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Elucidation of the Mechanism of Polysaccharide Cleavage by Chondroitin AC Lyase from *Flavobacterium heparinum*

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Abstract: Chondroitin AC lyase from *Flavobacterium heparinum* degrades chondroitin sulfate glycosaminoglycans via an elimination mechanism resulting in disaccharides or oligosaccharides with Δ4,5-unsaturated uronic acid residues at their nonreducing end. Mechanistic details concerning the ordering of the bond-breaking and -forming steps of this enzymatic reaction are nonexistent, mainly due to the inhomogeneous nature of the polymeric substrates. The creation of a new class of synthetic substrates for this enzyme has allowed the measurement of defined and reproducible k_{cat} and K_m values and has expanded the range of mechanistic studies that can be performed. The primary deuterium kinetic isotope effect upon k_{cat}/K_m for the abstraction of the proton α to the carboxylic acid was measured to be 1.67 ± 0.07, showing that deprotonation occurs in a rate-limiting step. Using substrates with leaving groups of differing reactivity, a flat linear free energy relationship was produced, indicating that the C4−O4 bond is not broken in a rate-determining step. Taken together, these results strongly suggest a stepwise mechanism. Consistent with this was the measurement of a secondary deuterium kinetic isotope effect upon k_{cat}/K_m of 1.01 ± 0.03 on a 4-{^{2}H}-substrate, indicating that no sp^2 character is developed at C4 during the rate-limiting step, thereby ruling out a concerted syn-elimination.

Introduction

Glycosaminoglycans (GAGs) are highly sulfated, unbranched polysaccharide chains built of a sequence of repeating disaccharide units consisting of hexosamine and uronic acid residues. These markedly heterogeneous polymers play a variety of important roles intracellularly (usually in secretory granules), at the cell surface, or, more commonly, in the extracellular matrix. These biological roles include simple mechanical support, the lubrication and cushioning of joints, the modulation of cell signals, cell adhesion, motility and proliferation, and acting as reservoirs for the specific binding of proteins in order to regulate or stabilize their activity.[1,2] There are four main classes of GAGs: chondroitin sulfate and dermatan sulfate; hyaluronic acid; heparin and heparan sulfate; and keratan sulfate. With the exception of hyaluronic acid, all GAGs are linked covalently to a protein core, forming a proteoglycan. Of these proteoglycans, chondroitin sulfates are the most common type of GAG chain, consisting of three major classes: chondroitin sulfate A (chondroitin 4-sulfate), chondroitin sulfate B (dermatan sulfate), and chondroitin sulfate C (chondroitin 6-sulfate). The chondroitin sulfates consist of an *N*-acetyl-D-galactosamine residue (usually O-sulfated at C4 or C6) attached through a β-(1,4) linkage to D-glucuronic acid or L-iduronic acid (dermatan sulfate) that is in turn attached via a β-(1,3) linkage (α-(1,3) for dermatan sulfate) to the next hexosamine residue.

GAG degradation is effected via two classes of enzymes: (i) polysaccharide hydrolases (eukaryotic) and (ii) lyases (prokaryotic). The hydrolases may be either inverting or retaining glycosidases, whereas the lyases act via an elimination mechanism, resulting in disaccharides or oligosaccharides with Δ4,5-unsaturated uronic acid residues at their nonreducing end (Figure 1). Although the substrate specificity and product compositions for a number of lyases have been characterized,[1,3−7] little is known about the lyase mechanism at the molecular level. X-ray crystal structures have been solved for several different types of polysaccharide lyases including chondroitin AC lyase,[8] chondroitin B lyase,[9] hyaluronate lyase,[10] alginate lyase,[11] and pectate lyase.[12] Other representative structures of these lyases can be found on the Web at http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html. Putative catalytic residues of these polysaccharide lyases have been suggested by site-directed mutagenesis studies, often in combination with X-ray structures of various enzyme−oligosaccharide complexes.[9,10,13−19] A wide variety of

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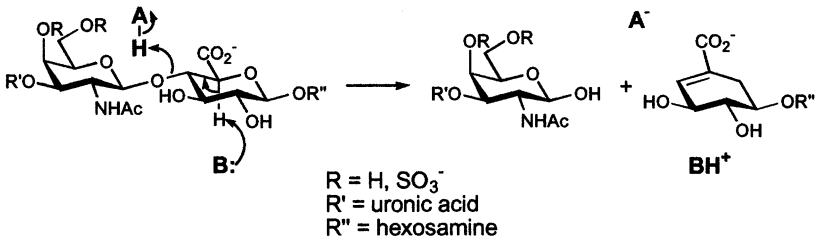


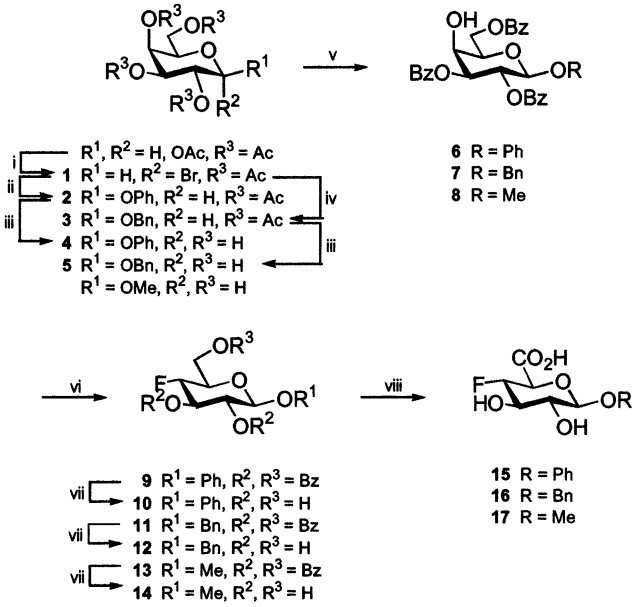
Figure 1. Proposed elimination mechanism of polysaccharide lyases.

general-base residues responsible for the abstraction of the relatively acidic H5 proton of the uronic acid moiety have been implicated from these studies including histidine, tyrosine, glutamate, and cysteine. General-acid catalysis also plays a major role in the lyase mechanism by neutralizing the negative charge on the carboxylate as well as the developing charge of the enolic intermediate and also by assisting the departing leaving group saccharide moiety. Divalent metal ions have often been found to play a part in the first two roles; however, not all polysaccharide lyases (including chondroitin AC lyase) have been shown to possess this cationic metal center at the active site. Consequently, a variety of amino acids have also been implicated in these roles, such as lysine, arginine, histidine, tyrosine, and aspartic acid.

Aside from these structural details and implications of important catalytic residues, no work has been published on deciphering the chemical mechanism of the polysaccharide lyase reaction. The inhomogeneous nature of the polymeric substrates acted upon by these enzymes is a major reason for the lack of mechanistic details. The introduction of defined chromogenic substrates for glycosidases not only considerably simplified the assay of these enzymes but also permitted a variety of mechanistic studies via the accurate measurement of kinetic isotope effects and linear free energy relationships produced by measuring the rates of hydrolysis of a series of substrates of differing reactivity. Through the design of defined substrates for chondroitin AC lyase, the equivalent analyses of this polysaccharide lyase are now possible.

Chondroitin AC lyase from the Gram-negative soil bacterium *Flavobacterium heparinum* (EC 4.2.2.5) cleaves GAG chains in a random endolytic fashion. It cleaves a variety of GAGs, including chondroitin sulfates A (chondroitin 4-sulfate) and C (chondroitin 6-sulfate), as well as the unsulfated chondroitin, and hyaluronic acid. The proposed mechanism, suggested by Gacesa,[20] is composed of three steps: (i) neutralization of the negative charge on the carboxylate anion of the glucuronic acid moiety, either by a divalent metal ion or a positively charged amino acid; (ii) general-base-catalyzed abstraction of the C5 proton and finally; (iii) the β-elimination of the 4-O-glycosidic bond. The means of lowering the pK_a of the protons adjacent to acid moieties (pK_a = 22) so that they can be abstracted by an active site base (p$K_a \sim 7$) is a matter of some contention.[21−26] However, the intent of this report on chondroitin AC lyase is not to determine the specific mode of stabilization of the proposed enolic intermediate but to decipher the order and importance to catalysis of the bond breaking and forming steps.

Scheme 1[a]



[a] Conditions: (i) HBr/AcOH, CH_2Cl_2, 0 °C → room temperature. (ii) Phenol, TBAHS, 1 M NaOH, CH_2Cl_2. (iii) NaOMe, MeOH. (iv) Benzyl alcohol, Ag_2CO_3, I_2, CH_2Cl_2. (v) Benzoyl chloride, pyridine, −40 °C → room temperature. (vi) Methyl DAST, CH_2Cl_2, −30 °C → room temperature. (vii) NaOMe, MeOH. (viii) For **17** and **16**: TEMPO, NaOCl, NaBr, TBAB, $NaHCO_3$, EtOAc/H_2O, 0 °C. For **15**: TEMPO, *t*-BuOCl, H_2O.

Results

I. Synthesis of the Fluorinated Substrates. *Phenyl 4-Deoxy-4-fluoro-β-D-glucopyranosiduronic Acid (**15**), Benzyl 4-Deoxy-4-fluoro-β-D-glucopyranosiduronic Acid (**16**), and Methyl 4-Deoxy-4-fluoro-β-D-glucopyranosiduronic Acid (**17**) (Scheme 1).* Three 4-fluoro monosaccharide substrates were prepared, differing only in their anomeric substituent. Phenyl β-D-galactopyranoside (**4**) was prepared in a fashion similar to that reported in the literature.[27] Briefly, this consisted of making the α-bromide **1**[27] from per-O-acetylated galactose, followed by a modified Königs−Knorr reaction in a biphasic system of

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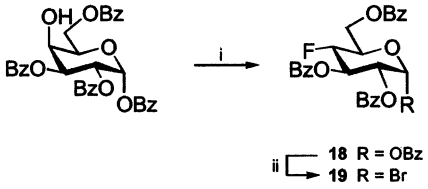
Scheme 2[a]



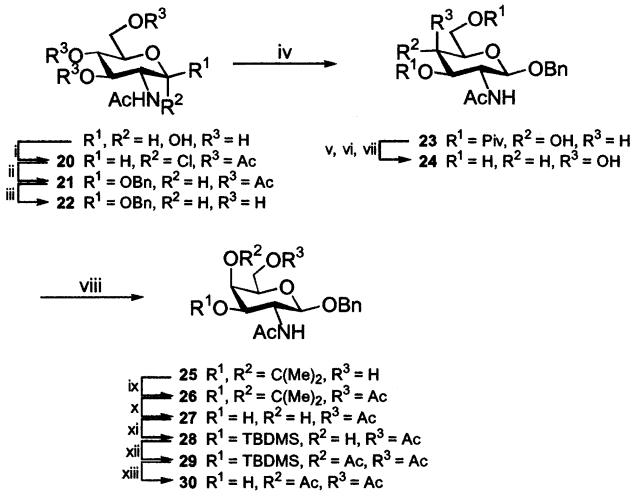
[a] Conditions: (i) Methyl DAST, CH_2Cl_2, −30 °C → room temperature. (ii) HBr/AcOH, CH_2Cl_2, 0 °C → room temperature.

CH_2Cl_2 and 1 M NaOH with tetrabutylammonium hydrogensulfate (TBAHS) as a phase-transfer catalyst to introduce the phenyl aglycon **2**, and finally a Zemplén deprotection of the acetates (39% from per-O-acetylated galactose). Benzyl β-D-galactopyranoside (**5**) was synthesized in a similar sequence but utilized benzyl alcohol and Ag_2CO_3 in the Königs−Knorr reaction (79% from per-O-acetylated galactose). The methyl β-D-galactopyranoside was purchased from a commercial source. Selective benzoylation[28] of the galactosides at −40 °C afforded **6**[29] (28%), **7**[30] (40%), and **8**[28] (52%), as white solids. Over-benzoylated products could be deprotected and rebenzoylated to give additional material. The 4-fluoro substituent was then easily introduced with methyl DAST in CH_2Cl_2 to give **9**, **11**, and **13**,[31]in yields of 83%, 60%, and 54%, respectively. After Zemplén deprotection of the benzoates, the primary hydroxyl was selectively oxidized to the carboxylic acid using 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO).[32] The oxidation of the benzyl and methyl glucosides proceeded smoothly using NaOCl as the primary oxidant, giving **16** (93%) and **17** (74%) as white solids. However, under the same conditions, the phenyl glycoside (**10**) underwent an undesired side reaction, resulting in the para chlorination of the aromatic ring, and giving an inseparable mixture of products. This was overcome by using *tert*-butyl hypochlorite (*t*-BuOCl)[33,34] as the primary oxidant, to give **15** as a white solid in a yield of 40%.

*Benzyl O-(4-Deoxy-4-fluoro-β-D-glucopyranosiduronyl)−(1→4)-2-acetamido-2-deoxy-β-D-galactopyranoside (**34**) (Schemes 2−4).* 1,2,3,6-Tetra-*O*-benzoyl-α-D-galactopyranose was synthesized according to the literature procedure.[35,36] The 4-fluoro substituent was then easily introduced with methyl DAST in CH_2Cl_2 to give **18** as a white solid (63%), followed by treatment with HBr in acetic acid to give the α-bromide donor **19** as a white solid in a yield of 72% (Scheme 2).

The obvious protection strategy to provide an acceptor *N*-acetylhexosamine sugar with the 3-hydroxyl free is through the one-step benzylidenation of C4 and C6. However, the glycosylation of this compound with the donor **19** proved to be problematic due to the low solubility of the acceptor and the resulting cleavage of the *p*-methoxybenzylidene ring under the acidic coupling conditions (AgOTf), affording several products, including trisaccharides. An alternative protection strategy was

Scheme 3[a]



[a] Conditions: (i) Acetyl chloride. (ii) Benzyl alcohol, Ag_2CO_3, I_2, CH_2Cl_2. (iii) NaOMe, MeOH. (iv) Pivaloyl chloride, pyridine, CH_2Cl_2, 0 °C. (v) Tf_2O, pyridine, −15 °C. (vi) H_2O, 90 °C. (vii) NaOMe, MeOH. (viii) 2,2-Dimethoxypropane, *p*-TsOH, then acetic acid, MeOH:H_2O (10:1), 45 °C. (ix) Ac_2O, pyridine. (x) Acetic acid:H_2O (4:1), 50 °C. (xi) *tert*-Butyldimethylsilyl chloride, imidazole, DMF, 80 °C. (xii) Ac_2O, pyridine, 4-(dimethylamino)pyridine. (xiii) TBAF, THF.

employed (Scheme 3) that involved many more steps. However, a lot of the steps were high yielding and the products easily crystallized, thus eliminating the need for extensive chromatography. Due to the enormous cost difference between glucosamine and galactosamine sugars, the synthesis of the acceptor molecule started with the less expensive gluco epimer and involved a conversion to the desired galacto epimer as part of the synthesis. 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**20**) was synthesized according to the literature procedure[37] and then reacted with benzyl alcohol and Ag_2CO_3 in a Königs−Knorr[38] reaction to produce **21**,[39] followed by a Zemplén deprotection with sodium methoxide, affording **22**[39] as a white solid. The conversion of the glucopyranoside derivative **22** into the galactopyranoside derivative **24** proceeded smoothly as described in the literature.[40] This involved the selective protection of the 3- and 6-hydroxyls as the pivaloyl esters to give **23**.[40] A triflate was then introduced at the remaining 4-hydroxyl group, which was subsequently displaced by water at 90 °C to give the product of inverted stereochemistry, followed by the deprotection of the pivaloyl esters with sodium methoxide in methanol to afford the desired benzyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**24**)[40] as a white solid. The protection of the 3- and 4-hydroxyls with an isopropylidene group to give **25** proceeded smoothly as described in the literature,[40] followed by the protection of the remaining 6-hydroxyl with an acetate to give **26** in an overall 60% yield from **20** (eight steps). Removal of the isopropylidene moiety with aqueous acetic acid at 50 °C (80%) followed by the selective protection of the more reactive equatorial hydroxyl with *tert*-butyldimethylsilyl chloride and imidazole in DMF at 80 °C gave crystalline **28** (79%). Acetylation of the remaining

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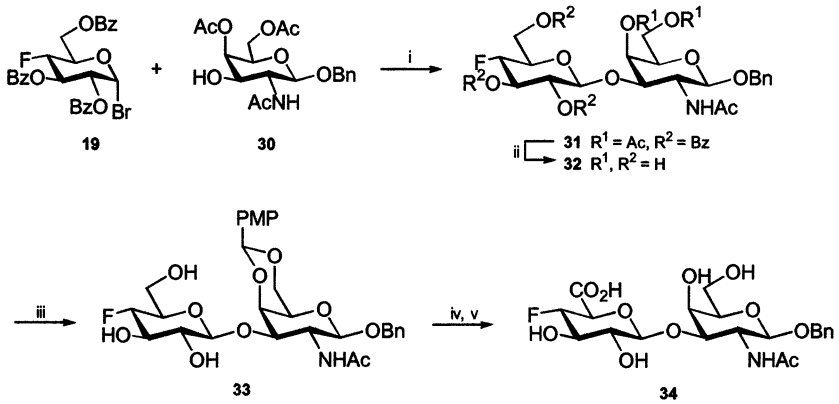
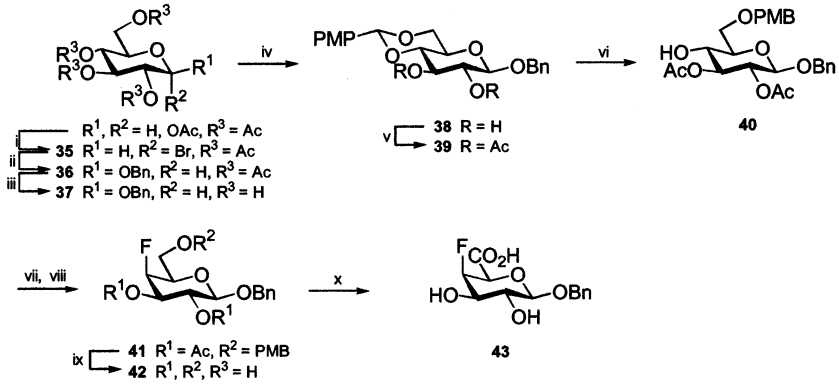
Scheme 4[a]



[a] Conditions: (i) AgOTf, CH_2Cl_2. (ii) NaOMe, MeOH. (iii) *p*-Anisaldehyde dimethyl acetal, *p*-TsOH, DMF, 50 °C. (iv) TEMPO, NaOCl, NaBr, TBAB, $NaHCO_3$, EtOAc/H_2O, 0 °C. (v) Acetic acid, MeOH.

Scheme 5[a]



[a] Conditions: (i) HBr/AcOH, CH_2Cl_2, 0 °C → room temperature. (ii) Benzyl alcohol, Ag_2CO_3, I_2, CH_2Cl_2. (iii) NaOMe, MeOH. (iv) *p*-anisaldehyde dimethyl acetal, camphorsulfonic acid, $CHCl_3$, reflux. (v) Ac_2O, pyridine. (vi) $NaCNBH_3$, TFA, THF/CH_2Cl_2. (vii) Tf_2O, pyridine, −15 °C → room temperature. (viii) TBAF, THF. (ix) Acetyl chloride, MeOH, 4 °C. (x) TEMPO, NaOCl, NaBr, TBAB, $NaHCO_3$, EtOAc/H_2O, 0 °C.

4-hydroxyl group required a catalytic amount of 4-(dimethylamino)pyridine (DMAP) in the reaction mixture to afford **29** as fine white crystals in a yield of 95% The silyl protecting group was removed with tetrabutylammonium fluoride (TBAF) in THF to give the acceptor **30** as a white crystalline solid in a moderate 49% yield. The yield of this final step was low due to the partial migration of the C4 acetate to C3.

Glycosylation of the donor **19** and the acceptor **30** was effected with silver triflate in CH_2Cl_2 to give the disaccharide **31** as a white crystalline solid in a 32% yield (Scheme 4). After a global Zemplén deprotection to give **32** (84%), a *p*-methoxybenzylidene group was installed using *p*-anisaldehyde dimethyl acetal and *p*-toluenesulfonic acid in DMF at 50 °C, affording **33** as a white crystalline solid (88%). The oxidation of the remaining primary hydroxyl group was accomplished with TEMPO and NaOCl under phase-transfer conditions with TBAB in $NaHCO_{3(aq)}$ and EtOAc.[32] Under the reaction and workup conditions employed, the cleavage of the *p*-methoxybenzylidene protecting group also occurred to some extent. The crude material was then dissolved in methanol with a drop of acetic acid to complete the deprotection, affording the desired disaccharide substrate **34** as a white solid in a modest 25% yield.

*Benzyl 4-Deoxy-4-fluoro-β-D-galactopyranosiduronic Acid (**43**) (Scheme 5).* Benzyl β-D-glucopyranoside (**37**) was synthesized from peracetylated glucose in a yield of 67% by first making the α-bromide **35** with HBr/AcOH, followed by glycosylation using a modified Königs−Knorr[38] procedure with benzyl alcohol and Ag_2CO_3 to give **36**, and finally a Zemplén deprotection with sodium methoxide.[41] A benzylidene protecting group was installed across C4 and C6 using *p*-anisaldehyde dimethyl acetal and a catalytic amount of camphorsulfonic acid in refluxing $CHCl_3$,[42] followed by the protection of the remaining hydroxyls with acetic anhydride and pyridine to give **39** as a white solid (57%). The benzylidene protecting group was selectively opened to the C6 position using sodium cyanoborohydride and trifluoroacetic acid (TFA) to give **40** as a colorless syrup (91%). The DAST reagent was unsuccessful at introducing an axial fluorine at C4 in order to produce the 4-fluorogalactopyranoside series of sugars. Consequently, a C4-triflate was displaced with TBAF starting from the suitably protected glucopyranoside **40** to give the desired 4-fluorogalactopyranoside, **41**, as a syrup (81% from **40**). After a facile global deprotection with acetyl chloride in methanol, the primary hydroxyl group was selectively oxidized with TEMPO and NaOCl to give **43** as a white solid (30% from **41**).

*Phenyl 4-Deoxy-4,4-difluoro-β-D-xylo-hexopyranosiduronic Acid (**49**) (Scheme 6).* Commercially available phenyl β-D-glucopyranoside was reacted with *p*-anisaldehyde dimethyl acetal and *p*-toluenesulfonic acid in DMF at 50 °C, followed by the protection of the remaining hydroxyls as benzyl ethers

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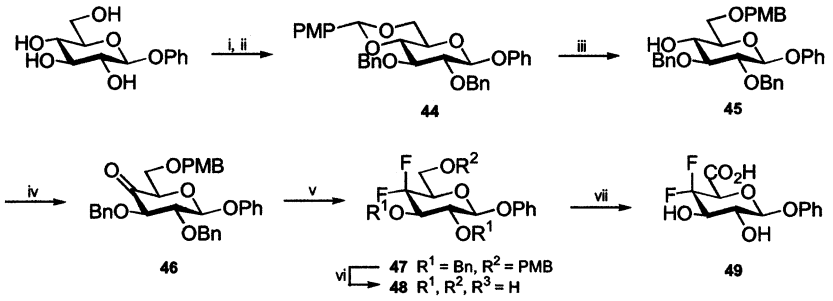
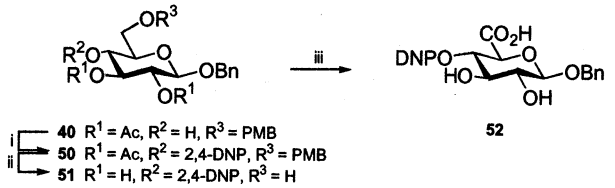
Scheme 6[a]



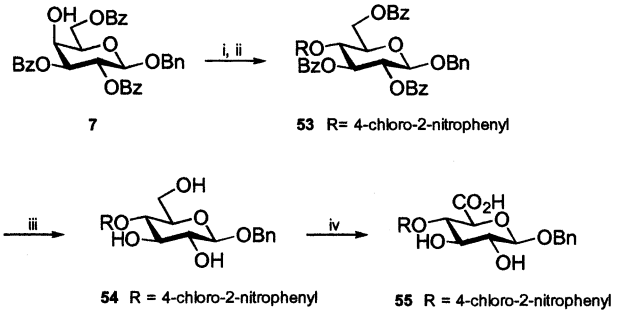
[a] Conditions: (i) *p*-Anisaldehyde dimethyl acetal, *p*-TsOH, DMF, 50 °C. (ii) NaH, benzyl bromide, DMF. (iii) $NaCNBH_3$, TFA, THF/CH_2Cl_2. (iv) Ac_2O, DMSO. (v) Methyl DAST, CH_2Cl_2, −30 °C → room temperature. (vi) H_2, Pd−C, EtOAc/EtOH, followed by CAN, CH_3CN/H_2O. (vii) TEMPO, *t*-BuOCl, H_2O.

Scheme 7[a]



[a] Conditions: (i) 2,4-Dinitrofluorobenzene, DABCO, DMF. (ii) acetyl chloride, MeOH, 4 °C. (iii) TEMPO, NaOCl, NaBr, TBAB, $NaHCO_3$, $EtOAc/H_2O$, 0 °C.

Scheme 8[a]



[a] Conditions: (i) Tf_2O, pyridine, −15 °C → room temperature. (ii) 4-Chloro-2-nitrophenol (K^+ salt), DMF, 80 °C. (iii) NaOMe, MeOH. (iv) TEMPO, NaOCl, NaBr, TBAB, $NaHCO_3$, $EtOAc/H_2O$, 0 °C.

using sodium hydride and benzyl bromide in DMF, to afford **44** as a white solid (39% isolated). After selectively opening the *p*-methoxybenzylidene ring to leave the 4-OH free, the oxidation of this hydroxyl with DMSO and acetic anhydride[43] gave the 4-keto compound **46** as a crystalline solid (75%). The introduction of two fluorines at C4 was accomplished by reacting the desired 4-keto compound **46** with DAST to give **47** in an 88% yield. The benzyl ethers were then removed by hydrogenation over Pd−C, and the removal of the *p*-methoxybenzyl ether was completed by a short treatment with ceric ammonium nitrate (CAN) to yield **48** (63%). As with the other substrates, the final step was the selective oxidation of the remaining primary hydroxyl with TEMPO and *t*-BuOCl to give **49** as a white solid in a moderate 27% yield.

II. Synthesis of the Chromogenic Substrates. *Benzyl 4-O-(2′,4′-Dinitrophenyl)-β-D-glucopyranosiduronic Acid (**52**) (Scheme 7).* The installation of the dinitrophenol moiety at C4 was accomplished via a nucleophilic aromatic substitution reaction with 2,4-dinitrofluorobenzene and DABCO in DMF to afford **50** as a pale yellow foam (80%). After deprotection of the acetates and the *p*-methoxybenzyl ether using acetyl chloride in methanol (89%), the primary hydroxyl group was selectively oxidized using TEMPO and NaOCl under phase-transfer conditions with tetrabutylammonium bromide (TBAB) in $NaHCO_{3(aq)}$ and EtOAc affording **52** as a white solid (52%).[32]

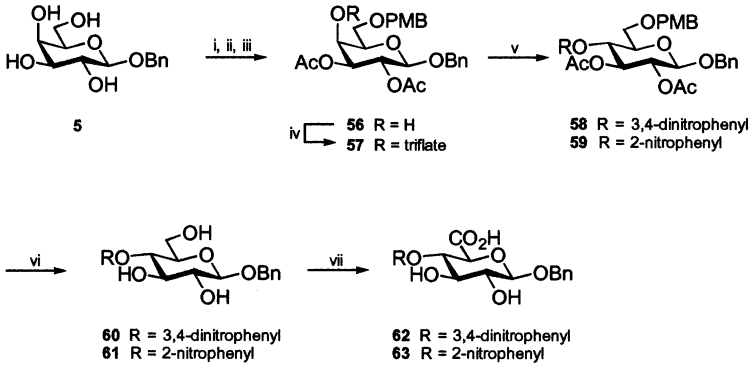
*Benzyl 4-O-(4′-Chloro-2′-nitrophenyl)-β-D-glucopyranosiduronic Acid (**55**) (Scheme 8), Benzyl 4-O-(3′,4′-Dinitrophenyl)-β-D-glucopyranosiduronic Acid (**62**), and Benzyl 4-O-(2′-Nitrophenyl)-β-D-glucopyranosiduronic Acid (**63**) (Scheme 9).* Coupling of the aryl moiety to the 4-position through a nucleophilic aromatic substitution reaction proved impractical for less electron deficient reagents than 2,4-dinitrofluorobenzene. These other phenol moieties were therefore introduced via the S_N2 displacement of a C4-triflate (prepared with triflic anhydride and pyridine) using the potassium salt of the desired phenol to give **53**, **58**, and **59** in yields of 37%, 38%, and 37%, respectively. This required an appropriately protected galactoside, and for comparison purposes, two protecting group strategies were used in the synthesis of these chromogenic substrates. Selective benzoylation of benzyl β-D-galactopyranoside (**5**)[44] provided the appropriately protected intermediate **7**[30] in one step in a moderate yield of 40%. Overbenzoylated products could be easily deprotected and subjected to further benzoylation to yield additional product. The alternative protection strategy included the installation of a *p*-methoxybenzylidene group, acetylation of the remaining hydroxyls, and the selective opening of the benzylidene to afford the C6 *p*-methoxybenzyl ether using $NaCNBH_3$ and TFA to give **56** in an overall yield of 43% (Scheme 9). Chromatography was only necessary after the final ring-opening step. Zemplén deprotection of the benzoates of **53** with NaOMe proved problematic, with the phenol moiety migrating to the C3 position, leaving the desired compound **54** in a yield of only 53%. A higher yield could have been achieved using the acidic global deprotection strategy (acetyl chloride in methanol) employed for **58** and **59**, which gave the desired **60** and **61** in yields of 82% and 84%, respectively. The final step for all three compounds was the selective oxidation of the primary hydroxyl with TEMPO and NaOCl under phase-transfer conditions with TBAB in $NaHCO_{3(aq)}$ and EtOAc to give **55**, **62**, and **63** in yields of 78%, 87%, and 53%, respectively.

III. Synthesis of the Fluorogenic Substrate. *Phenyl 4-Methylumbelliferyl-β-D-glucopyranosiduronic Acid (**69**) (Scheme 10).* The preparation of **65** from phenyl galactoside (**4**) proceeded

(43) Albright, J. D.; Goldman, L. *J. Am. Chem. Soc.* **1967**, *89*, 2416−2423.

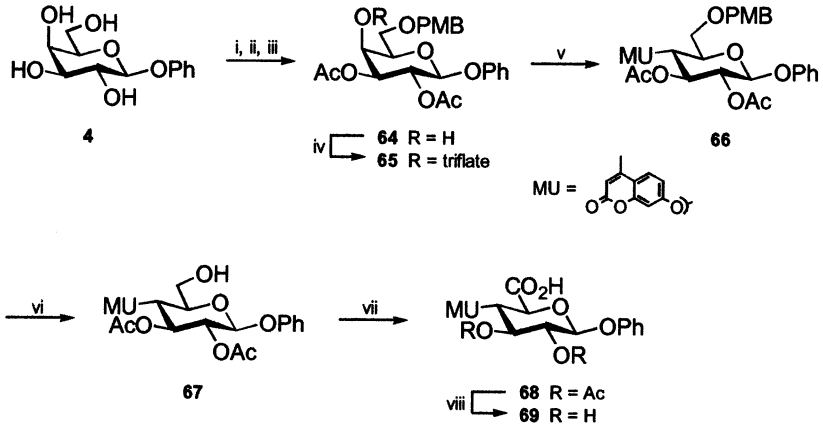
(44) Stoffyn, A.; Stoffyn, P. *J. Org. Chem.* **1967**, *32*, 4001−4006.

Scheme 9[a]



[a] Conditions: (i) *p*-Anisaldehyde dimethyl acetal, *p*-TsOH, DMF, 50 °C. (ii) Ac_2O, pyridine. (iii) $NaCNBH_3$, TFA, THF/CH_2Cl_2. (iv) Tf_2O, pyridine, −15 °C → room temperature. (v) K^+ salt of appropriate phenol, DMF, 80 °C. (vi) Acetyl chloride, MeOH, 4 °C. (vii) TEMPO, NaOCl, NaBr, TBAB, $NaHCO_3$, EtOAc/H_2O, 0 °C.

Scheme 10[a]

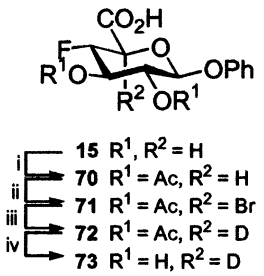


[a] Conditions: (i) *p*-Anisaldehyde dimethyl acetal, *p*-TsOH, DMF, 50 °C. (ii) Ac_2O, pyridine. (iii) $NaCNBH_3$, TFA, THF/CH_2Cl_2. (iv) Tf_2O, pyridine, −15 °C → room temperature. (v) 7-Hydroxy-4-methylcoumarin (K^+ salt), DMF, 80 °C. (vi) CAN, CH_3CN:H_2O (9:1). (vii) CrO_3, H_2SO_4, acetone/H_2O, 35 °C, sonication. (viii) NaOMe, MeOH.

in a manner similar to that described for **57** in the synthesis of the chromogenic substrates **62** and **63**. The C4 triflate **65** was displaced with the potassium salt of 7-hydroxy-4-methylcoumarin (4-methylumbelliferone) in DMF at 80 °C to produce **66** as a white foam (29%). Selective deprotection of the *p*-methoxybenzyl group with CAN gave **67** (87%) followed by a sonicated Jones oxidation[45] with CrO_3 and H_2SO_4 in acetone/H_2O to afford the acid **68** as glassy white solid. The Jones oxidation was used in place of the TEMPO oxidation employed in the other syntheses due to the fact that the methylumbelliferyl group was not compatible with the conditions of the TEMPO oxidation. The facile Zemplén deprotection of the acetates afforded the desired compound, **69**, as a white solid (94%).

IV. Synthesis of the Deuterated Substrates. *Phenyl 4-Deoxy-4-fluoro-5-{*^{2}*H}-β-*D*-glucopyranosiduronic Acid (73) (Scheme 11).* Compound **15** was acetylated with acetic anhydride and sulfuric acid (79%) followed by radical bromination with *N*-bromosuccinimide (NBS) in CCl_4, directed by the adjacent carbonyl, to give the 5-bromo compound, **71**, as a white solid (39%). Deuterium incorporation at C5 proceeded smoothly with tributyltin deuteride in refluxing toluene to give both the D-gluco- and L-ido-configured sugars in roughly equal amounts. ^{1}H NMR analysis confirmed the expected $^4C_1$ chair conformation for the desired D-gluco compound and $^1C_4$ chair for the L-ido compound. Purification difficulties reduced the isolated yield of **72** to 5%, which was then deprotected with NaOMe-d_3 in CD_3OD to give the desired substrate **73** as a white solid (92%).

Scheme 11[a]

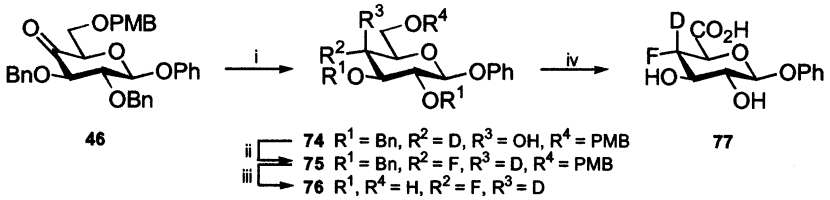


[a] Conditions: (i) Ac_2O, pyridine. (ii) NBS, *hν*, CCl_4. (iii) Tributyltin deuteride, toluene, reflux. (iv) NaOMe-d_3, CD_3OD.

Phenyl 4-Deoxy-4-fluoro-4-{^{2}*H}-β-*D*-glucopyranosiduronic Acid (77) (Scheme 12).* Sodium borodeuteride in methanol was used to reduce the ketone of **46** and introduce the deuterium functionality at C4, giving the galactoside **74** as a white crystalline solid (94%). The stereochemistry at C4 was confirmed by analysis of the ^{1}H NMR signals arising from the protio compound also present as a result of the less than 100% deuterium incorporation. After the incorporation of fluorine at C4 with DAST to give **75**, global deprotection was effected by hydrogenolysis over Pd−C in EtOAc/EtOH. However, the

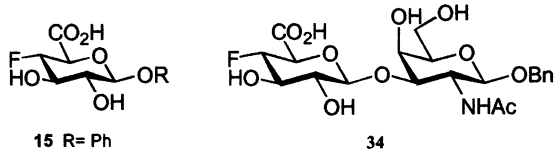
(45) Allanson, N. M.; Liu, D. S.; Chi, F.; Jain, R. K.; Chen, A.; Ghosh, M.; Hong, L. W.; Sofia, M. J. *Tetrahedron Lett.* **1998**, *39*, 1889−1892.

Scheme 12[a]



[a] Conditions: (i) Sodium borodeuteride, MeOH. (ii) Methyl DAST, CH_2Cl_2, −30 °C → room temperature. (iii) H_2, Pd−C, EtOAc/EtOH, followed by CAN, CH_3CN/H_2O. (iv) TEMPO, *t*-BuOCl, H_2O.

Table 1. Kinetic Data for Fluorinated Substrates



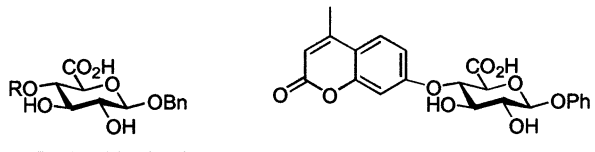
substrate (aglycon)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
15 (Ph)	2.3	114	20
16 (Bn)	0.16	28	5.9
17 (Me)	no saturation observed		0.22
34 (GalNAc)	0.011	12.5	0.85

presence of trace amounts of sulfur from the previous DAST reaction poisoned the hydrogenation catalyst and made the deprotection problematic, despite extensive purification. The *p*-methoxybenzyl ether was removed with CAN in CH_3CN/H_2O to afford **76** as a white solid (58%). The final step was the selective oxidation of the primary hydroxyl with TEMPO and *t*-BuOCl to give the desired **77** as a white solid in a yield of 50%

V. Enzymology: Effect of Varying Substrate Structure on Reactivity. The importance of the anomeric substituent of the glucuronic acid moiety on kinetic parameters was first analyzed using substrates with a fluoride leaving group, since these are the most readily synthesized. The first substrates studied were those with a methyl (**17**), benzyl (**16**), or phenyl (**15**) group at the anomeric center: the kinetic data are summarized in Table 1. The preference for an aromatic substituent is clear from the fact that no saturation was observed for the substrate with the methyl anomeric group. The k_{cat}/K_m for this substrate is 91 and 27 times smaller, respectively, than those of the substrates with a phenyl or benzyl group at the anomeric center. The substrate with a phenyl group at the anomeric center has a significantly larger k_{cat} than did the benzyl-containing substrate; however, its binding is poorer as reflected in its larger K_m value. To investigate the contribution to catalysis of the sugar residue in the +2 binding site, a disaccharide substrate (**34**) was synthesized that incorporated an *N*-acetylgalactosamine moiety at the reducing end. As expected, the binding was improved with this disaccharide substrate, although not as much as had been hoped. Surprising was the fact that the k_{cat} value was reduced significantly from that of the monosaccharide substrates with a phenyl or benzyl group at the anomeric center, resulting in a much poorer substrate as indicated by the lower k_{cat}/K_m value (Table 1).

VI. Chromogenic and Fluorogenic Substrates. Substrates incorporating phenol leaving groups of differing reactivity were synthesized in order to perform a Brønsted analysis of the

Table 2. Kinetic Data for Chromogenic and Fluorogenic Substrates[a]



substrate	phenol pK_a	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
52	3.96	0.019	7.0	2.7
62	5.36	0.019	9.0	2.1
55	6.45	0.035	12.2	2.8
63	7.22	0.027	16.9	1.6
69	7.8	0.0016	1.0	1.5

[a] Note that analyses of **52**, **55**, **62**, and **63** were done at pH 6.8, whereas that for **69** was performed at pH 8.

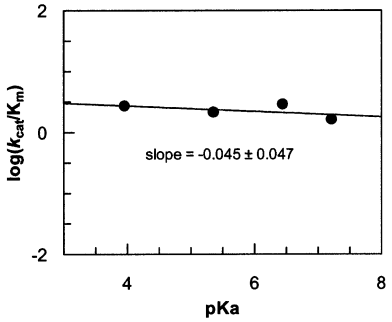


Figure 2. Linear free energy relationship for substrates with phenol leaving groups of varying pK_a values.

enzymatic turnover of these substrates. Four competent substrates, **52**, **55**, **62**, and **63**, with nucleofugalities (leaving-group abilities) defined by their pK_as were synthesized and tested as substrates. The resultant kinetic data are summarized in Table 2 and the linear free energy relationship is presented in Figure 2. The second-order rate constant (k_{cat}/K_m) is independent of phenol pK_a as shown by the flat linear free energy relationship. This strongly suggests that the breaking of the C4−O4 bond does not occur in a rate-limiting step and essentially rules out a concerted syn-elimination.

A synthetic substrate with a fluorescent 4-methylumbelliferyl leaving group (**69**) was synthesized in order to create a more sensitive assay. The overall catalytic efficiency of this fluorogenic substrate as measured by k_{cat}/K_m (1.5 M^{-1} s^{-1}) is similar to that of the chromogenic substrates (Table 2). Indeed, the slightly lower value can be explained by the higher pH used in this assay to facilitate aglycon detection. An increase in pH has been shown not only to decrease k_{cat} but also to increase K_m (vide infra).

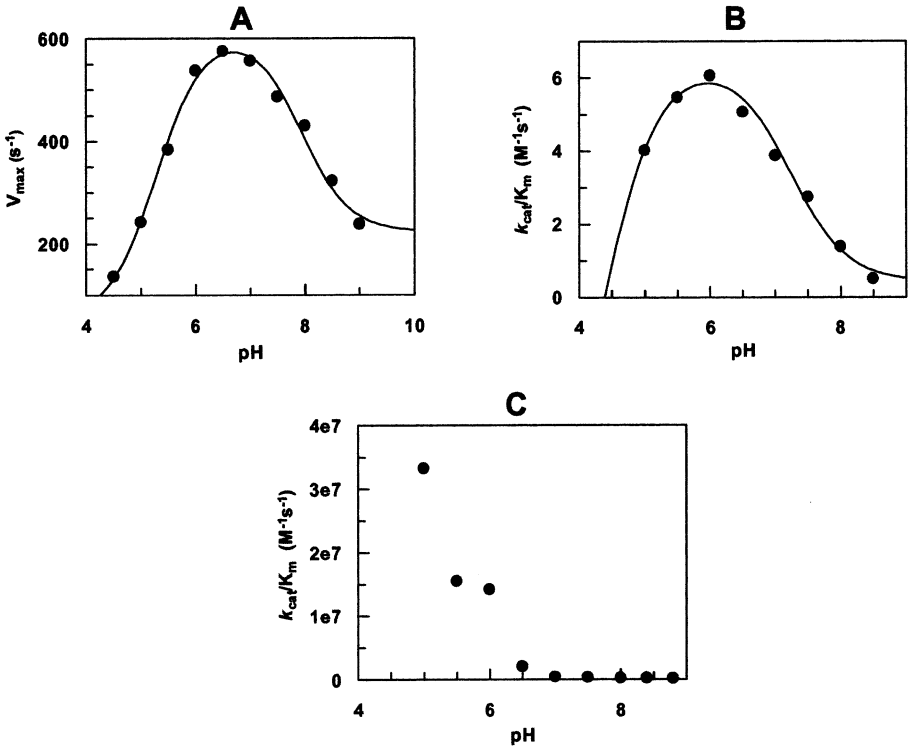


Figure 3. pH activity profiles for chondroitin AC lyase. (A) V_{max} vs pH for chondroitin 6-sulfate. An extinction coefficient of $\epsilon = 3800\ M^{-1}\ cm^{-1}$ for the unsaturated products was used to calculate the rates.[3] (B) k_{cat}/K_m vs pH for the synthetic substrate **16**. (C) k_{cat}/K_m vs pH for chondroitin 6-sulfate.

VII. Inhibitors. Two compounds were synthesized that contain fluorine at C4 in the axial position in the hope that these might serve as improved substrates by virtue of an anti-elimination reaction mechanism (E2). Benzyl 4-deoxy-4-fluoro-β-D-galactopyranosiduronic acid (**43**) was not turned over by the enzyme, yet was found to be a competitive inhibitor with a K_i value of 3 mM. Similarly, the 4,4-difluoro compound **49** showed no activity ($k_{cat} < 1 \times 10^{-5}\ s^{-1}$) and was also a competitive inhibitor, with a K_i value of 24 mM.

VIII. pH Dependence. pH profiles for the reaction catalyzed by chondroitin AC lyase were constructed for the natural substrate (chondroitin 6-sulfate) as well as for one of the synthetic monosaccharide substrates, the phenyl 4-deoxy-4-fluoroglucuronide (**16**). A simple analysis of V_{max} versus pH for chondroitin 6-sulfate revealed the rate to be maximal at pH 6.8 and seemingly dependent on two ionizations of $pK_{a1} = 5.3$, and $pK_{a2} = 7.9$ (Figure 3A). A similar analysis of V_{max} versus pH was not possible with the synthetic substrate due to its high K_m value. Instead, a k_{cat}/K_m versus pH analysis was performed by measurement of initial rates at low substrate concentrations and dividing by enzyme concentration. This analysis showed the second-order rate constant to be maximal at a pH of ~6, slightly below the optimum found for the V_{max} profile for the natural substrate (Figure 3B). The one apparent pK_a value obtainable from this curve is $pK_{a2} = 7.2$. When the k_{cat}/K_m versus pH analysis was performed with chondroitin 6-sulfate, remarkable changes in K_m were observed with changes in pH. The K_m value decreased by a factor of 175 when the pH was changed from 8.8 to 5.0. This resulted in a steadily increasing k_{cat}/K_m value with decreasing pH, as shown in Figure 3C.

IX. Kinetic Isotope Effects. Substrates with deuterium incorporated at C5 (**73**) and C4 (**77**) were synthesized in order to probe the enzyme mechanism via primary and secondary deuterium kinetic isotope effects (KIEs). Using substrate concentrations well below K_m, the primary deuterium KIE on k_{cat}/K_m for abstraction of the proton α to the carbonyl was measured to be 1.67 ± 0.07. The presence of a primary deuterium KIE demonstrates that the C5−H5 bond is broken in a rate-limiting step and also rules out an E1 elimination mechanism. The secondary deuterium KIE on k_{cat}/K_m was measured to be 1.01 ± 0.03. This supports a stepwise mechanism where the bond to the C4 leaving group is not broken in a rate-limiting step and is consistent with the flat linear free energy relationship.

X. Solvent Deuterium Exchange. The ^1H NMR spectrum of remaining 4-fluoro substrate **16** and product after partial conversion by chondroitin AC lyase in D_2O does not reveal any incorporation of deuterium into the C5 position of the substrate (Figure 4). While this result does not prove that deuterium exchange is not occurring within the active site, which may be inaccessible to bulk solvent, this result is however consistent with a mechanism that involves the rapid elimination of the C4 substituent after a rate-limiting proton abstraction ($(E1cb)_{irr}$).

Discussion

Substrate Structure. The use of heterogeneous polymeric substrates places immense limitations on the type of mechanistic studies that can be utilized to elicit the details of an enzymatic reaction. Chondroitin sulfates are commercially available; however, the sulfation patterns are not consistent throughout the polymer, and the molecular weight is not accurately known and most likely varies between individual chains. Further, the structures of these substrates cannot easily be manipulated to allow the use of mechanistic probes such as kinetic isotope effects. The synthetic substrates described herein have allowed

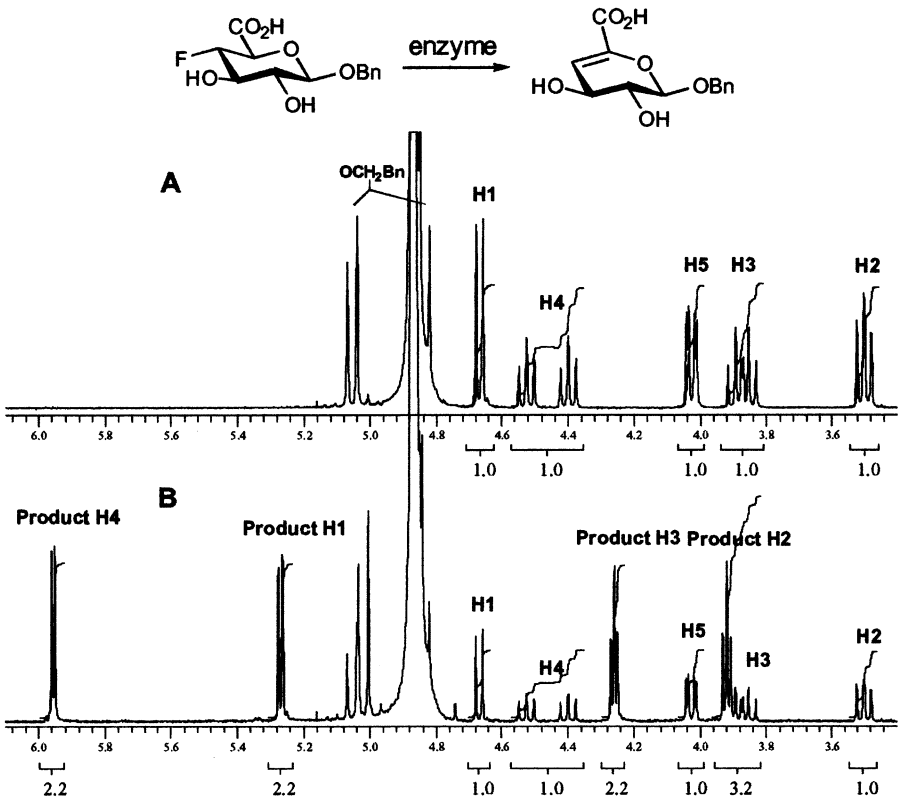


Figure 4. Deuterium exchange experiment. Partial 400-MHz ^{1}H NMR spectra of (A) the monosaccharide substrate **16** and (B) its partial enzymatic digestion to product by chondroitin AC lyase in D_2O.

the measurement of defined and reproducible k_{cat} and K_m values, previously unattainable with the natural polymeric substrates, as summarized in Tables 1 and 2. The use of a variety of leaving groups allows a range of different detection techniques to be used in their analysis, as described elsewhere.[46] Interestingly, the chromogenic substrates bind more tightly than do the fluoride-releasing substrates, presumably because of hydrophobic interactions between the aromatic leaving groups and hydrophobic residues in the enzyme active site, since residues such as tyrosine and tryptophan are very commonly found at the active sites of carbohydrate-binding proteins. In fact, the three-dimensional structure of various enzyme−oligosaccharide complexes of chondroitin AC lyase reveals tyrosine, histidine, phenylalanine, and several tryptophan residues arranged throughout the active site.[14] Of mechanistic importance is the finding of higher k_{cat} values for the fluoride-releasing substrates than for substrates with oxygen-based leaving groups. This presumably arises from the stabilization of the developing negative charge by the highly electronegative fluorine atom in the rate-limiting deprotonation step. This preference of the enzyme for substrates with aromatic appendages is also reflected in the results shown in Table 1 in which the structure of the anomeric substituent was varied. Compromises must be made in the choice of anomeric substituent: phenyl glycosides have the highest k_{cat} values, but bind poorly, while the converse is true for benzyl glycosides.

Since the natural substrates for chondroitin AC lyase are long polysaccharide chains, one would expect that increasing the number of sugar units in the synthetic substrate would increase the binding interactions with the enzyme, thereby yielding faster turnover. Disappointingly, a disaccharide substrate with an *N*-acetylgalactosamine residue at the reducing end (**34**) binds only slightly tighter than does the monosaccharide substrate, and with a k_{cat} value at least 10-fold lower, resulting in a much less efficient substrate as measured by the low k_{cat}/K_m value (Table 1). Perhaps binding interactions toward the nonreducing end of the active site are more important for binding and catalysis. Unfortunately the addition of extra sugar units to occupy the −1 and −2 enzyme-binding sites is not compatible with the design of this class of substrates. Based upon the findings, the phenyl aglycon was chosen to be used in all substrates for further mechanistic analyses.

The finding of higher k_{cat} values for the fluoride-releasing substrates than for the chromogenic or fluorogenic substrates, possibly due to the stabilization of the developing negative charge by the highly electronegative fluorine atom, suggested that a substrate bearing two fluorines at that position, a 4,4-difluoro substrate, might prove a better substrate still, especially given the trans relationship of the axial fluorine with the C5 proton. A fluorine substituent is only slightly larger than a hydrogen; thus, it is reasonable to expect that this substrate might bind. However, surprisingly, no cleavage whatsoever of this compound (**49**) was observed, nor was the 4-fluoro-galactopyranoside analogue (**43**) a substrate for the enzyme. A trivial explanation for this otherwise surprising finding would be that the compounds with an axial fluorine substituent do not bind to the active site. However, both of these compounds were shown to be competitive inhibitors of the enzyme with K_i values of 24 and 3 mM for the 4,4-difluoro- and 4-fluorogalacto compounds, respectively. Despite the small difference in size of fluorine and hydrogen atoms, the introduction of the axial

(46) Rye, C. S.; Withers, S. G. *Anal. Biochem.*, in press.

C4-fluorine must either introduce enough additional steric bulk or result in an unfavorable dipolar interaction of sufficient magnitude to cause an unproductive mode of binding. An alternative explanation for the lack of activity shown by the difluoro **49** may rest at least partly with the greater than 10 kcal/mol ground-state stabilization of the carbon−fluorine bond afforded by geminal fluorine atoms.[47,48] The greater inhibition by **43** is most likely due to the fact that monosaccharide substrates with a benzyl aglycon have been shown to bind tighter to the enzyme than do those with a phenyl aglycon moiety, such as **49**.

pH Dependence. Previous studies[3,49] of the pH dependence of chondroitin AC lyase unfortunately contradict each other; thus, further analyses of pH dependence were performed using both the enzyme's natural substrate (chondroitin 6-sulfate) and a synthetic monosaccharide substrate (**16**). A simple analysis of V_{max} versus pH with chondroitin 6-sulfate (Figure 3A) revealed the V_{max} to be maximal at a pH of 6.8, and this was the pH chosen for subsequent enzymatic analyses. Two pK_as can be extracted from this curve, 5.3 and 7.9, which may represent two ionizable groups important for catalysis in the enzyme such as the general-acid and general-base residues. These pK_as could also reflect the ionization of the substrate within the enzyme−substrate complex. However, the pK_a values of the substrate carboxylic acid moieties have been shown to be much lower than either of these values (vide infra), rendering this interpretation less likely. Consistent with this interpretation is the very similar pH dependence of k_{cat}/K_m for **16** (Figure 3B), which is maximal at around pH 6. The one clear pK_a value that can be abstracted from this curve is 7.2, reasonably close to that deduced from the study with the natural substrate and implying the presence of an essential group that must remain protonated for activity, possibly the acid catalyst. From X-ray crystallographic data, a tyrosine residue has been implicated as the acid catalyst.[14,15] The pK_a of a tyrosine residue is normally around 10; however, a decrease of 2−3 pK_a units is not unprecedented for amino acids located at the active site of enzymes. Perhaps the most surprising finding was the remarkable change in K_m for the natural substrate with changing pH. The K_m was measured to be 1.23 mg/mL at pH 8.8 and only 7.0×10^{-3} mg/mL at pH 5.0, corresponding to a 175-fold change. Conversely, for the synthetic substrate **16**, the K_m dropped by only one-third between pH 6.8 and pH 5.2. The substantial decrease in K_m observed with chondroitin 6-sulfate resulted in a significantly different profile of k_{cat}/K_m versus pH from that seen with the synthetic substrate or from the profile of V_{max} with the same substrate (Figure 3C and A). The origin of this difference must lie in the differences between the synthetic and natural substrates, which are threefold: (i) the synthetic substrate has a fluoride leaving group that does not require acid catalysis, (ii) the synthetic substrate is not sulfated, and (iii) the synthetic substrate is a monosaccharide, whereas the natural substrate is a large polysaccharide. The dependence of the leaving group on acid catalysis is not expected to affect the binding of the substrate to the enzyme and thus should not result in a dependence of K_m upon pH. The pK_a of the sulfate group is expected to be sufficiently low that the ionization of this group should not affect the results in the pH range of 5−9. With the use of Gran plots,[50−52] the pK_a values of chondroitin 6-sulfate and the synthetic substrate **16** were measured to be 2.9 and 2.7, respectively. This small difference in the pK_a values of the carboxylic acid groups of these two compounds is not sufficient to explain the different effects of pH on the K_m. Inspection of the three-dimensional structure of chondroitin AC lyase reveals that the proposed binding cleft on the enzyme is positively charged, presumably in order to bind this polyanionic substrate.[8,14] It therefore seems reasonable that a decrease in pH may afford a more positively charged binding pocket on the enzyme that may well lead to enhanced binding only of a long polysaccharide chain such as the natural substrate, but not the monosaccharide synthetic substrate. It is also conceivable that chondroitin 6-sulfate undergoes a pH-dependent conformational change that affects its binding to the enzyme active site[1,53] and thus might be expected to change the K_m value.

Toward a Mechansim. To probe the identity and nature of the rate-determining step(s) for the cleavage of these substrates, a study of the dependence of the rate upon leaving-group ability was performed. The resulting flat linear free energy relationship (Figure 2) shows that there is no significant charge development on the C4 oxygen at the transition state of the rate-limiting step. There are two main possibilities to explain this result. The first is that there is highly effective proton donation to the departing phenolate by a general-acid catalyst at the transition state, which neutralizes any charge development at the C4 oxygen. If this is the case, it is impossible to tell whether the reaction is concerted or stepwise. The second possibility is that the reaction is stepwise with the breaking of the C4−O4 bond not occurring in a rate-limiting step, thus ruling out a concerted syn-elimination. This second explanation is preferred not only by the rarity of concerted syn-eliminations but also by its agreement with the near-unity secondary deuterium KIE that was measured with a substrate containing deuterium at C4 (vide infra).

The primary deuterium KIE on k_{cat}/K_m for abstraction of the proton α to the carbonyl was measured using **73** to be $k_H/k_D = 1.67 \pm 0.07$. The presence of a primary deuterium KIE demonstrates that the C5−H5 bond is broken in a rate-limiting step, ruling out an E1 elimination mechanism. This result, along with the flat linear free energy relationship, suggests a stepwise mechanism with rate-limiting proton abstraction, conforming to an $(E1cb)_{irr}$ reaction scheme. This is consistent with the fact that proton transfers to and from carbon acids are usually much slower than those to and from oxygen, nitrogen, and sulfur.[54] This slow transfer has been attributed to the need for structural reorganization accompanying the delocalization of the negative charge, solvent reorganization, and from the poor hydrogen-bonding capability of carbon acids and of the carbanionic carbon.[55] The Hammond postulate tells us that the transition state leading to the formation of a high-energy species, such as the enolic intermediate proposed for this elimination mechanism, occurs late on the reaction coordinate and resembles the enolic intermediate. Consequently, the extent of proton transfer from

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the substrate at the transition state is large, and one might expect a KIE much less than the maximal value, as was observed. In addition, the chemical step in an enzymatic reaction may well not be rate-limiting, or only partially so, as a result of evolutionary pressure on the enzyme to accelerate chemical steps until they are just slightly faster than the release of product.[56,57] Small primary KIEs may also arise from a nonlinear arrangement of the proton donor, proton, and the proton acceptor, in the transition state for the transfer of the proton. Such an occasion is conceivable if the catalytic base residue responsible for the abstraction of the proton is the same residue responsible for delivering a proton to the leaving group. This is postulated to be the case with alginate lyase A1-III from *Sphingomonas* species, an enzyme that degrades a poly-β-D-mannuronic acid substrate. From the three-dimensional structure of the enzyme complexed with a trisaccharide product, a tyrosine residue has been implicated as both the catalytic base that abstracts the C5 proton and the general acid that protonates the leaving sugar unit.[16] Less than maximal primary deuterium KIEs have been measured with other enzyme systems undergoing elimination reactions. For enolase, the primary KIE for the abstraction of the proton α to the carbonyl group is dependent on both the pH and Mg^{2+} concentration and ranges from $\sim$1.2 to $\sim$3.3.[58,59] The crotonase-catalyzed dehydration of 3-hydroxybutyrylpantetheine shows a primary KIE of 1.60[60] and that for *o*-succinylbenzoate synthase has been measured to be 2.7.[61] The reaction catalyzed by UDP-*N*-acetylglucosamine 2-epimerase also shows a small primary KIE of 1.8 for the C2 hydrogen.[62] Although not an overall elimination process, there is strong evidence for the elimination of the UDP moiety as part of the proposed reaction mechanism. The primary KIE for an antibody-catalyzed elimination of HF adjacent to a ketone has been measured to be 2.35.[63]

Using **77** with a deuterium incorporated at C4, the secondary deuterium KIE was measured to be 1.01 $\pm$ 0.03. A secondary KIE value close to unity would be expected if the rate-limiting step were solely the proton abstraction and formation of the enolic intermediate since C4 does not acquire any sp^2 character during this step. On the other hand, a partially rate-limiting departure of the leaving group where C4 takes on partial sp^2 character at the transition state is expected to show a secondary KIE greater than 1, with values of $k_H/k_D = 1.3-1.4$ as maximal values. The elimination of water by the non-carbohydrate-degrading enzymes fumarase and crotonase has been shown to involve the kinetically significant departure of the leaving group and shows secondary deuterium kinetic isotope effects ranging from 1.13 to 1.23, illuminating the significant sp^2 character at this center.[60,64] The low value of the secondary deuterium KIE measured for chondroitin AC lyase at pH 6.8 and 30 °C therefore supports a stepwise mechanism in which the bond to the C4 leaving group is not broken in a rate-limiting step.

The primary and secondary deuterium KIEs, combined with the flat linear free energy relationship, suggest that the rate-limiting step is the abstraction of the C5 hydrogen and the formation of the enolic intermediate. Once this intermediate is formed, the C4 substituent is eliminated in a non-rate-limiting step. Further evidence that is consistent with this proposal would be the absence of deuterium exchange at the C5 position before the elimination of the C4 substituent. Other enzymes, such as enolase,[58] show rapid exchange of the proton α to the carbonyl group with solvent and are believed to use a stepwise mechanism in which proton abstraction and leaving-group departure are both partially rate-limiting. On the other hand, the crotonase-catalyzed β-elimination,[60] which is thought to be concerted, shows almost no deuterium exchange (= 3%). Figure 4 shows the partial ^1H NMR spectra of a monosaccharide substrate (**16**) and that of the substrate and product mixture after partial conversion by chondroitin AC lyase in D_2O. If deuterium exchange at C5 occurred faster than the elimination of the 4-fluoro group, then the integration of H5 in the starting material would decrease relative to that of H1, H2, and H3. However, this is clearly not the case, as shown by the integrations in Figure 4. This is consistent with a mechanism that involves the rapid elimination of the C4 substituent after a rate-limiting proton abstraction ($(E1cb)_{irr}$). However, the absence of exchange does not constitute proof that this process is not occurring since enzyme active sites have long been proposed to be sequestered from bulk solvent, and thus, the residues located there may not be accessible to the D_2O solvent as required for deuterium exchange. The use of modified substrates also has its limitations in that it is not absolutely required that the lyase-catalyzed elimination of the polysaccharide substrate occur in the same manner as that deduced with the synthetic substrates. However, these studies do at least provide general mechanistic insights into turnover of these artificial substrates by the enzyme. Further, it is important to note that the mechanistic studies presented in this paper would not be possible without the modified substrates.

Conclusions

The synthesis and kinetic evaluation of several simple substrates for chondroitin AC lyase has allowed the determination of defined and reproducible k_{cat} and K_m values, previously unattainable with the inhomogeneous and polymeric natural substrates. Evidence has been gathered from linear free energy relationships, primary and secondary deuterium KIEs, and isotope exchange experiments in order to elucidate the catalytic mechanism of this enzyme. The flat linear free energy relationship produced using substrates with leaving groups of differing reactivity combined with the low secondary deuterium KIE strongly suggests that the breaking of the C4-leaving group bond does not occur in a rate-limiting step, thus ruling out a concerted mechanism. The primary deuterium KIE of 1.67 $\pm$ 0.07 shows that the abstraction of the C5 proton occurs in a rate-limiting step and that the transition state for this step is late on the reaction coordinate, resembling the proposed enolic intermediate.

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Supporting Information Available: Experimental procedures and characterization data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org. See any current masthead page for ordering information and Web access instructions.

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