts on 3-sulfoglucosamine residues in heparan sulfate. Its inactivation in mice resulted in a new form of LSD, which we designated MPS IIIE.^{17–19}

The substrate specificities of the three remaining arylsulfatases, ARSI, ARSJ, and ARSK, remain unknown. Arylsulfatase K was originally identified through bioinformatics on the basis of the conserved sequence C-S/T/C/A-P-S-R common to all sulfatases.^{13,14} We recently showed that ARSK, but not an active site mutant lacking formylglycine (ARSK-C/A), exhibits activity toward the arylsulfate pseudosubstrates *p*-nitrocatechol sulfate and *p*-nitrophenyl sulfate.¹⁶ The enzyme also has an acidic pH optimum, colocalizes with the lysosomal marker LAMP1, and carries mannose-6-phosphate tagged *N*-linked oligosaccharides, which facilitate lysosomal sorting via mannose-6-phosphate receptors.¹⁶ Thus, ARSK most likely acts on lysosomal substrates. However, the identity of the natural substrate on which ARSK acts has not been determined.

To explore the substrate specificity of ARSK, we synthesized two disaccharides that are derived from heparan sulfate and contain a 2-sulfoglucuronate moiety (1, G2A0; 2, G2S0) and two disaccharides that are part of dermatan sulfate and have a 2-sulfoiduronate (3, I2a4; 4, I2a6; Figure 1). It was anticipated

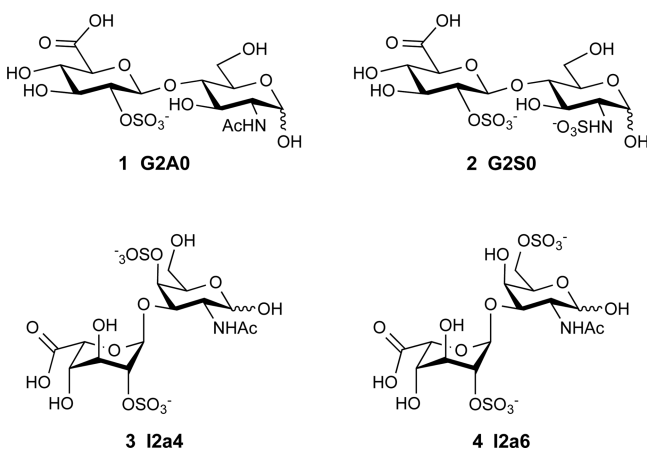


Figure 1. Disaccharides G2A0, G2S0, I2a4, and I2a6. To simplify the representation of constituent disaccharides, we use a disaccharide structure code.²⁷ In this code, a uronic acid is designated as G or I for D-glucuronate or L-iduronate, respectively, and the hexosamine is designated as A or a for *N*-acetylglucosamine or *N*-acetylgalactosamine, respectively. The *N*-substituent on glucosamine is either A or S for acetate or sulfate, respectively. The presence and location of ester-linked sulfate groups are depicted by the number of the carbon atom on which the sulfate group is located or by 0 if absent. For example, G2S0 refers to a disaccharide composed of 2-sulfoglucuronate-*N*-sulfoglucosamine.

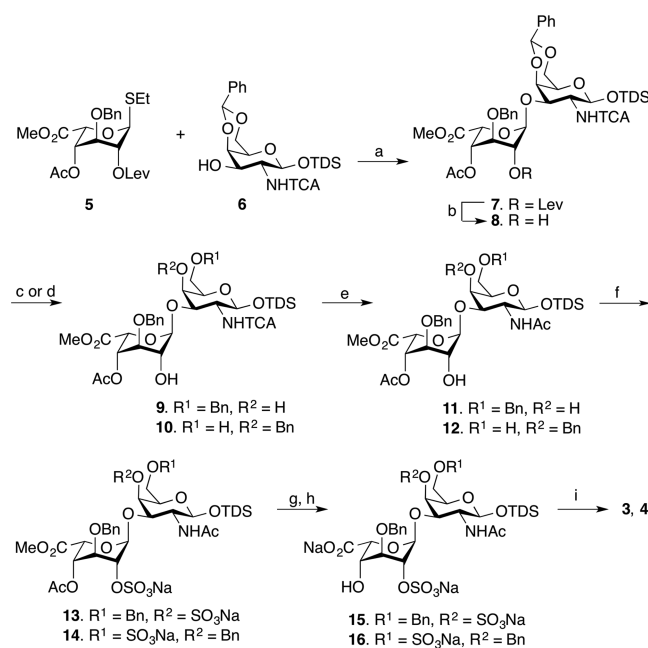
that compounds 1 and 2 might be substrates for ARSK because the genes encoding enzymes that catalyze the hydrolysis of all sulfates of GAGs have been cloned with the exception of 2-*O*-sulfate esters from 2-sulfoglucuronate. I2a4 and I2a6 were prepared as part of a larger collection of nonreducing end disaccharides as potential biomarkers for iduronate-2-sulfatase deficiency.²⁰

Previously, we described a modular approach for the preparation of heparan sulfate oligosaccharides in which a small number of strategically chosen monosaccharide building blocks were employed to prepare a set of disaccharides that can be converted into glycosyl donors and acceptors for assembly of

a large number of oligosaccharides.²¹ Compounds 1 and 2 were prepared using this strategy; however, the commonly employed thexylidimethyl silyl (TDS) ether for anomeric protection had to be replaced by a benzyl glycoside because the removal of the TDS ether using hydrofluoric acid (HF)-pyridine resulted in a loss of the sulfate group at the amino function (see the SI for synthetic details).

We employed a similar modular approach for the preparation of dermatan sulfate derived saccharides 3 and 4 (Scheme 1).

Scheme 1. Chemical Synthesis of Standards 3 and 4^a



^aReagents and conditions: (a) NIS, TMSOTf, DCM, 0°C. (b) NH₂NH₂·HOAc, toluene/EtOH; 92%. (c) Et₃SiH, TfOH, −78 °C (61%, 9). (d) Et₃SiH, PhBCl₂, −78 °C (81%, 10). (e) Zn–Cu couple, AcOH, r.t. (64%, 11, 72%, 12). (f) Py·SO₃, DMF, (81%, 13, 92%, 14). (g, i) LiOH, H₂O₂, THF. (ii) 4M NaOH, MeOH (93% and 74%). (h) HF·Py, pyridine, r.t. (81%, 15; 75%, 16). (i) Pd(OH)₂/C, H₂, H₂O (78%, 3; 81%, 4).

Thus, glycosylation of thioglycosyl donor 5 with acceptor 6 in the presence of *N*-iodosuccinimide (NIS) and a catalytic amount of triflic acid (TfOH) gave disaccharide 7 as only the β-anomer due to the presence of the participating levulinoyl (Lev) ester (yield 83%). Donor hydrolysis was observed when a glycosyl donor was used having the amine protected as a trichloroethoxy carbamate. The application of an allyl glycoside as acceptor also gave a rather low yield of coupling product.

Disaccharide 7 can be converted into a panel of differently sulfated disaccharide standards. For example, hydrolysis of the benzylidene acetal will give a diol that can be employed for the preparation of a di-*O*-sulfated derivative. On the other hand, regioselective reductive opening of the benzylidene acetal can give a C-4 or C-6 hydroxyl for sulfation. The Lev ester can also be selectively removed to give entry into another range of sulfated compounds. For the purpose of preparing compounds 3 and 4, the Lev ester of 8 was selectively cleaved using hydrazine acetate in a mixture of toluene and methanol to give alcohol 8. Next, the benzylidene acetal of 8 was opened regioselectively using trimethylsilane in the presence of triflic acid or PhBCl₂ to provide compounds 9 and 10, respectively.

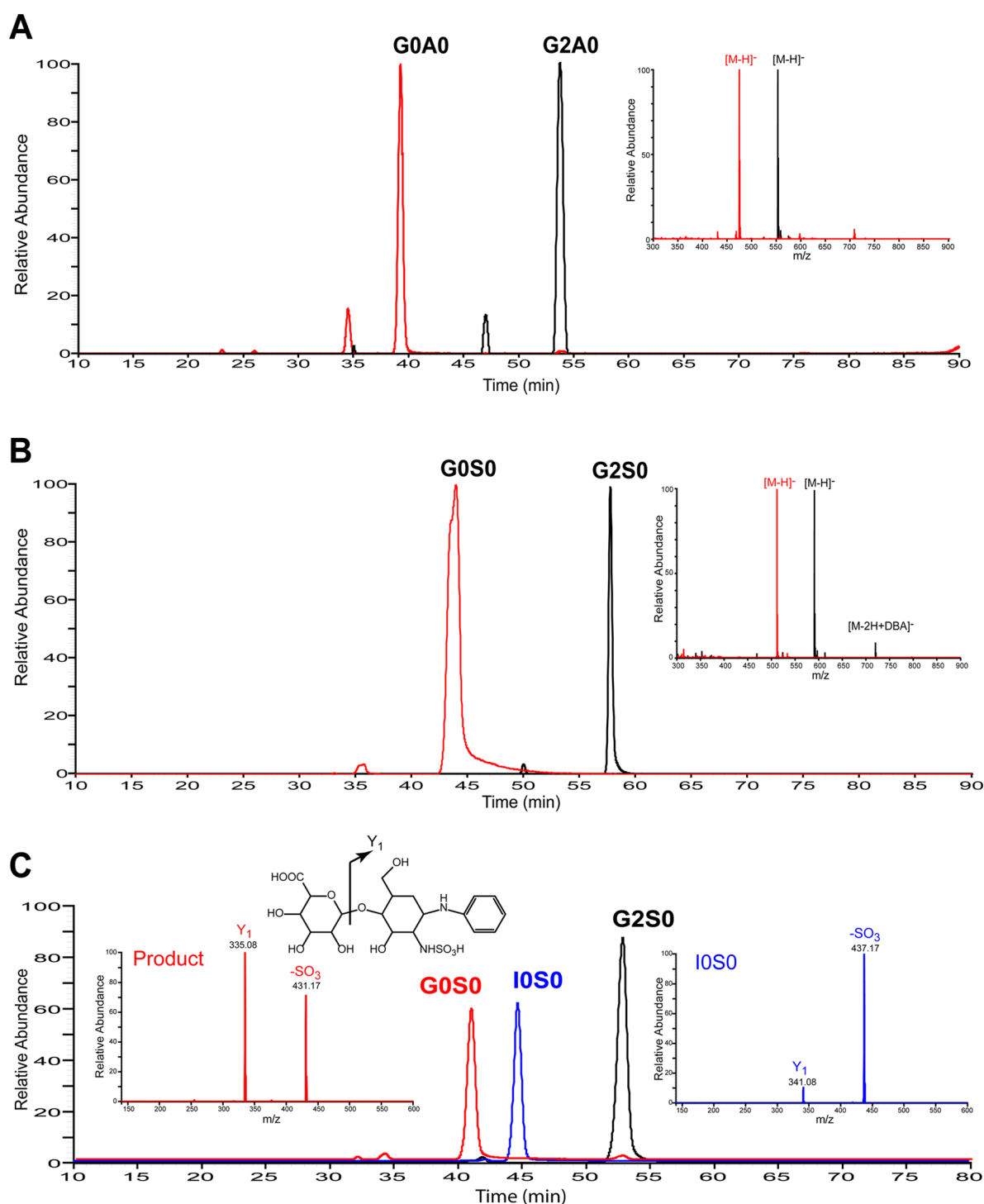


Figure 2. ARSK desulfates G2A0 and G2S0. G2A0 and G2S0 (2 nmol) were treated with ARSK (5 ng) as described in the [Methods](#). Prior to LC/MS analysis, reactions were labeled with [$^{12}\text{C}_6$]aniline. Shown is the extracted ion current for both substrate (black trace) and the corresponding desulfation products (red trace). (A) G2A0 was treated with ARSK. Inset: mass spectrum for unreacted substrate ($m/z = 553$) and the product ($m/z = 473$), consistent with the loss of one sulfate group. (B) G2S0 was treated with ARSK. Inset: mass spectrum for unreacted substrate ($m/z = 591$) and the product ($m/z = 511$), consistent with the loss of one sulfate group. (C) [$^{13}\text{C}_6$]aniline labeled standards G2S0 and I0S0 were added to the [$^{12}\text{C}_6$]aniline labeled product of G2S0 after treatment with ARSK. The product disaccharide and I0S0 are isobaric but elute differently (red trace and blue trace, respectively). Tandem mass spectrometry shows that these isobaric disaccharides both generated the same product ions, but in distinct proportions. The detection of a strong Y₁ ion in both the product and the I0S0 standard verifies that both are N-sulfated.

The reduction of the trichloroacetamido group of **9** and **10** to the corresponding acetamido containing derivatives **11** and **12** proved troublesome, and the conventional use of Bu₃SnH/azobisisobutyronitrile (AIBN) did not proceed to completion,

and a mixture of products was obtained in which varying numbers of chlorides had been reduced. Fortunately, the use of the Zn–Cu²² couple in acetic acid gave the desired compounds **11** and **12** in acceptable yields. The disaccharides were then

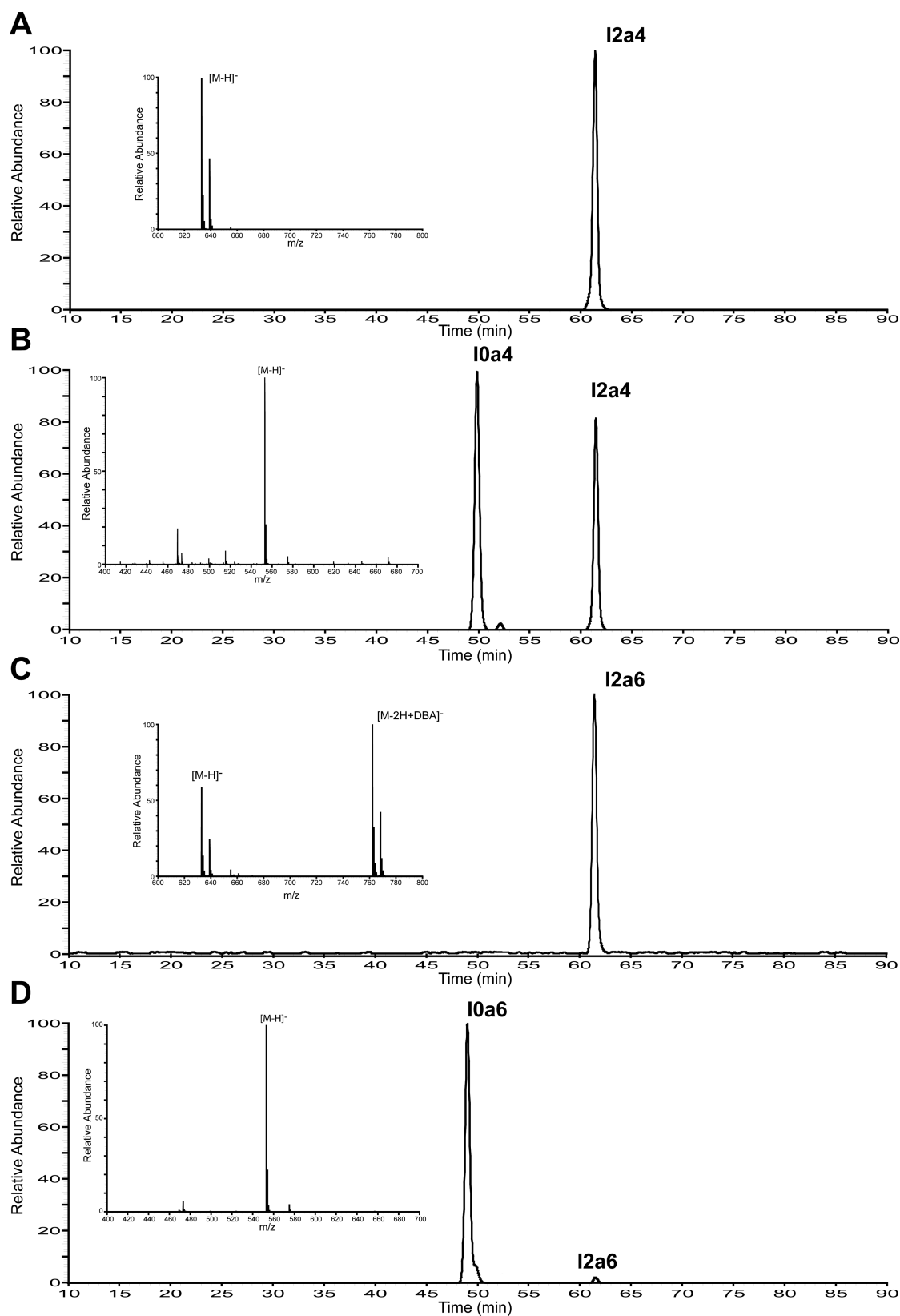


Figure 3. Comparison of I2a4 and I2a6 as substrates for iduronate-2-sulfatase (IDS). For each reaction, 2 nmol of substrate disaccharide was reacted with 50 ng of recombinant human IDS in reaction buffer and incubated for 24 h at 37 °C before aniline labeling and analysis by LC/MS. (A) Elution profile and mass spectrum of I2a4. (B) Product formed from I2a4 by IDS. (C) Elution profile and mass spectrum of I2a6. (D) Product formed from I2a6 by IDS.

sulfated with pyridine sulfonate in dimethylformamide (DMF) to give **13** and **14** in excellent yields, respectively. After the

installation of *O*-sulfates at the desired positions, the methyl ester was hydrolyzed using LiOH/H₂O₂, and the anomeric

TDS of the resulting compounds was cleaved using a HF–pyridine complex in the presence of pyridine to give disaccharides **15** and **16**. Controlling the amount of HF–pyridine was critical as excess reagent resulted in degradation of the compounds. Finally, removal of benzyl ethers was accomplished by hydrogenation in the presence of Pd/C in a mixture of methanol and water to give the target disaccharides **3** and **4** in 78% and 81% yield, respectively. Analysis of the various disaccharides and intermediates is described in the SI.

Recombinant human ARSK was produced in HEK293 cells and purified as described recently.¹⁶ Enzyme (5 ng) was added to 2 nmol of each of the four disaccharides and incubated overnight at 37 °C. The reaction products were lyophilized and then labeled with [¹²C₆]aniline by reductive amination as described.²² All samples were then analyzed by reversed phase ion-pairing liquid chromatography–mass spectrometry. The extracted ion currents were monitored for masses corresponding to those predicted for the unreacted substrates and the expected 2-O-desulfated products. Characteristic elution profiles for G2A0 (*m/z* = 553) and G2S0 (*m/z* = 591) are shown in Figure 2A and B, respectively. Incubation with ARSK resulted in complete conversion of G2A0 and G2S0 to products with masses expected for the products, G0A0 (*m/z* = 473) and G0S0 (*m/z* = 511), respectively. The collision induced dissociation profile of the product of G2S0 treated with ARSK gave a strong signal for a Y1 ion with *m/z* = 335, which demonstrates that the product was 2-O-desulfated (Figure 2C). For comparison, we also analyzed standard I0S0, which separated from G0S0 and fragmented less efficiently (Figure 2C and inset).

Complementary experiments were performed with iduronate containing disaccharides I2a4 and I2a6 as substrates. Incubation with IDS resulted in desulfation of these substrates to the expected 2-O-desulfated products (Figure 3). Interestingly, under these conditions, IDS converted both I2a6 and I2a4 to I0a6 and I0a4, respectively, albeit with different overall yields (compare Figure 3B and D). ARSK had no effect on iduronate containing substrates, I2a4 and I2a6 (Supporting Information Figure S1A).

Finally, we examined ARSK-C/A in which the active site cysteine at residue 80 was converted to alanine and thus cannot be converted into the active form containing a formylglycine residue by FGE, resulting in inactivation of enzyme activity toward an arylsulfate substrate.¹⁶ As expected, ARSK-C/A had no measurable activity toward G2S0 compared to wildtype enzyme (Supporting Information Figure S1B). Similarly, IDS and arylsulfatase G (ARSG), the most recently characterized heparan sulfate degrading arylsulfatase,¹⁹ did not react with G2S0 (Supporting Information Figure S1B).

Thirty years ago, Shaklee *et al.* demonstrated that lysosomal enzyme preparations from human fibroblasts derived from Hunter patients lacking IDS catalyzed the removal of the 2-O-sulfate groups from 2-sulfoglucuronates in disaccharides derived from heparan sulfate and dermatan sulfate.²³ The measured glucuronate-2-sulfatase activity exhibited optimal activity at pH 4, suggesting a lysosomal origin, and the enzyme was eventually purified to homogeneity.^{24,25} More recently, Wiegmann *et al.* demonstrated that ARSK localizes to lysosomes, contains mannose 6-phosphate, and has an acidic pH optimum towards artificial sulfatase substrates, indicating a crucial role in lysosomal degradation of sulfated substrates.¹⁶ The data reported here provide evidence that ARSK is the glucuronate-2-O-sulfatase. ARSK likely acts not only on heparan sulfate but

also on chondroitin/dermatan sulfate that contains 2-sulfoglucuronate based on the work by Shaklee *et al.*^{23,26} We propose to rename ARSK as glucuronate-2-sulfatase (GDS) by analogy to IDS for iduronate-2-sulfatase. The identification of ARSK/GDS completes the identification of all the genes for this group of hydrolytic enzymes required for degradation of heparan sulfate and dermatan sulfate, as is shown in Figure 4 for the catabolism of heparan sulfate.

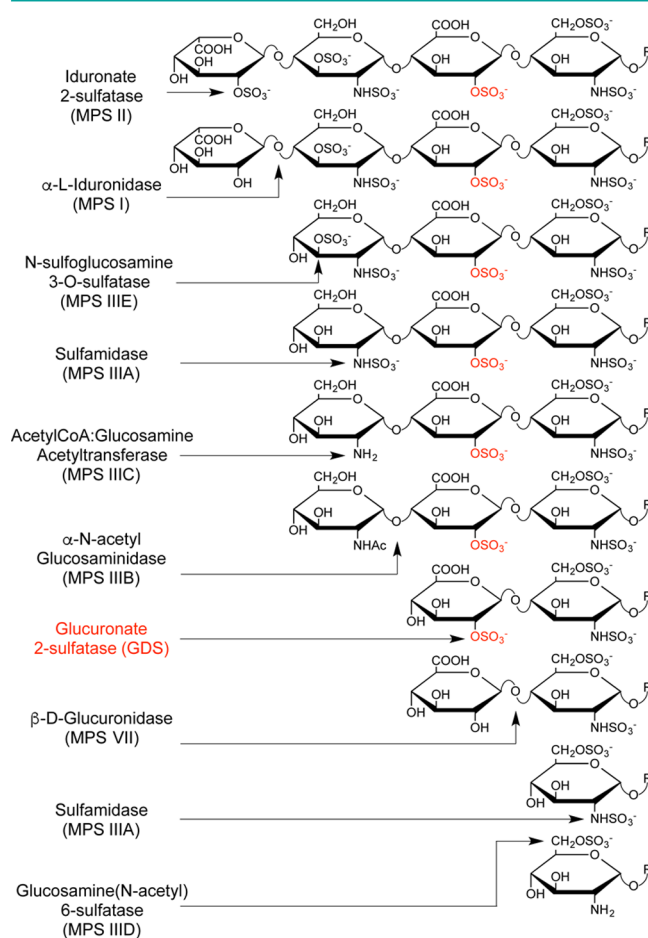


Figure 4. Pathway of lysosomal heparan sulfate degradation. GDS catalyzes the desulfation of 2-sulfoglucuronate residues. All of the enzymes required to degrade sulfated GAGs are now known.

Deficiencies of sulfatases involved in GAG degradation result in lysosomal storage of undegraded substrates. Thus, we predict that a deficiency of GDS should result in accumulation of GAGs characterized by a terminal 2-sulfoglucuronate residue at the nonreducing end. We recently reported a facile method for typing MPS disorders based on release of the nonreducing end biomarkers characteristic of each disorder.²⁰ We therefore predict that some undiagnosed cases of MPS may result from GDS deficiency and should manifest the NRE biomarkers containing 2-sulfoglucuronate, such as G2S0 or G2A0 after enzymatic depolymerization of lysosomal GAGs. The standards will allow further characterization of this enzyme in cells and tissues and make possible the analysis of enzyme activity in animals and perhaps humans lacking this enzyme. ARSK deficiency may be the genetic cause in mucopolysaccharidosis patients of unknown etiology, for which diagnosis now is possible with the help of the new disaccharide standards using the GRIL-LC/MS method and recombinant ARSK enzyme for

in vitro digestion of patient material. In order to unravel the physiological relevance of ARSK in mammals, we are currently establishing an *Arsk/Gds*-deficient mouse model which might also help to identify human patients as we expect a MPS-like phenotype in these animals.

METHODS

Enzyme Assays. ARSK or ARSK-C/A (5 ng), 30 ng of ARSG, and 50 ng of IDS (kind gift from Shire Human Genetic Therapies, Inc.) were diluted into 55 μ L of 200 mM NaAc (pH 4.6) and incubated with 2 nmol of synthetic disaccharide for 24 h at 37 °C (total volume of 60 μ L).¹⁶ Samples were lyophilized and subjected to GRIL-LC/MS analysis. Representative data are shown for each experiment, which was repeated at least three times.

GRIL-LC/MS Analysis. Samples were analyzed by LC/MS and quantified using the GRIL-LC/MS method using an LTQ Orbitrap Discovery electrospray ionization mass spectrometer (Thermo Scientific) equipped with an Ultimate 3000 quaternary HPLC pump (Dionex) and a C-18 reverse phase 1 mm i.d., 150 mm, 5 μ m microbore column as previously described.²² Dibutylamine (DBA) was used as an ion pairing reagent, and disaccharides were separated with an increasing methanol step gradient for LC/MS. All enzymatic reactions were dried and subjected to reductive amination labeling with [¹²C₆]aniline prior to the addition of standards and subsequent LC/MS analysis. For quantitative GRIL-LC/MS, 10–20 pmol of differentially isotope labeled standards ([¹³C₆]aniline tagged) were added prior to LC/MS analysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b01033.

Synthesis of substrates (PDF)

Supporting spectra (PDF)

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Notes

The authors declare no competing financial interest.

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